cAMP Regulation of ERKs and Cell Growth

by

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CERTIFICATE OF APPROVAL

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ABSTRACT

Hormonal stimulation of G protein-coupled receptors which signal to the heterotrimeric G protein Gs, cAMP, and PKA exhibit cell-type specific effects on ERKs. Stimulation of the β_2-adrenergic receptor (β_2AR) with isoproterenol results in rapid activation of cAMP and PKA. The signaling mechanism by which PKA activates ERKs has not been well characterized. Recent evidence has shown that ERKs can be activated by cAMP/PKA via the activation of the small G protein Rap1 and the serine/threonine kinase, B-Raf. B-Raf is expressed in a cell-type specific manner and is closely related to Raf-1 and when active, phosphorylates and activates MEK/ERK. Therefore, B-Raf expression may provide a pathway for hormones which elevate cAMP/PKA to activate ERKs. Experiments presented in this thesis suggest that isoproterenol can stimulate ERKs in Hek293 cells, which express both endogenous β_2AR and B-Raf. Moreover, isoproterenol stimulates ERKs through a Gsα/cAMP/PKA/Rap1 and B-Raf pathway.

In many cell types, cAMP/PKA inhibits the physiological actions of growth factors to stimulate ERKs and cell proliferation. Stimulation of ERKs by growth factors are required for a number of biological processes including, cellular proliferation. A candidate protein that may function to antagonize Ras-dependent activation of ERKs and cell proliferation is Rap1. Rap1 can be activated by cAMP/PKA however, the role of Rap1 in cAMP-dependent inhibition of ERKs and cell growth has not been examined. Results presented here demonstrate that Rap1 is required for cAMP's inhibition of both ERK activation and cell growth and proliferation in fibroblasts.

The mechanism by which PKA activates Rap1 is unclear. Previous studies have suggested that PKA may utilize the Rap1 exchanger, C3G, its adapter protein Crk-L, and the scaffold protein Cbl to indirectly activate Rap1. However, the ability of PKA to utilize a C3G/Crk-L/Cbl pathway to activate Rap1 has not been directly tested. We show that cAMP/PKA stimulation of fibroblasts resulted in Rap1 activation which requires membrane recruitment and activation of a C3G/Crk-L/Cbl complex. Interestingly, cAMP but not EGF stimulated Rap1 activation and the association of C3G, Crk-L, and Cbl, which could be blocked by inhibitors of PKA or Src family kinases. Furthermore,

cAMP/PKA's ability to activate Rap1 and inhibit ERKs and cell growth was also blocked in fibroblasts derived from mice deficient of Src (SYF) but not fibroblasts derived from mice which were wild type at the Src locus (Src++). Data presented within this thesis demonstrates for the first time that cAMP activates Rap1 in a PKA/Src/C3G/Crk-L/Cbl-dependent manner to inhibit ERKs and cell growth in fibroblasts.

In 1978, Erickson and colleagues demonstrated an increase in phospho-serine within Src's amino-terminus following cell treatment with cAMP which increased Src kinase activity. A consensus PKA site at serine 17 of Src was proposed to be the major site of serine phosphorylation but no physiological role for phosphorylation at serine 17 has been proposed. Therefore, we examined the mechanism of PKA's activation of Src and the significance of Src's activation by PKA in fibroblasts.

Work presented in this thesis reveals that PKA stimulation resulted in Src kinase activity by phosphorylating Src on serine 17. A Src mutant which blocks PKA phosphorylation of Src at serine 17 blocked cAMP's activation of Src and Rap1, and inhibition of ERKs and cell growth. A Src mutant containing aspartate at position 17 showed elevated kinase activity, activated Rap1, and inhibited growth factor-mediated activation of ERKs and cell growth. This novel pathway of cAMP/PKA/Src/Rap1 mediates inhibition of growth factor activation of ERKs and cell proliferation in fibroblasts. This is the first example of the regulation of Src signaling by PKA and, significantly, it identifies an anti-proliferative role for Src in the physiological regulation of cell growth by cAMP.

CHAPTER ONE

INTRODUCTION

cAMP's Regulation of Rap1, ERKs, and Cell Growth

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INTRODUCTION

Stimulation of cell surface G protein-coupled receptors (GPCRs) triggers a number of biochemical and physiological cellular effects. A variety of stimuli including lipids, photons, odorants, calcium ions, neurotransmitters, and hormones can act through GPCRs giving rise to diverse physiological functions (Ji et al., 1998). Hormonal stimulation of the intracellular heterotrimeric G protein, Gs, results in increases in intracellular cAMP. The intracellular second messenger cyclic adenosine monophosphate (cAMP) has become a central molecule which mediates hormone action on the intracellular pathways to regulate cellular metabolism (Gottesman and Fleischmann, 1986). Moreover, cAMP is a key signaling molecule which integrates hormonal stimulation with cell growth and proliferation (Gottesman and Fleischmann, 1986; Puck, 1977). Because of the importance of cAMP in cell proliferation, both cAMP and its target the cAMP-dependent protein kinase (PKA) have been recognized as important molecules in medicine (Aukrust et al., 1999; Ciardiello et al., 1996). Examples of therapeutic uses include hormones (Maruno et al., 1998) and cAMP analogues in anti-cancer treatments (Carlson et al., 2000; Ciardiello et al., 1996; Tortora et al., 1995) and PKA activators to inhibit restenosis following coronary angioplasty (Bonisch et al., 1998).

One of the key aspects of cAMP's cellular growth effects is the cell-type specific character of its effects. While cAMP inhibits the growth of a large group of cells, it also cooperates in the proliferative actions of a number of hormones and growth factors. The mitogen-activated protein (MAP) kinase (also called extracellular signal-regulated kinases, or ERK) intracellular signaling cascade can be regulated by cAMP/PKA and ERKs play a central role in cellular growth and proliferation (see Figure 1.1). Data

presented in this thesis examines the cell-type specific effects of cAMP on ERKs and cell growth. Several areas of cAMP's regulation of ERKs are discussed and addressed by this thesis work. Both the positive and negative biochemical pathways that regulate ERKs and its relation to cAMP's cell-type specific actions on cell proliferation are addressed. Second, the specific intracellular signaling mechanisms that allow for the regulation of ERKs by cAMP are also considered. These pathways may provide for the cell-type specificity of cAMP's actions. We will describe systems where cAMP's activation of ERKs utilizes a Rap1/B-Raf signaling pathway. We will also examine model systems where cAMP-dependent inhibition of ERKs and cell growth depends on Rap1's inhibition of Raf-1 in fibroblasts. In addition, data demonstrating the requirement for Src in cAMP's activation of Rap1 and its cell-type specific actions will also be presented. Taken together, work described in this thesis demonstrates the ability of cAMP signaling to cross talk with growth factor signaling pathways to regulate ERK activation and cell growth. This work provides a significant contribution to understanding the molecular basis for cell growth within the field of cell biology.

cAMP and the inhibition of cell growth

Hormones activate intracellular cAMP through stimulation of plasma membrane-associated adenylyl cyclases. This occurs through G protein-coupled receptors (GPCRs) that link hormones to the heterotrimeric G protein, Gαs (Masters et al., 1988). The ability of Gαs to inhibit cell growth has been demonstrated in Rat-1 fibroblasts and NIH3T3 cells using a constitutively active mutant of Gαs (Chen and Iyengar, 1994). Most of cAMP's intracellular actions can be explained via the activation of PKA.

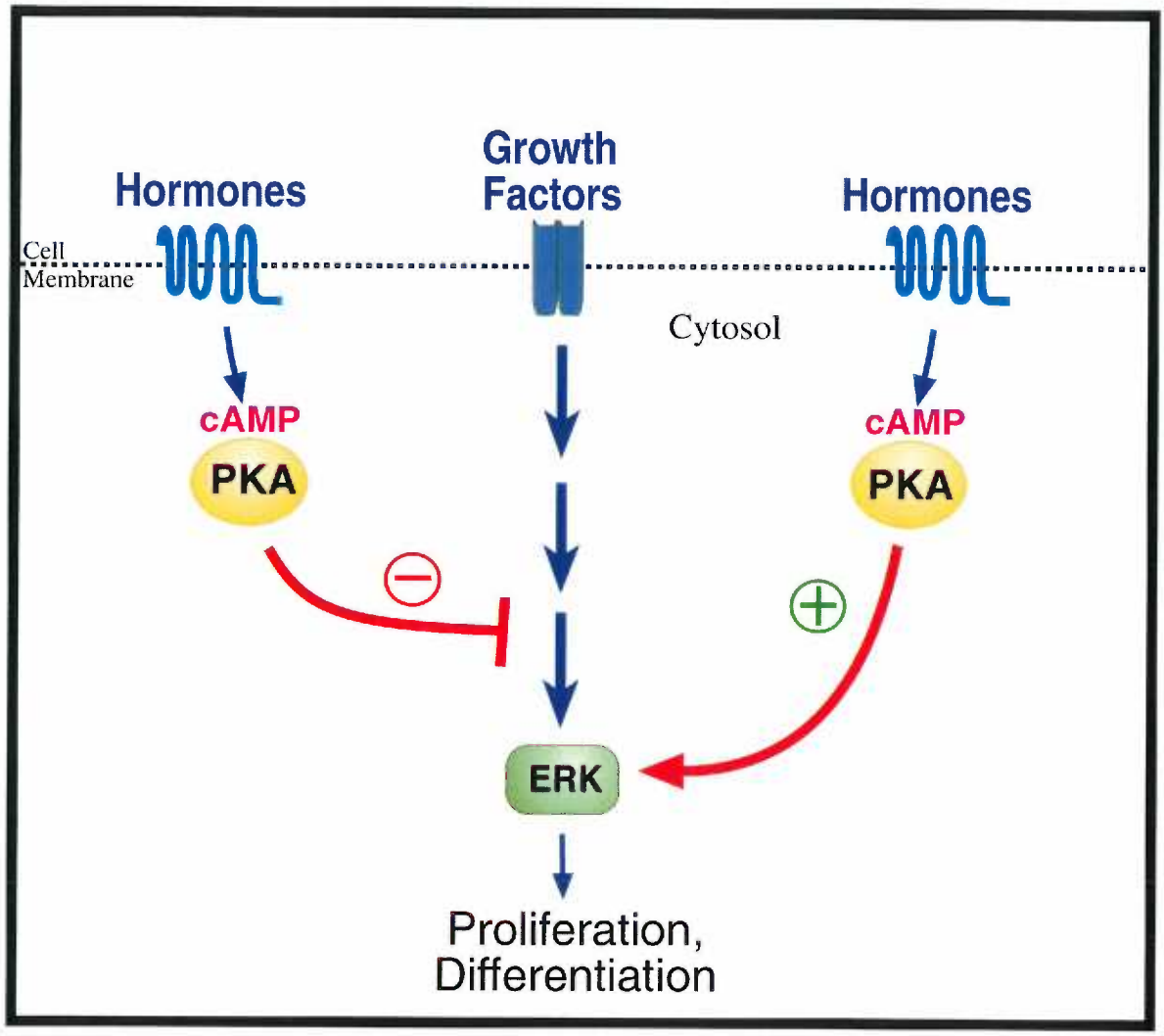

Figure 1.1. **cAMP's regulation of ERKs and proliferation.**
Stimulation of cells with growth factors results in activation of the extracellular signal-regulated kinase, ERK. ERK can stimulate either proliferation or differentiation depending on the stimulus and cell type. Hormonal stimulation of cells can activate Gαs and adenylyl cyclases to stimulate the production of cAMP. cAMP activates the cAMP-dependent protein kinase, PKA. In some cells, PKA activation stimulates ERKs and cell proliferation (right path). In other cells, PKA activation inhibits growth factor-dependent activation of ERKs and cell proliferation (left path).

Indeed, data presented within this thesis and elsewhere, suggests that cAMP mediates its anti-proliferative effects via stimulation of PKA.

A broad mechanism by which PKA inhibits cell proliferation induced by growth factors is to block one of multiple points in the cell cycle. For example, PKA has been shown to inhibit cell proliferation by either decreasing the levels of cyclins (Stuart et al., 2000; van Oirschot et al., 2001) or increasing cell cycle inhibitor proteins $p21^{cip1}$ (Lee et al., 2000) or $p27^{kip1}$ (Stuart et al., 2000; van Oirschot et al., 2001).

cAMP inhibits cell proliferation in a broad array of cell types, which are discussed below. A number of mesenchymally derived cells can be inhibited by stimulation of cAMP including, smooth muscle cells (Bornfeldt and Krebs, 1999), cardiac myocytes (Gupta et al., 1996), osteoblasts (Siddhanti et al., 1995; Verheijen and Defize, 1995), and chondrocytes (Hirota et al., 2000). Work presented in this thesis and elsewhere also demonstrates the ability of cAMP to inhibit proliferation of fibroblasts (Dubey et al., 2001; Marienfeld et al., 2001; Schmitt and Stork, 2002).

Several other cell types whose growth is also inhibited by cAMP include, but are not limited to hepatocytes (Thoresen et al., 1999), colo-rectal cells (Hopfner et al., 2001), endothelial cells (Kim et al., 2001), astrocytes (Metz and Ziff, 1991), and adrenal cortical cells (Lepique et al., 2000). Interestingly, adult neuronal cells which have already undergone mitosis can also be inhibited by cAMP during early stages of differentiation (Herman et al., 1994; Vogt Weisenhorn et al., 2001).

In addition, cAMP inhibits the proliferation of white blood cells including macrophages, leukocytes (Tortora et al., 1988), and both T lymphocytes (Naderi et al., 2000; Tamir and Isakov, 1994; Torgersen et al., 2002) and B lymphocytes

(Venkataraman et al., 1998). Taken together, the above data demonstrates that cAMP stimulation inhibits cell growth in a broad range of cell types and suggests that cAMP is an important molecule in regulating cellular phenotypes. The use of cAMP in anti-cancer therapy may also prove a useful tool under certain scenarios.

Inhibition of ERK may mediate cAMP's growth inhibitory effects

The growth-inhibitory actions of hormones, cAMP, and PKA have been linked to inhibition of the ERK kinase cascade in many cell types. Growth factor stimulation of cell surface receptors activates the small G protein Ras which leads to ERK activation (Marshall, 1999). Active Ras (GTP loaded) recruits the MAP kinase kinase kinase (MAPKKK), Raf-1, to the membrane where it is activated (Marais et al., 1995; Mason et al., 1999). Activated Raf-1 phosphorylates and activates the MAPKK, MEK, which in turn phosphorylates and activates ERK kinase (Huang et al., 1993; Jelinek et al., 1994; Macdonald et al., 1993). ERK activation can trigger proliferation and cell growth via a number of pathways including increasing cyclin D1 expression (Kerkhoff and Rapp, 1998) and stimulating both protein and DNA synthesis (Graves et al., 2000).

Classically, hormones which activate Gαs stimulate cAMP/PKA. Interestingly, ERK activation and transformation by Ras, in NIH3T3 cells, can be blocked by constitutively activated mutants of Gαs (Chen and Iyengar, 1994). This data would suggest that hormonal stimulation of Gαs utilizes the cAMP/PKA pathway to inhibit ERKs and cell growth (Chen and Iyengar, 1994). Indeed, work presented in this thesis demonstrates that both the β-adrenergic agonist, isoproterenol, and the hormone prostaglandin E_1 inhibit ERKs and cell growth via stimulation of cAMP/PKA (Schmitt

and Stork, 2002). However, GPCRs are not the only receptors that can elevate cAMP levels to inhibit ERKs. For example, stimulation of cAMP through estrogen receptors in breast cancer cell lines mediates cAMP's inhibition of EGF signals to ERKs (Filardo et al., 2002). This inhibition also requires PKA and may play an anti-proliferative role in these cells.

In contrast to work presented within this thesis, cAMP may also inhibit cell growth without inhibiting ERKs (Kahan et al., 1992; McKenzie and Pouyssegur, 1996). It should be noted that examining single or selected timepoints of ERK regulation by cAMP may fail to recognize subtle regulatory effects. For example, in CCL39 fibroblast cells growth factor stimulation of ERKs is not inhibited by cAMP but is delayed (McKenzie and Pouyssegur, 1996). Depending on the cell-type and stimulus cAMP may inhibit either early or late ERK activation (Cospedal et al., 1999). Interestingly, stimulation of PC12 with cAMP resulted in an early inhibition of ERKs but an enhancement of the late phase of ERK activation by NGF leading to differentiation (Arslan and Fredholm, 2000).

Mechanisms of cAMP inhibition of ERKs

cAMP inhibits ERKs via PKA and Rap1

Contemporary studies by a number of research groups have examined the ability of cAMP/PKA to inhibit ERKs. Molecular and biochemical studies by Krebs (Graves et al., 1993), Sturgill (Wu et al., 1993), Bos (Burgering et al., 1993), and McCormick (Cook and McCormick, 1993) suggested that the target of cAMP's inhibition of ERKs was downstream of Ras activation. Subsequently, it was demonstrated that in cAMP-treated

fibroblasts Ras was activated normally in response to growth factors, but was unable to bind and activate Raf-1 which resulted in ERK inhibition (Cook and McCormick, 1993; Wu et al., 1993). Therefore, the site of cAMP's action was mapped downstream of Ras and at the level of Raf-1 signaling. The requirement for PKA in cAMP's effects was later confirmed using a genetic approach in cells expressing mutant PKA (Sevetson et al., 1993) however, the exact mechanism of PKA's inhibition has remained elusive.

The mechanism of inhibition of Raf-1 by PKA is still a focus of recent studies as well as work presented in this thesis (Piiper et al., 2000; Ramstad et al., 2000; Schmitt and Stork, 2001). PKA can phosphorylate Raf-1 at a number of sites and evidence that PKA phosphorylation directly inhibits Raf-1 activity has been shown (Hafner et al., 1994; Mischak et al., 1996). The ability of PKA to phosphorylate serine 43 of Raf-1 has been proposed to uncouple Raf-1 from Ras which can be achieved *in vitro* (Wu et al., 1993). Moreover, phosphorylation of serine 43 has been proposed to account for PKA's inhibition of Raf-1 activation in fibroblasts (Wu et al., 1993) and T cells (Ramstad et al., 2000). However, mutagenesis of this site failed to block PKA from inhibiting ERKs in both NIH3T3 and Hek293 cells *in vivo* (Sidovar et al., 2000). Additional mechanisms for PKA's ability to uncouple Raf-1 from Ras must exist.

One possible mechanism involves the Ras family member Rap1. The small G protein Rap1 is also a target of PKA activation, and has become a focus of attention over the last several years (Altschuler et al., 1995). Rap1 is thought to be ubiquitously expressed and exists as one of two isoforms, Rap1a and Rap1b which mediate similar physiological functions (Pizon et al., 1990; Pizon et al., 1994). Rap1 was first identified as an antagonist of Ras-induced ERK activation and cell transformation in NIH3T3 cells

(Cook et al., 1993; Kitayama et al., 1990). Interestingly, although Rap1 can block signals from constitutively activated Ras, it cannot block signals from constitutively active Raf-1 (Sakoda et al., 1992). Similar to cAMP, Rap1's inhibitory action targets upstream of Raf-1 and downstream of Ras signaling (Burgering et al., 1993). Data presented in this thesis, suggests that Rap1 antagonizes Ras activation of Raf-1 and ERKs by binding to and sequestering Raf-1 away from Ras (Schmitt and Stork, 2001). Rap1's ability to bind and inhibit Raf-1 requires Rap1 activation by cAMP/PKA.

Studies presented here and elsewhere demonstrate that the role of Rap1 in ERK regulation can be examined by inhibiting Rap1 activation through expression of either the interfering mutant RapN17 (Chen et al., 1999; Dugan et al., 1999; Schmitt and Stork, 2000) or the Rap1 inhibitor, Rap1GAP1 (Mochizuki et al., 1999; Schmitt and Stork, 2001). Indeed, we have shown that in NIH3T3 cells, inhibition of endogenous Rap1 blocked cAMP/PKA's inhibitory effects on both ERKs and cell growth (see Figure 1.2A) (Schmitt and Stork, 2001). While Rap1 has been well characterized as an antagonist of ERKs and cell growth in cell lines, this has not been demonstrated in animal models.

PKA activates Rap1 via Src

Rap1 is activated by hormones which signal to cAMP and PKA (Altschuler et al., 1995; Schmitt and Stork, 2002) and can inhibit Ras-dependent signals therefore, Rap1 is a potential target of cAMP's inhibition of Ras/Raf-1-dependent signals to ERKs (see Figure 1.2A). PKA activation of Rap1 has been demonstrated in a number of cell types including, neurons (Vossler et al., 1997), glia (Dugan et al., 1999), fibroblasts (Altschuler et al., 1995; Schmitt and Stork, 2001), neutrophils (Quilliam et al., 1991), and platelets

(Quilliam et al., 1991). Both Rap1a and Rap1b are targets of PKA phosphorylation (serines 179 and 180 in Rap1b) (Quilliam et al., 1991; Siess et al., 1990). However, early studies revealed that these sites were dispensable for Rap1 activation by cAMP (Altschuler and Lapetina, 1993). Additional studies have also suggested either negative or positive roles for PKA phosphorylation of Rap1 on Rap1 activity itself (Hu et al., 1999; Tsygankova et al., 2001). From these and other studies, it is clear that direct phosphorylation of Rap1 can not account for PKA's activation of Rap1, and that PKA's activation of Rap1 must be indirect.

Potential indirect mechanisms by which PKA could activate Rap1 include activation of specific exchangers or inhibition of specific inhibitors. Inhibition of Rap1, like other G proteins, is achieved via specific GTPase activating proteins (GAPs). The Rap1-specific GAP, Rap1GAP1, is a substrate of PKA, however PKA does not appear to effect Rap1GAP1's ability to inhibit Rap1 activity (Polakis et al., 1992). PKA may also regulate upstream exchangers/activators of Rap1. The first Rap1-specific exchanger identified was C3G, or Crk SH3 guanine nucleotide exchanger (Gotoh et al., 1995). The possibility that PKA could activate C3G exchange activity is suggested by studies presented in this thesis in which PKA's activation of Rap1 was blocked by the expression of an interfering mutant of C3G, in Hek293 cells (Schmitt and Stork, 2000). Subsequent work has confirmed a requirement for C3G in PKA's activation of Rap1 in NIH3T3 cells and embryonic fibroblasts (Schmitt and Stork, 2002).

cAMP/PKA stimulation recruited C3G to the membrane fraction of cells in a complex with the adaptor Crk-L and the scaffold protein Cbl. C3G/Crk-L is recruited to Cbl following the phosphorylation of a specific tyrosine residue on Cbl that serves as a

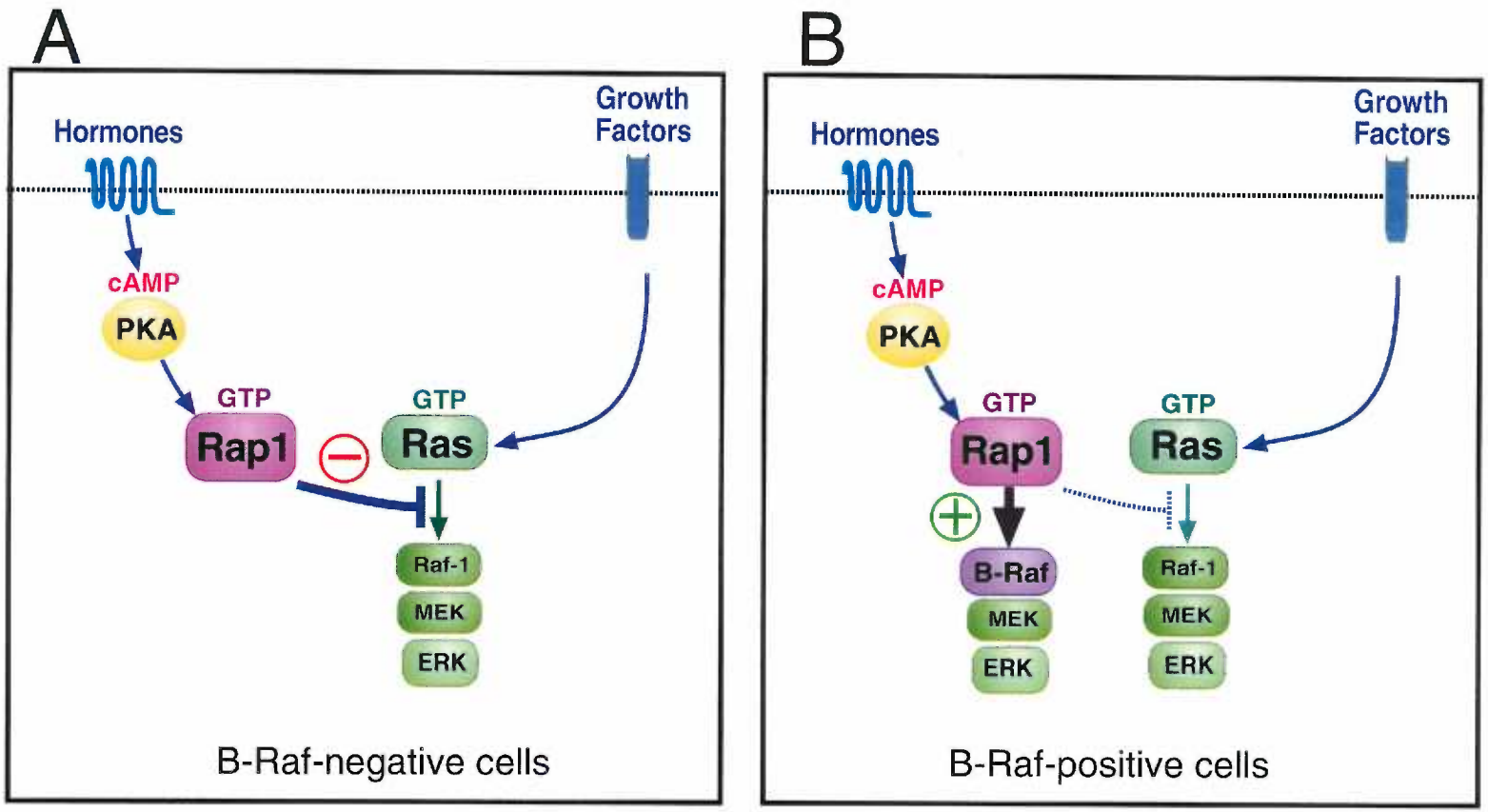

Figure 1.2. **Rap1 activation by cAMP regulates ERKs in a cell type-specific manner.**
(A) Rap1 activation by cAMP inhibits ERKs. Hormonal stimulation of a Gαs/cAMP/PKA module leads to Rap1 activation (GTP loading). Many cells express Raf-1 as the major Raf isoform. In these cells, GTP-loaded Rap1 blocks Ras' activation of Raf-1, thereby inhibiting growth factor activation of ERKs and cell growth. (B) Some cells express B-Raf as well as Raf-1. In these cells, GTP-loaded Rap1 can activate B-Raf and hormonal stimulation of cAMP/PKA/Rap1 in these cells activates ERKs. Rap1 may also antagonize Ras activation of Raf-1, as described in 1.2A. Rap1's activation of B-Raf often predominates over the inhibition of Raf-1 resulting in net effect of ERK activation.

docking site for the SH2 domain of Crk-L (Schmitt and Stork, 2002; Thien and Langdon, 2001). This tyrosine phosphorylation of Cbl requires both PKA and the Src tyrosine kinase (Schmitt and Stork, 2000). Interestingly, PKA activates Src via a direct phosphorylation at serine 17 (Gottesman and Fleischmann, 1986; Roth et al., 1983). Work presented here demonstrates that PKA phosphorylation of Src induces the formation of the Cbl/Crk-L/C3G complex which is required for PKA's activation of Rap1. Furthermore, Src is required for PKA's inhibition of ERKs and cell growth in both NIH3T3 cells and mouse embryonic fibroblasts (Schmitt and Stork, 2002). The requirement of Src for PKA to inhibit ERKs identifies a unique anti-proliferative function for Src one that is distinct from the well studied proliferative actions of this proto-oncogene. It will be important to define how PKA phosphorylation of Src dictates Src's activation of Rap1.

cAMP utilizes additional mechanisms to inhibit ERKs

PKA's activation of Rap1 supplies a potential mechanism for cAMP/PKA to disrupt Ras/Raf-1 signaling in multiple cell types. However, other mechanisms by which cAMP/PKA inhibit ERKs have been proposed (see Figure 1.3). These mechanisms may include non-adherent cell growth, additional kinase targets of cAMP, and MAP kinase phosphatases.

Activation of ERKs by growth factors is blunted in non-adherent cells, and a role for PKA in this block has been demonstrated (Howe and Juliano, 2000). While the mechanism of PKA activation following loss of adherence is not well established, the inhibition by PKA was associated with an inhibitory phosphorylation of the p21-

associated kinase (PAK) (Howe and Juliano, 2000). PAK is a candidate Raf-1 kinase whose phosphorylation of Raf-1 is required for Raf-1 to be fully activated by Ras (Sun et al., 2000).

Additional kinase targets have been proposed. Recent studies have identified AKT (PKB) as a potent negative regulator of the two major MAPKKK's, Raf-1 (Scheid and Woodgett, 2000) and B-Raf (Guan et al., 2000). Rap1 has been proposed to activate AKT through the activation of PI3-K (Tsygankova et al., 2001). In addition, direct phosphorylation of AKT by PKA has been suggested (Sable et al., 1997). It is possible that either mechanism of activation of AKT by cAMP may limit activation of Raf-1 and ERKs.

The family of dual specificity phosphatases, MKPs, can also regulate ERKs. MKP-1 and MKP-2 are immediate early genes whose activity is regulated by transcription (Misra-Press et al., 1995). cAMP is a potent inducer of MKP transcription (Misra-Press et al., 1995) and this induction by cAMP limits ERKs' activation (Plevin et al., 1997). This is one of several mechanisms which may account for the delayed inhibition of ERKs following cAMP treatment that occurs in a time frame compatible with transcriptional regulation. Taken together, these studies would suggest that additional mechanisms exist for cAMP to inhibit ERKs however, these studies have not examined the physiological role of these pathways which will be important to determine.

cAMP's inhibition of cell growth can be independent of ERK inhibition

In many cell types the role of ERKs has not yet been examined. However, studies have identified cell types where inhibition of ERK signaling is not involved in PKA's anti-

proliferative effects. As mentioned previously, the examination of single or selected timepoints of ERK regulation by cAMP may fail to recognize subtle regulatory effects as can be seen in CCL39 fibroblast cells where growth factor stimulation of ERKs is not inhibited by cAMP but is delayed (McKenzie and Pouyssegur, 1996). Additional studies with ACTH, a pituitary hormone that signals via cAMP/PKA, inhibits FGF2 proliferation in mouse Y1 adrenocortical cells without inhibiting ERKs (Lepique et al., 2000). While the exact mechanism of inhibition was not determined, it was suggested that cAMP may be inhibiting cell growth by blocking the expression of key cell cycle transription factors and AKT activation.

The role of cAMP's inhibition of ERKs in T cells is unclear. Limited reports have suggested that PKA inhibited the activation of both T lymphocytes (Ramstad et al., 2000) and B cells (Myklebust et al., 1999) via inhibition of ERKs. However other pathways have also been proposed, including cAMP inhibition of JAK-STAT-mediated immune responses (Ivashkiv et al., 1996; Sengupta et al., 1996). In addition, cAMP can also block T lymphocyte proliferation by inhibiting the transcription of the IL-2 cytokine (Rao et al., 1997), through the action of PKA to disrupt the function of NFAT, NF-kB, and Elk-1 transcription factors (Chow and Davis, 2000; Whitehurst and Geppert, 1996; Zhong et al., 1997). Clearly, studies examining PKA's inhibition of ERKs in lymphocytes is in its infancy and additional studies will need to be carried out to address the role of PKA as well as Rap1 both *in vitro* and *in vivo* model systems.

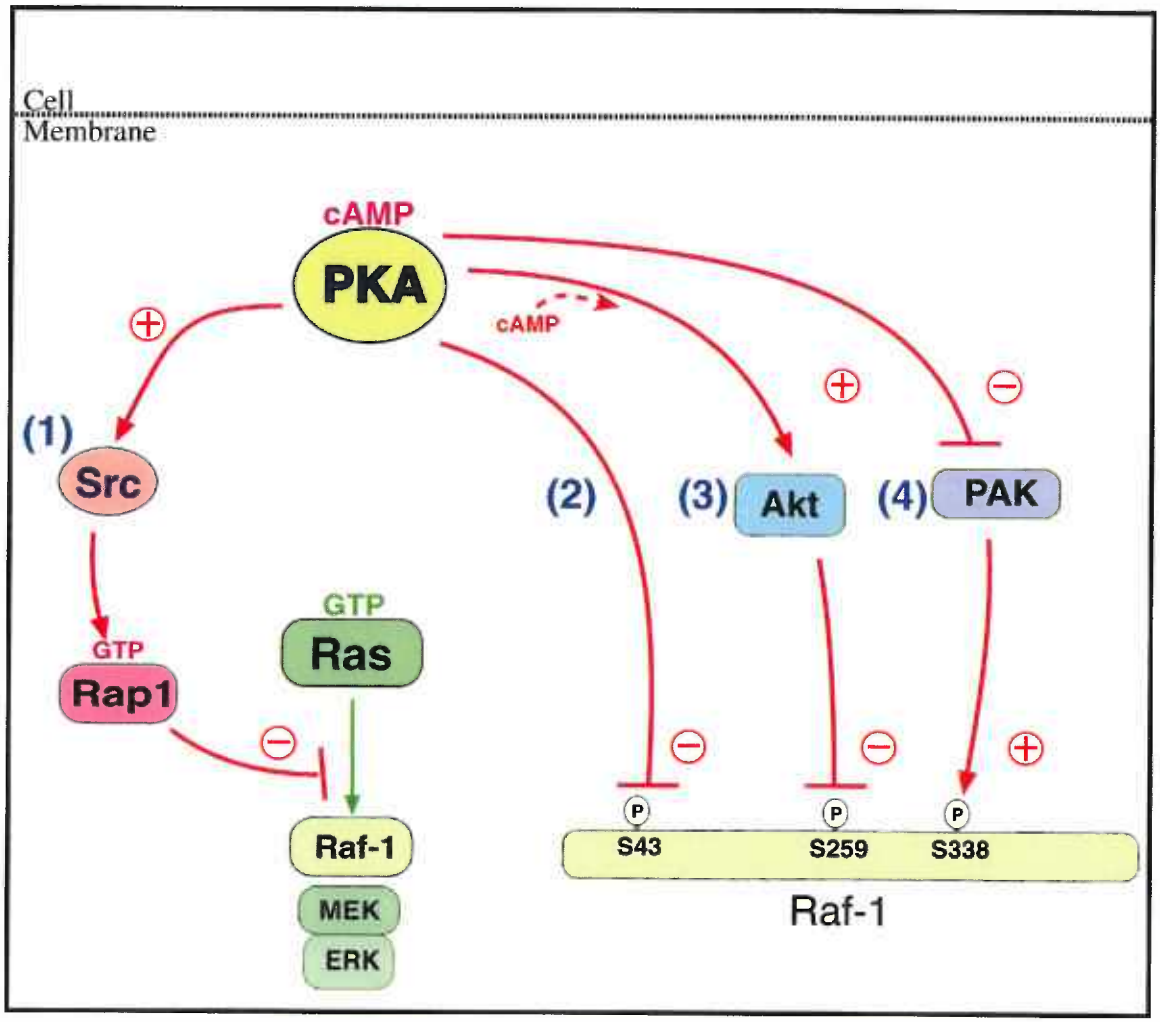

Figure 1.3. **Mechanisms of cAMP/PKA inhibition of ERK activation.** (1) cAMP can activate Rap1, to antagonize Ras signaling to Raf-1. cAMP activation of PKA activates Rap1 via a Src-dependent pathway. (2) PKA may also inhibit Raf-1 via phosphorylations on Raf-1. PKA phosphorylation of serine 43 can inhibit Raf-1's ability to bind to GTP-loaded Ras. (3) cAMP/PKA may also inhibit Raf-1 by activating the serine/threonine kinase AKT. AKT phosphorylation of Raf-1 at serine 259 blocks Raf-1 activation. (4) PKA can also interfere with the activation of Raf-1 by the serine/threonine kinase PAK. Following recruitment to Ras, Raf-1 requires phosphorylation at serine 338 by a putative PAK kinase to be fully activated. PKA may inhibit PAK activity by direct phosphorylation, thereby inhibiting Raf-1 activation by Ras.

cAMP and cell proliferation/differentiation

Hormones coupled to cAMP have also been shown to stimulate ERKs and cell growth (Ariga et al., 2000; Iacovelli et al., 2001). To understand cAMP's cell-type specific effects it is important to determine whether the signaling mechanisms mediating cAMP's anti-proliferative effects are similar to those mediating cAMP's proliferative effects. We will also describe a diverse group of cells for which cAMP activates ERKs and cell growth. The ERK-dependence of cAMP's activation of differentiation will also be discussed.

cAMP activates ERKs and cell proliferation

cAMP not only inhibits cell growth but stimulates cell growth as well. Many of the cell types in which cAMP stimulates proliferation belong to the endocrine system of tissues. In these cells, proliferation is induced by hormones and GPCRs coupled to Gαs and cAMP production. For prostate cells, cAMP's effects can be synergistic with growth factors (Chen et al., 1999) and cytokines (Deeble et al., 2001). Additional examples include, TSH stimulation in thyroid cells (Ariga et al., 2000; Kimura et al., 2001), GHRH in somatotrophs (Mayo et al., 2000), VIP and PACAP in lacto-somatotrophs (Le Pechon-Vallee et al., 2000), FSH in Sertoli cells (Crepieux et al., 2001), and VIP and CRF in keratinocytes (Mitsuma et al., 2001). In many cases, ERK signaling is required for cAMP's proliferative effects including, brown fat (Lindquist and Rehnmark, 1998), preadipocytes (Yarwood et al., 1996), pituitary (Le Pechon-Vallee et al., 2000), Sertoli cells (Crepieux et al., 2001), and kidney (Yamaguchi et al., 2000). Interestingly, polycystic epithelium is also stimulated by cAMP. A role for ERKs in this action of

cAMP has also been proposed (Hanaoka and Guggino, 2000; Sutters et al., 2001; Yamaguchi et al., 2000). Clearly cAMP and ERKs play an important role in stimulating cell proliferation. It is interesting to speculate that Rap1 may also play a role in mediating cAMP's proliferative effects in certain cell types, although an oncogenic role for Rap1 has not been reported.

cAMP-dependent cell differentiation can be triggered by PKA and ERK

cAMP activation of ERKs has been associated with both proliferation and differentiation, and the ERK cascade can mediate both proliferation and differentiation within the same cell (Vossler et al., 1997). This phenomenon has been demonstrated in the neuronal-like cell line, PC12, where transient activation of ERKs by EGF triggers proliferation, while sustained activation of ERKs by NGF and FGF trigger differentiation (York et al., 1998). In PC12 cells, differentiation by cAMP requires sustained ERK activation (Okumura et al., 1994; Vossler et al., 1997).

The ERK-dependence of cAMP-induced cellular differentiation has been well studied in neuronal cells lines. For example, cAMP stimulation can differentiate pluripotent embryonic carcinoma cells along a neuronal lineage (Sharma et al., 1990) as well as induce differentiation of immortalized neurons (Cibelli et al., 2001). Interestingly, cAMP can also potentiate NGF's stimulation of ERKs and PC12 differentiation (Calleja et al., 1997). Neuronal differentiation is evident by the cellular phenotypes of increased neuronal activity, depolarization induce changes in gene expression, and increased synaptic plasticity (Martin et al., 1997). Interestingly, some of these differentiated phenotypes of neurons have been shown to require both PKA and ERK activities (Grewal

et al., 2000b; Vincent et al., 1998; Waltereit et al., 2001; Winder et al., 1999). Differentiation of endocrine-responsive prostatic tumor cells by cAMP also requires ERKs (Deeble et al., 2001). A number of other cell types can also be differentiated by cAMP stimulation through PKA and ERK including, cardiac myocytes, where isoproterenol induces cellular hypertrophy (Zou et al., 1999). VIP stimulation of retinal pigment epithelial cells also results in melanogenesis and regulates fluid transport capacity (Koh, 2000). Interestingly, the role for Src in the cellular differentiation of retinal pigment epithelial cells by cAMP was suggested. An additional example can be seen in granulosa cells, where cAMP stimulates steroidogenesis and requires both PKA and ERK (Seger et al., 2001).

One aspect of the cAMP-mediated differentiation phenotype is the induction of specific genes. cAMP activates gene expression via the binding of phosphorylated CREB to cAMP-responsive elements (CREs) within the promoters of specific genes. In neurons, activation of CRE-containing genes mediates both neurotrophic and differentiation functions (Ahn et al., 1998; Riccio et al., 1999). Interestingly, a requirement for ERKs has been identified in PKA dependent-activation of CREB transcription, downstream of CREB phosphorylation (Grewal et al., 2000b). Similar requirements for ERKs have been shown for cAMP induction of several genes including, the dopamine beta-hydroxylate gene in PC12 cells (Swanson et al., 1998) and the myelin basic protein gene (Clark et al., 1998).

Mechanisms by which cAMP stimulates ERK signaling

Rap1 activation of B-Raf

cAMP's activation of ERKs was originally described in PC12 cells (Frodin et al., 1994). These and other studies identified the target of cAMP stimulation to be upstream of MEK and ERKs. Careful examination of the signaling pathway from cAMP to ERKs revealed that cAMP's effects might be Ras-independent. This was confirmed by studies examining the regulation of ERKs by parathyroid hormone (PTH) and cAMP in Chinese hamster ovary cells (Verheijen and Defize, 1997) and by Forskolin stimulation of cAMP in PC12 cells (Vossler et al., 1997). In both systems, Ras-independent activation of ERKs by cAMP was demonstrated by the absence of inhibition on ERKs by the interfering mutant of Ras, RasN17. A requirement for Rap1 in cAMP's activation of ERKs was demonstrated using interfering mutants of Rap1 (Vossler et al., 1997), and validated using a genetic approach (Wan and Huang, 1998).

Despite Rap1's ability to inhibit Ras/Raf-1 signaling to ERKs, work presented in this thesis and elsewhere demonstrate that Rap1 activates ERKs in several cell types (Faure and Bourne, 1995; Schmitt and Stork, 2000; Vossler et al., 1997). This is because Rap1 can activate ERKs via B-Raf (Schmitt and Stork, 2000; Vossler et al., 1997). This is depicted in Figure 1.2B. B-Raf is a member of the Raf family of MAPKKKs that is highly expressed in the brain (Morice et al., 1999) and other tissues (Barnier et al., 1995). It is highly homologous to Raf-1 within both its kinase and Ras-binding domains, and, like Raf-1, has only one known substrate: the MAPKK, MEK.

Rap1 activation of B-Raf was first demonstrated *in vitro*, using B-Raf purified from brain extracts (Ohtsuka et al., 1996) and was shown to be dependent on Rap1 activation (Liao et al., 2001; York et al., 2000). Recently, a role for the B-Raf binding partner 14-3-3 in Rap1 activation of B-Raf by cAMP has also been demonstrated (Qiu et

al., 2000). Furthermore, studies in B-Raf-expressing cells have shown that cAMP activation of ERKs requires Rap1 and B-Raf (Dugan et al., 1999; Schmitt and Stork, 2000; Zanassi et al., 2001). Rap1's activation of B-Raf and ERKs has been shown in multiple cell types including PC12 cells (Vossler et al., 1997), neurons (Zanassi et al., 2001), cortical astrocytes (Schinelli et al., 2001), testes (Berruti, 2000), and Hek293 cells (Schmitt and Stork, 2000).

The physiological consequences of the PKA/Rap1/B-Raf pathway is dependent on cell type. For example, PKA/Rap1/B-Raf is utilized by certain cells to regulate proliferation (Chen et al., 1999), and by other cells to regulate differentiation (York et al., 1998). Rap1/B-Raf signaling may also be involved in specific pathophysiological responses such as susceptibility to infection (de Magalhaes et al., 2001; Wessler et al., 2002) and cancer (Yamaguchi et al., 2000). It is interesting to speculate that in situations where ERKs are oncogenic the PKA/Rap1/B-Raf pathway may play a key role.

cAMP activation of ERKs is regulated by B-Raf expression

cAMP's activation of Rap1 may provide a model for cAMP's cell-type specific actions. Depending on the cell-type, active Rap1 may stimulate B-Raf/ERK and/or inhibit Raf-1/ERK. In this model, the consequence of cAMP's action on ERKs depends on B-Raf expression (see Figure 1.2). Work presented here suggests that concurrent activation of B-Raf and inhibition of Raf-1 by both cAMP and Rap1 occurs in the same cells (see Figure 1.2B). For example, in Hek293 cells, which express B-Raf, Rap1 activation by either isoproterenol or cAMP antagonizes Ras activation of Raf-1 but activates ERKs via B-Raf. The consequence of isoproterenol/cAMP stimulation in these cells is the

activation of ERKs via a Rap1-dependent, but Ras-independent pathway (Schmitt and Stork, 2000). This dual action of cAMP is also seen in purinergic neurons, where PKA activates B-Raf while inhibiting Raf-1 (Gao et al., 1999). Other groups have confirmed similar effects in PC12 cells where adenosine stimulation of cAMP inhibits the early phase of ERK activation by NGF but enhances the late effects (Arslan and Fredholm, 2000).

In B-Raf-negative cells, like NIH3T3 cells and glial cells, cAMP activation of Rap1 inhibits growth factor activation of ERKs and cell growth (Dugan et al., 1999; Schmitt and Stork, 2001). Transfection of B-Raf into these cells converts Rap1 into an activator of ERKs (Dugan et al., 1999; Vossler et al., 1997). Surprisingly, expression of B-Raf also converts integrin signaling from inhibition to activation of ERKs and a Rap1-dependent mechanism has been proposed (Barberis et al., 2000). One important area for future study is to determine whether changes in B-Raf expression levels account for the reversal of cAMP regulation of ERKs and cell growth in physiological systems.

Role of Src in PKA activation of ERKs via Rap1/B-Raf

The requirement of PKA for cAMP's activation of Rap1 in many studies has confirmed the importance of PKA in Rap1 activation (Altschuler et al., 1995; Schmitt and Stork, 2000; Schmitt and Stork, 2001). We have recently revealed, a pathway from PKA to Rap1 through Src activation in fibroblasts (see Figure 1.3). These cells do not express B-Raf and Rap1 blocks Ras activation of Raf-1 and ERKs (Schmitt and Stork, 2002). It is possible that Src also mediates PKA's activation of Rap1 in B-Raf-positive cells as well (see Figure 1.4). Previous studies have proposed a requirement for Src family kinases in

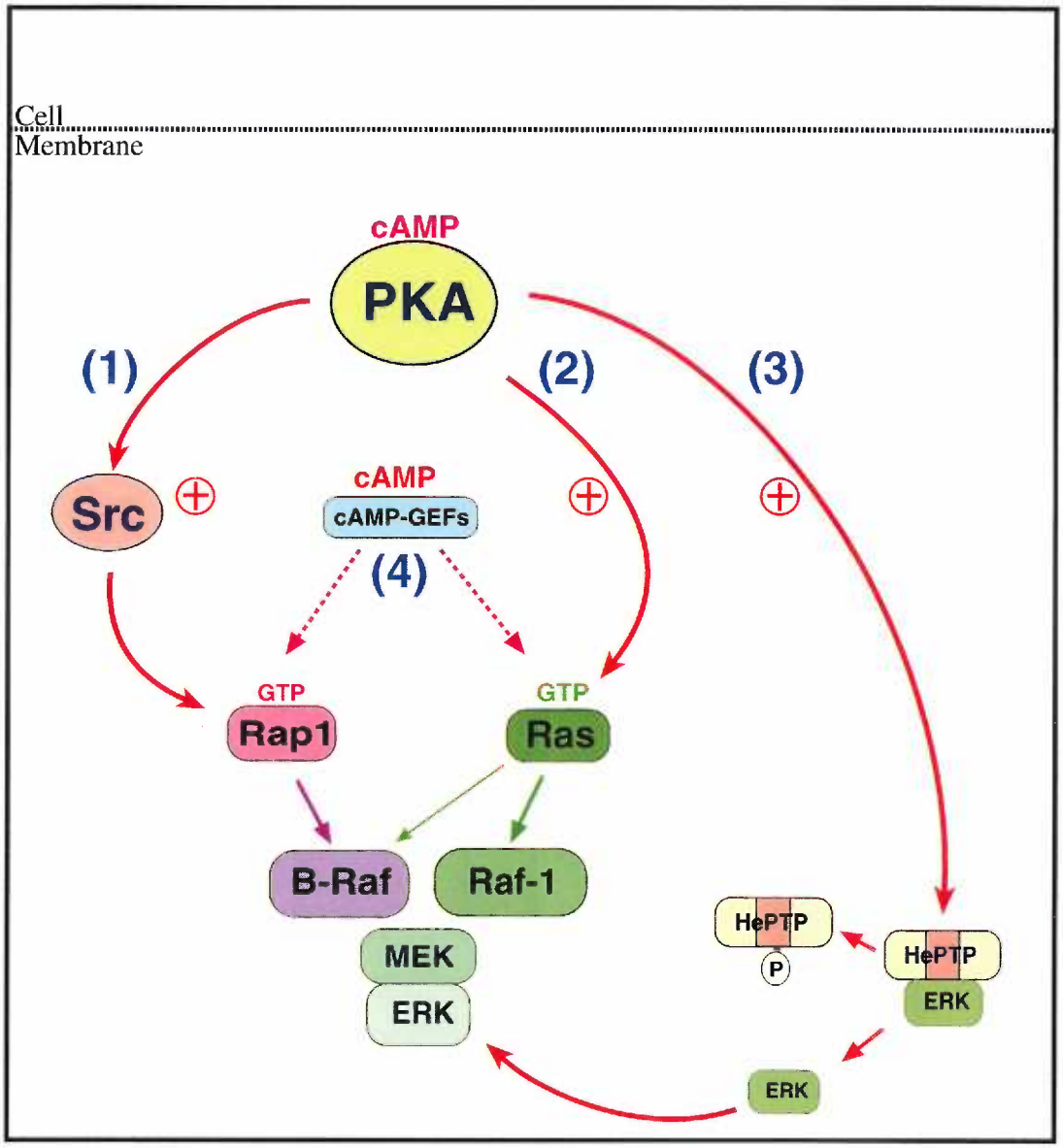

Figure 1.4. **Mechanisms by which cAMP/PKA can activate ERKs.**
(1) In B-Raf-expressing cells, hormonal stimulation of PKA may activate ERKs via the Src-dependent activation of Rap1, which can activate ERKs through B-Raf. (2) PKA may also stimulate Ras activation in response to G protein-coupled receptor stimulation which results in ERK activation through Ras activation of either B-Raf or Raf-1. (3) PKA may activate ERKs by phosphorylating the ERK phosphatase, HePTP, which releases ERK from inhibition by the phosphatase. (4) cAMP may also activate Rap1 and Ras through PKA-independent pathways involving cAMP-GEFs.

PKA's activation of ERKs which may reflect specific examples of the use of the PKA/Src/Rap1 pathway (Crepieux et al., 2001; Gentili et al., 2001; Kobierski et al., 1999; Lindquist et al., 2000). For example, VIP stimulation of retinal pigment epithelia requires a pathway through cAMP and Src (Koh, 2000). It is also possible that both NGF and norepinephrine actions on PC12 cells may also utilize signals from Src to Rap1 and ERKs (D'Arcangelo and Halegoua, 1993; Zhong and Minneman, 1999). Signaling via β-adrenergic receptors to ERKs has also been shown to require PKA, Src (Cao et al., 2000; Daaka et al., 1997; Lindquist et al., 2000), and Rap1 (Schmitt and Stork, 2000). It should be noted that in some cases Src has been shown to play a proliferative role independent of ERK activation (Angel Fresno Vara et al., 2001). Selective pharmacological inhibitors of Src family kinases (Hanke et al., 1996) make it possible to test the requirement of Src in cAMP's regulation of ERK and cell growth in these and other systems.

cAMP can activate Rap1 in the absence of PKA activity

Work from this thesis suggests that cAMP exerts most of its effects through PKA however, PKA-independent actions of cAMP may also exist, including the activation of Rap1. In dog thyroid cells, Rap1 is activated by cAMP via both PKA-dependent and PKA-independent mechanisms (Dremier et al., 2000). Rap1 activation by TSH and cAMP, in rat thyroid cells, does not require PKA (Tsygankova et al., 2001). Studies in leukemic cells have also identified a PKA-independent activation of Rap1 (von Lintig et al., 2000). The recent identification of a family of cAMP-binding proteins that are guanine nucleotide exchange factors (GEFs) for Rap1, Rap2 and Ras, have opened the door for cAMP's actions on Rap1, Ras, and cell growth. These GEFs, called cAMP-

GEFI and II (Kawasaki et al., 1998a) (or Epac1 and 2 (de Rooij et al., 1998)), show increased GEF activity towards Rap1, and the related Rap2 (Schmidt et al., 2001a), upon their binding to cAMP (Kraemer et al., 2001). In addition, a Ras GEF called CnrasGEF can also be activated by cAMP (Pham et al., 2000). These, or related GEFs, may play a role in the cAMP-dependent, PKA-independent activation of small G proteins in thyroid cells (Tsygankova et al., 2000) (see Figure 1.4). It is possible to speculate that these GEFs may also play a role in cAMP-stimulated ERK-dependent cell growth and differentiation. These GEFs appear to be expressed in a cell-type specific manner and may represent a growing family of GEFs that have yet to be discovered. However, the physiologic role of these GEFs has yet to be determined.

cAMP activation of ERKs can require Ras

As suggested above, Ras may also be a target of cAMP signaling to ERKs in certain cell types and depending on the stimulus (see Figure 1.4). β-adrenergic receptor signaling has been shown to involve a PKA-dependent switch of β-adrenergic receptor coupling from Gαs to Gαi, and subsequent activation of Ras via the βγ subunits of Gi (Daaka et al., 1997). Ras activation following GPCR stimulation utilizes signals generated from the βγ subunits of the heterotrimeric G proteins (Crespo et al., 1994; Lopez-Llasaca et al., 1997). Interestingly, these studies have also identified a role for Gαs and PKA signaling in ERK activation (Daaka et al., 1997).

Ras activation by this pathway can proceed simultaneously with PKA's activation of Rap1 (Schmitt and Stork, 2000). For example, in Hek293 cells the β-adrenergic receptor can activate Rap1 and ERKs through Gαs /cAMP, and can activate Ras through

βγ (Schmitt and Stork, 2000). Interestingly, Rap1's activation appears to block the ability of Ras to bind to Raf-1 and activate ERKS. In COS-7 cells, isoproterenol has also been shown to utilize both Gαs and Gβγ to regulate ERK signaling. In these cells, the β-adrenergic receptor can inhibit ERKs via cAMP (possibly via Rap1), but can activate ERKs via βγ (Crespo et al., 1995).

It appears as though in some neuronal cells B-Raf is be the major Raf isoform activated by Ras (Jaiswal et al., 1994). In these cells, cAMP's activation of ERKs requires both Ras and B-Raf but not Rap-1. Interestingly, PKA was not required for cAMP's activation of Ras (Busca et al., 2000). In contrast, Ras has also been shown to be activated by cAMP in cortical neurons, in a PKA-dependent manner (Ambrosini et al., 2000). The above data suggest that Ras can be activated by cAMP however, the ability of cAMP to activate Ras appears to be cell-type and stimulus specific and will need further examination.

PKA can activate ERKs through additional mechanisms

The primary mechanisms that have been proposed to explain how cAMP can activate ERKs have focused on Rap1 or Ras activation lying upstream of ERK. However, there are reports of cAMP's activation of ERKs being independent of MEK (Lee and Esselman, 2001). Several possibilities exist including, decreasing ERK phosphatase activity or through an indirect pathway.

One mechanism that can account for PKA-dependent and G protein-independent activation of ERK is through the inhibition of downstream ERK phosphatases. Two studies examining PKA's inhibition of potential ERK directed PTPases, including HePTP

(Saxena et al., 1999), PTP-SL and STEP (Blanco-Aparicio et al., 1999). The hematopoietic protein tyrosine phosphatase HePTP was negatively regulated by PKA, by direct phosphorylation within the kinase interaction motif (KIM) (Saxena et al., 1999). Release of ERKs from HePTP upon PKA phosphorylation was associated with increased ERK activity (see Figure 1.4). This mechanism is similar to that proposed for protein tyrosine phosphatases PTP-SL and STEP (Blanco-Aparicio et al., 1999).

Recent studies in neurons have also suggested an indirect role for cAMP/PKA in ERK activation at the Schaffer collateral-CA1 synapse within the hippocampus. In these cells cAMP was shown to induce rapid activation of BDNF/TrkB signals (Patterson et al., 2001). Interestingly, although ERK activation by Forskolin was independent of BDNF, the nuclear localization of ERKs required BDNF signaling (Patterson et al., 2001). Thus, additional pathways exist for PKA to activate ERKs independent of those discussed in previous sections.

Conclusions

One important aspect of this thesis is that cAMP regulation of the ERK cascade provides an important cross talk between hormones and growth factor signaling. The cell-type specificity of cAMP's actions correlates with that of Rap1 activation. The specificity is determined by the effects of both Rap1's actions on Raf-1 and B-Raf (see Figure 1.2). Therefore, cAMP's activation of Rap1 can account for both cAMP's inhibition and activation of ERKs. Indeed, work presented in this thesis will suggest that cAMP and Rap1 can activate ERKs in cells that express B-Raf but not in cells that lack B-Raf. Because cAMP can activate Rap1 in a variety of cells, it will be important to examine the

expression of B-Raf when evaluating the mechanism of action of cAMP in specific cell types. It will also be important to examine the ability of Src to carry a stimulatory signal from PKA to ERKs through Rap1 and to examine the role of Src in developmental or physiological models of cell growth.

THESIS AIMS

The major focus of this thesis is to test the following:

1) Does stimulation of endogenous β-adrenergic receptors utilize a Rap1/B-Raf pathway to activate ERKs?

As discussed, stimulation of cells with the β-adrenergic receptor agonist, isoproterenol, may use a cAMP/PKA- and Ras-dependent mechanism to activate ERKs. Based on previous results from our lab and others, cAMP/PKA may also activate ERKs via Rap1 in B-Raf expressing cells. This hypothesis will be tested in Hek293 cells which express both endogenous β_2-adrenergic receptors as well as B-Raf.

2) Is Rap1 required for cAMP/PKA's ability to inhibit ERKs and cell growth?

cAMP/PKA has been shown to inhibit ERK activation and cell growth and proliferation in NIH3T3 fibroblasts. Rap1 is activated by cAMP/PKA and can antagonize ERK activation by Ras. Therefore, the possibility that Rap1 may mediate cAMP/PKA's inhibitory effects on ERKs and cell growth will be examined in NIH3T3 cells.

3) What is the mechanism for PKA's activation of Rap1?

PKA activates Rap1 to inhibit ERKs and cell growth in fibroblasts. Previous work suggests that PKA activates Rap1 indirectly via the guanine nucleotide exchange factor, C3G. C3G associates with Crk-L and Cbl following Cbl's tyrosine phosphorylation. It is possible that PKA may activate the tyrosine kinase, Src, to activate C3G/Crk-L/Cbl. Depending on the cell-type, Src activation by PKA may lead to inhibition of ERKs and cell proliferation. Therefore, the role of these molecules in Rap1 activation and inhibition of ERKs and cell growth will be examined in NIH3T3 cells. Parallel studies will be carried out in mouse embryonic fibroblasts derived from mice expressing only wild type endogenous Src (Src++) or mice deficient of all Src family kinases (SYF).

CHAPTER TWO

β_2-adrenergic Receptor Activates Extracellular Regulated Kinases (ERKs) via the Small G Protein Rap1 and the Serine/Threonine Kinase B-Raf

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ABSTRACT

G protein-coupled receptors can induce cellular proliferation by stimulating the MAP kinase cascade. Heterotrimeric G proteins are composed of both α and $\beta\gamma$ subunits that can signal independently to diverse intracellular signaling pathways including those that activate MAP kinases. In this study, we examined the ability of isoproterenol, an agonist of the β_2-adrenergic receptor (β_2AR), to stimulate ERKs. Using Hek293 cells, which express endogenous β_2AR, we show that isoproterenol stimulates ERKs via β_2AR. This action of isoproterenol requires PKA and is insensitive to pertussis toxin (PTx) suggesting that Gsα activation of PKA is required. Interestingly, β_2AR activates both the small G proteins Rap1 and Ras, but only Rap1 is capable of coupling to Raf isoforms. β_2AR inhibits the Ras-dependent activation of both Raf isoforms Raf-1 and B-Raf, whereas Rap1 activation by isoproterenol recruits and activates B-Raf. β_2AR's activation of ERKs is not blocked by expression of RasN17, an interfering mutant of Ras, but is blocked by expression of either RapN17 or Rap1GAP1, both of which interfere with Rap1 signaling. We propose that isoproterenol can activate ERKs via Rap1 and B-Raf in these cells.

INTRODUCTION

Cell proliferation is regulated by extracellular signals including growth factors and hormones. Growth factors activate receptor tyrosine kinases (RTKs) to stimulate a number of intracellular signaling cascades. One cascade, the MAP kinase (or ERK) cascade triggers cellular proliferation through multiple mechanisms including inducing stimulation of progression through the G1/S transition of the cell cycle and by activating rate-limiting proteins involved in both DNA and protein synthesis (Graves et al., 2000; Whitmarsh and Davis, 2000). ERKs are activated in cancerous cells through the action of proto-oncogenes like Ras that lie upstream of the MAP kinase cascade. Hormones can also activate the MAP kinase cascade to stimulate proliferation in many cell types (Dhanasekaran et al., 1995). Some hormones, like insulin, act like growth factors to activate RTKs to stimulate intracellular cascades leading to ERK (Avruch, 1998; Boulton et al., 1991). However, most hormones act via serpentine (or seven-transmembrane receptors), and couple to heterotrimeric GTP binding proteins (G proteins) to elicit their effects (Bourne, 1997; Ginell and Brown, 1996).

Heterotrimeric G proteins are composed of two functional units, an alpha (α) subunit and a beta-gamma ($\beta\gamma$) subunit. Both α and $\beta\gamma$ are released from hormone receptors upon ligand binding and can directly bind to and activate specific effectors. For α, one of these effectors is adenylate cyclase. Historically α subunits that stimulate adenylate cyclase are called $\alpha\underline{s}$ for stimulatory, while those that inhibit adenylate cyclase are termed $\alpha\underline{i}$, for inhibitory. Over the past five years, cross-talk between G protein-coupled signaling pathways have been identified for many G protein-coupled receptors (Dhanasekaran et

al., 1995; Sugden and Clerk, 1997). The activation of MAP kinase cascades has been established for G proteins of diverse classes, including Gs, Gi, and Gq (Budd et al., 1999; van Biesen et al., 1996; Vossler et al., 1997). For some of these, direct or indirect involvement of cytoplasmic tyrosine kinases has been shown (Florio et al., 1999; Lev et al., 1995; Luttrell et al., 1996; Tang et al., 1999; Wan et al., 1996). For others, association with regulatory molecules like RasGAP (Jiang et al., 1998) or Rap1GAP1 (Jordan et al., 1999; Mochizuki et al., 1999) provides the cross-talk necessary to modulate signals to the small G proteins Ras or Rap1, respectively, to regulate the MAP kinase cascade.

Perhaps the best studied mechanism of cross-talk between G proteins and the MAP kinase cascade involves the $\beta\gamma$ subunit of heterotrimeric G proteins. Activation of both Gq- and Gi-coupled receptors releases $\beta\gamma$ to activate the tyrosine kinase c-src which can activate Ras via the phosphorylation of the adaptor molecule Shc, which then recruits a complex consisting of Grb2 and SOS, the Ras-specific guanine nucleotide exchange factor (GEF), to the membrane where it can activate Ras (Hawes et al., 1996). In some cases, a role for PI3-Kγ in src activation has been shown (Lopez-Llasaca et al., 1997). In other cases, src is activated by a calcium-sensitive kinase PYK2 (Lev et al., 1995). Despite variations on the mechanisms used, all examples of $\beta\gamma$ signaling to ERKs require Ras activation.

Recently, the α subunits of heterotrimeric G proteins have also been shown to signal to the MAP kinase cascade. The α subunits of Gi and Go (which share extensive sequence

homology and PTx-sensitivity) both bind to Rap1GAP1, a GTPase activating protein specific for a distinct small G protein Rap1 (Mochizuki et al., 1999). Rap1 is a cell type-specific antagonist of Ras-dependent signaling and its inhibition by Rap1GAP1 can allow Ras to signal effectively to ERKs. The α subunit of Gs has also been implicated in MAP kinase activation. For example, constitutively activated mutants of Gsα are oncogenic (Faure et al., 1994; Landis et al., 1989; Lyons et al., 1990; Zachary et al., 1990). These mutants encode an oncogene called Gsp that can activate ERKs when expressed in transfected cells. Activated Gsα triggers the synthesis of the second messenger cAMP through direct association with specific adenylate cyclases (Masters et al., 1988; Pieroni et al., 1993). The major target of cAMP is the cAMP-dependent protein kinase PKA (Beavo et al., 1975; Butcher et al., 1968). PKA has cell type-specific actions on MAP kinase signaling. In many cell types, PKA antagonizes Ras-dependent activation of Raf-1, an ubiquitously expressed MAP kinase kinase kinase (Cook and McCormick, 1993; Graves et al., 1993; Sevetson et al., 1993; Wu et al., 1993) to inhibit cellular proliferation and Ras-dependent transformation (Chen and Iyengar, 1994). In other cell types, PKA can activate MAP kinase through a distinct pathway involving Rap1 and a cell type-specific isoform of Raf called B-Raf (Dugan et al., 1997; Vossler et al., 1997; Wan and Huang, 1998). Recently, a second enzyme target for cAMP, cAMP-GEF (or Epac), was identified as a Rap1-specific GEF (de Rooij et al., 1998; Kawasaki et al., 1998a). Therefore, in B-Raf-expressing cells, cAMP has at least two potential mechanisms to activate ERKs through Ras-independent pathways, one via PKA and another through direct activation of Rap1-GEFs.

The ability of hormones that couple to Gsα to activate Rap1 and ERKs has been examined in transfected cell lines over-expressing specific serpentine receptors. In CHO cells overexpressing the adenosine A_{2A}-receptor, adenosine has been shown to activate ERKs via Rap1 (Seidel et al., 1999). In Hek293 cells, a well-studied model of G protein coupling, over-expression of β_2-adrenergic receptor (β_2AR) was shown to couple to ERKs via a Ras-dependent pathway (Daaka et al., 1997; Della Rocca et al., 1997). The best studied receptor system coupled to Gsα is the β_2AR and its activation by the agonist isoproterenol. In this study, we examine the mechanism by which isoproterenol activates ERKs in Hek293 cells expressing endogenous levels of β_2AR.

EXPERIMENTAL PROCEDURES

Materials. Antibodies to Rap1, B-Raf, Raf-1, recombinant MEK-1 protein, and agarose-conjugated antibodies to ERK1, ERK2 (c-16), and myc-Erk were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Anti-Ras antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Phosphorylation-specific ERK antibodies (pERK) that recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2), at residues threonine 183 and tyrosine 185 were purchased from New England Biolabs (Beverly, MA). Isoproterenol, thrombin, carbachol, Flag (M2) antibody, and LPA were purchased from Sigma (St. Louis, MO). Forskolin, clonidine, PTx, alprenolol, atenolol, epidermal growth factor (EGF), AG1478, and N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89) were purchased from Cal Biochem (Riverside, CA). Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatswoth, CA.). Radioisotopes were from NEN-DuPont Life Science Products.

Cell culture. Hek293 cells were cultured in Dulbecco-Modified Eagle Medium (DMEM) plus 10% fetal calf serum at 37° C. in 5% CO_2. Cells were maintained in serum-free DMEM for 16 hours at 37° C. in 5% CO_2 prior to treatment with various reagents for both immune complex assays and western blotting. Cells pre-treated with PTx, (100 ng/ml) were incubated in serum-free media for 16 hours prior to stimulations. All inhibitors, unless otherwise indicated, were added to cells 20 minutes prior to treatment.

Western blotting. Cell lysates were prepared as described (Vossler et al., 1997). Cell lysate protein concentrations were quantified using Bradford protein assay. For detection of B-Raf, ERK2, myc-ERK, Rap1, Flag, Ras, and phospho-ERK1/2 (pERK), equal protein amounts of

cell lysate per treatment condition were resolved by SDS-PAGE, blotted onto PVDF (Millipore Corporation, Bedford, MA) membranes and probed with the corresponding antibodies according to the manufacturers guidelines.

Plasmids and Transfections. Seventy to eighty percent confluent Hek293 cells were co-transfected with the indicated cDNAs using a lipofectamine kit (Gibco BRL) according to the manufacturer's instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the same amount of DNA. Following transfection, cells were allowed to recover in serum containing media for 24 hr. Cells were then starved overnight in serum free DMEM before treatment and lysis.

Immune complex assays. For ERK assays, all cell treatments were lysed in ice-cold lysis buffer (50 mM Tris-HCL (pH 8.0), 10% glycerol, 1% nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). The lysates were centrifuged at low speed to remove nuclei and the supernatant was examined for ERK activity using myelin basic protein (MBP) as a substrate and [^{32}P]-γATP as a phosphate donor with equal protein amounts per assay condition as described (Vossler et al., 1997). For B-Raf assays, untreated and treated cells were lysed in ice-cold 1% NP-40 buffer containing 10 mM Tris pH 7.4, 5 mM EDTA, 50 mM NaCl, and 1 mM PMSF. Immune complex kinase assays were performed as described (Vossler et al., 1997) using MEK-1 as a substrate and [^{32}P]-γATP as a phosphate donor with equal protein amounts per assay. The reaction products of all kinase assays were

resolved by 10% SDS-polyacrylamide gel and analyzed with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Nickel affinity chromatography. Experiments utilizing polyhistidine-tagged Rap1 (His-Rap1 and His-RapV12) and Ras (His-Ras), were performed by transfecting Hek293 cells using lipofectamine reagent. Cells were lysed in ice-cold buffer containing 1% NP40, 10mM Tris, pH 8.0, 20 mM NaCl, 30 mM $MgCl_2$, 1mM PMSF, and 0.5mg/ml aprotinin. Supernatants were prepared by low speed centrifugation. Transfected His-tagged proteins were precipitated from supernatants containing equal amounts of protein using Ni-NTA Agarose and washed with 20mM imidazole in lysis buffer and eluted with 500 mM imidazole and 5mM EDTA in phosphate-buffered saline. One-half of the eluates containing His-tagged proteins were separated on SDS-PAGE and B-Raf or Raf-1 proteins were detected by western blotting (Vossler et al., 1997). The remaining His-Rap-1 eluates, of equal amounts, were immunoprecipitated with B-Raf antisera and B-Raf kinase activity was measured by immune complex assay. Equal amounts of His-Rap1 and His-Ras was confirmed by western blotting.

Affinity Assay for Rap1 Activation in Hek293 cells. GST fusion protein of the Rap1-binding domain of RalGDS was expressed in *Escherichia coli* following induction by isopropyl-1thio-b-D-galactopyranoside (GST-RalGDS was a gift from Dr. Bos, Utrecht University, The Netherlands to P. J. S. S.). Bacterial lysates were prepared and GST fusion proteins were immobilized by incubating lysates for 1 hour at 4° C. with glutathione-sepharose. Sepharose beads were washed three times in order to remove excess GST fusion protein. Hek293 cells were grown as described, and were stimulated at 37° C. for the indicated times and immediately

lysed in ice-cold lysis buffer (50 mM Tris-HCL (pH 8.0), 10% glycerol, 1% nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Active Rap1 was isolated using methods as described by Franke *et al.* (Franke et al., 1997a). Briefly, cell lysates were cleared by centrifugation, and equal amounts of supernatants were incubated with GST-RalGDS-Rap1 binding domain pre-coupled to glutathione beads. Following a 1 hour incubation at 4° C., beads were pelleted and rinsed threes times with ice-cold lysis buffer, protein was eluted from the beads using 2X Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane, blocked in 5% milk for 1 hour and, probed with α-Rap1/Krev-1 or Flag (M2) antibody overnight at 4° C., followed by an HRP-conjugated anti-rabbit secondary antibody. Proteins were detected using enhanced chemiluminescence.

Affinity Assay for Ras Activation in Hek293 cells. Hek293 cells were grown as described, and were stimulated at 37° for the indicated times and immediately lysed in ice-cold lysis buffer (50 mM Tris-HCL (pH 8.0), 10% glycerol, 1% nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Following the manufacturer's recommended protocol, activated Ras was isolated from stimulated lysates using agarose coupled GST-Raf1-RBD provided in the Ras Activation Assay Kit (Upstate Biotechnology, Lake Placid, N.Y.). Proteins were eluted from the beads using 2X Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane, blocked in 5% milk for 1 hour and, probed with α-Ras antibody overnight at 4° C., followed by an HRP-conjugated anti-mouse secondary antibody. Proteins were detected using enhanced chemiluminescence.

RESULTS

Isoproterenol activates ERK via endogenous β_2ARs - Isoproterenol treatment of Hek293 cells with the β-adrenergic agonist, isoproterenol, induces phosphorylation of MAP kinase ERK in a dose-dependent manner (Fig. 2.1A). Three minute stimulations with increasing concentrations of isoproterenol, revealed maximal ERK kinase activity at concentrations over 10 μM. Similar to previously published data, 10 μM isoproterenol induced endogenous ERK kinase activity maximally between 3 and 5 minutes (Fig. 2.1B) (Crespo et al., 1995). Isoproterenol-induced ERK kinase activation was completely blocked by pretreatment with the selective $\beta_{1,2}$-adrenergic antagonist alprenolol (Fig. 2.1C). Pretreatment with the selective β_1-adrenergic antagonist, atenolol, did not inhibit isoproterenol-mediated activation of MAP kinase. These results suggest that isoproterenol activates ERKs via endogenously expressed β_2ARs with maximal activation between 3 and 5 minutes.

β_2ARs mediate their intracellular signals via Gsα which, upon isoproterenol binding is released to activate adenylate cyclase. This results in the rapid elevation of intracellular cAMP levels and activation of the cAMP-dependent protein kinase PKA. To determine whether PKA plays a role in mediating endogenous ERK activation we utilized the selective PKA inhibitor H89 (Chijiwa et al., 1990). Pretreatment of serum-starved Hek293 cells with H89 completely eliminated the ability of isoproterenol to activate ERK kinase (Fig. 2.2). As a positive control, we treated cells with forskolin, an activator of adenylate cyclase. Forskolin activated ERKs and H89 abolished forskolin activation of ERKs (Fig. 2.2). Taken together, the above data demonstrate that isoproterenol activates

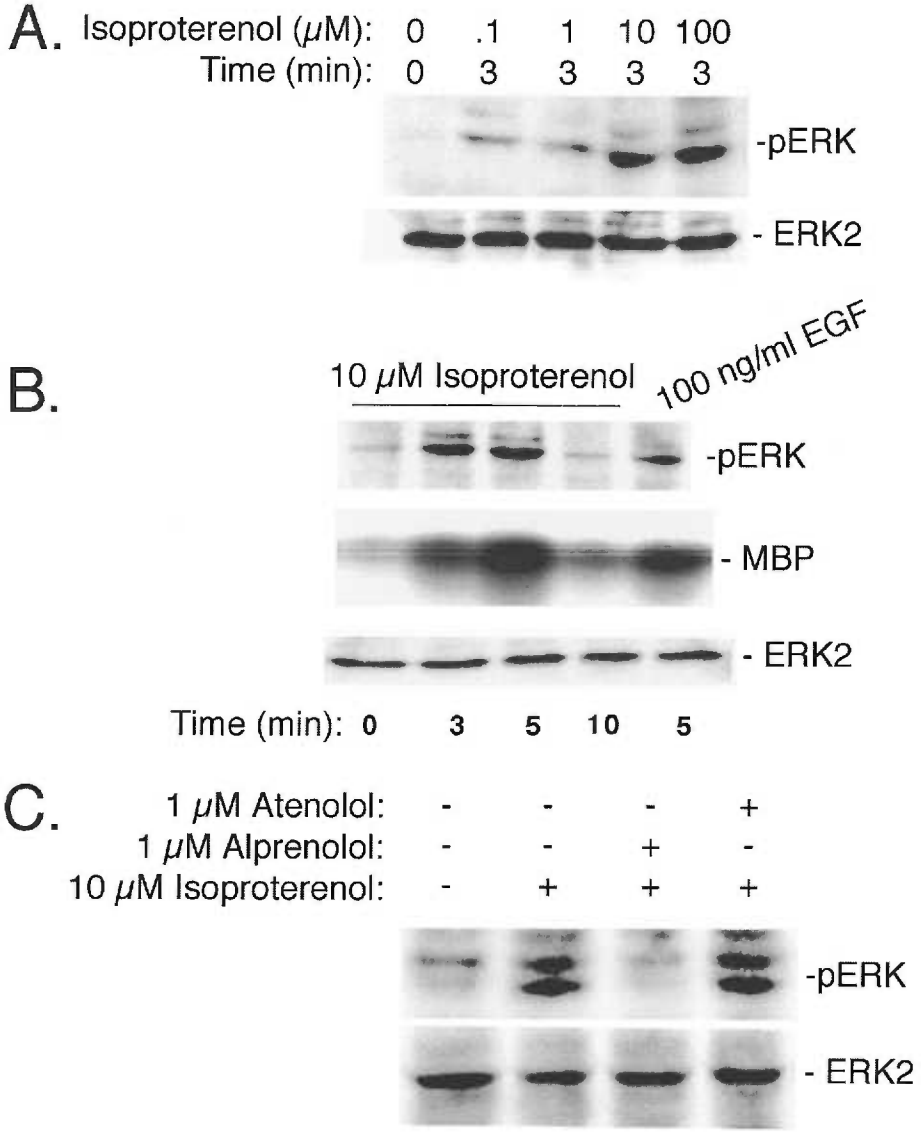

FIG. 2.1. **ERK activation by β2AR.** A, Isoproterenol dose-response of phosphorylated ERKs (pERK). Hek293 cells were serum-starved and treated with increasing concentrations of isoproterenol for 3 min. Cell lysates were prepared as detailed in the "experimental procedures". B, Time course of endogenous ERK activation following isoproterenol stimulation in Hek293 cells. Hek293 cells were harvested for either immune complex kinase assay using myelin basic protein (MBP) as a substrate or western blotting, using phospho-specific ERK1/2 (pERK) antibodies. Cells were treated with isoproterenol or EGF, as indicated. Upper panel: a representative western blot probed with pERK antibody. Middle panel: a representative autoradiogram with the position of MBP shown. Lower panel: western blotting showing equal loading of protein amounts within cell lysate was performed using ERK2 antibody. C, Blockade of isoproterenol stimulation of pERK. Serum-starved cells were treated with isoproterenol following a 10 minute pretreatment with either atenolol or alprenolol. Upper panel: a representative western blot probed for pERK antibody. Bottom panel: Equal amounts of protein were utilized as evidenced by the western blot probed with ERK2 antibody.

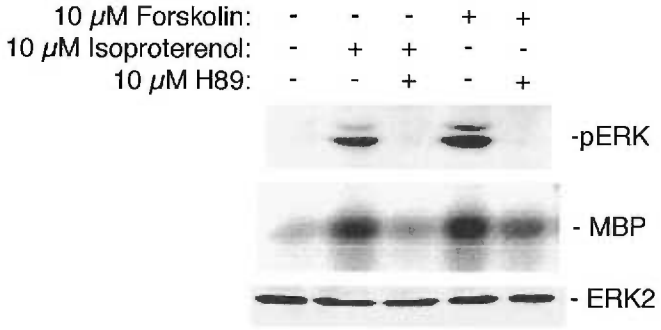

FIG. 2.2. **Endogenous β2-adrenergic receptors in Hek293 cells activate ERKs via PKA.** Serum-starved Hek293 cells were treated with isoproterenol for 3 minutes or forskolin for 5 minutes in the absence or presence of the PKA inhibitor H89 (10mM), as indicated. Cells were then lysed and equal protein amounts per-treatment condition were used for western blot with pERK or kinase assay using MBP as a substrate. A representative experiment showing both pERK (upper panel) and kinase activity (middle panel) is shown. The lower panel demonstrates equal protein levels as evidenced by western blot probed for ERK2.

endogenous signaling pathways which utilize both the β_2AR and the cAMP-dependent kinase PKA.

ERK activation by isoproterenol is insensitive to PTx treatment - Recent reports using Hek293 cells transiently transfected with cDNA encoding the β_2AR have shown that isoproterenol-induced activation of ERK was blocked by PTx (Daaka et al., 1997; Pierce et al., 2000). These data imply that ERK activation utilizes a Giα (or Goα) pathway to stimulate ERK activity. To investigate whether β_2AR can activate endogenous signaling pathways in the presence of PTx we pretreated Hek293 cells overnight with PTx and assessed the ability of isoproterenol to activate ERKs. In an extended time course measuring ERK activation by isoproterenol, no differences between PTx-treated cells and untreated cells were seen (Fig. 2.3A). ERK activation following treatment of Hek293 cells with both the muscarinic agonist carbachol (Fig. 2.3A) and lysophosphatidic acid (LPA, data not shown) was blocked by PTx, consistent with their ability to couple to Giα. To further confirm that the activation of ERKs by isoproterenol was insensitive to PTx, immune complex kinases assays were performed on endogenous ERK1/2. As can be seen in Fig. 2.3B, isoproterenol's activation of ERKs was not blocked by PTx. However, activation of ERKs by the α-adrenergic receptor agonist, clonidine, was blocked by PTx. As a negative control, we show that EGF-mediated activation of ERKs was not blocked by PTx (Fig. 2.3A). These results would indicate that β_2AR is able to activate endogenous ERKs via a Giα/Goα-independent pathway.

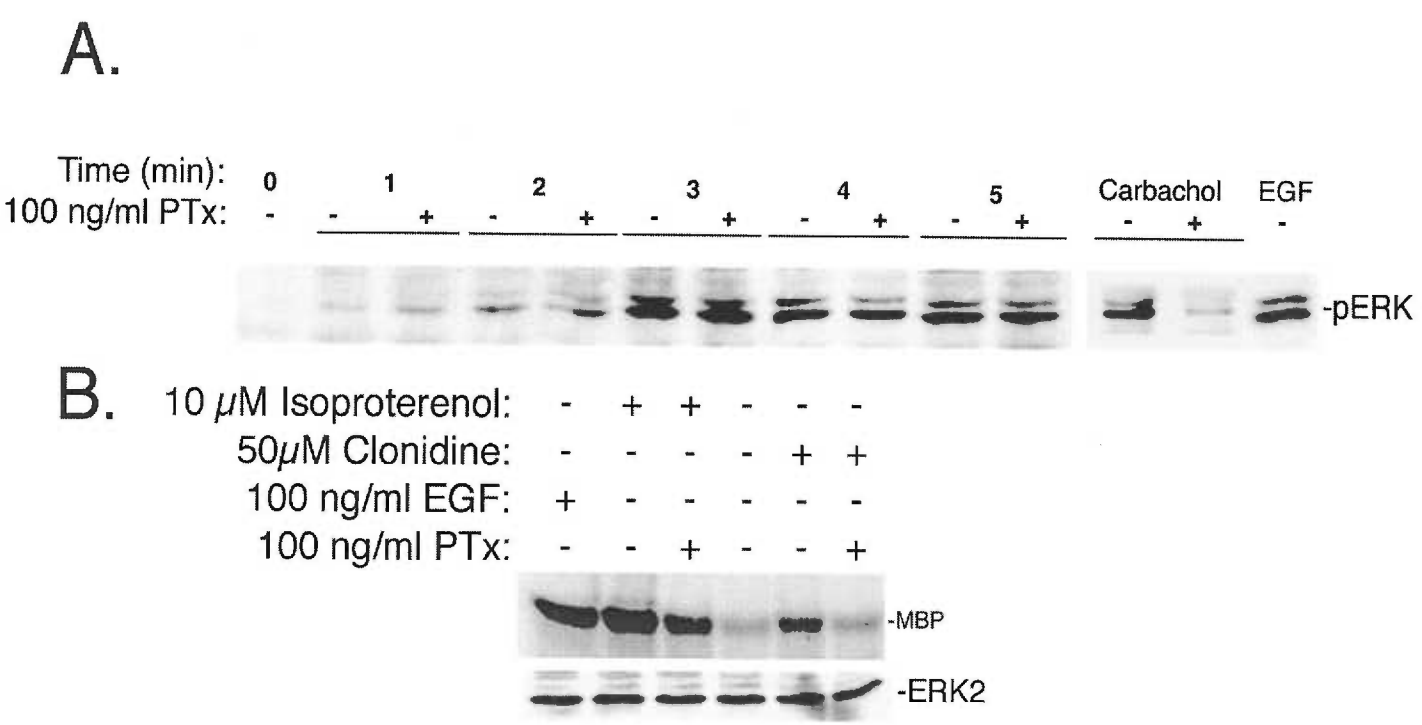

FIG. 2.3. **β2AR-mediated activation of ERKs via endogenous receptors is insensitive to PTx.** A, Hek293 cells were serum-starved and received either no pretreatment or pretreatment with 100 ng/ml PTx for 16 hours. Cells were then stimulated with 10 M isoproterenol for the indicated times. As negative and positive controls, respectively, Hek293 cells were also treated with 100 ng/ml EGF for 5 minutes and 10 M carbachol for 5 minutes in the presence or absence of PTx. Hek293 cells were lysed and equal amounts of protein were analyzed by western blotting with pERK antibody (upper panel). B, Hek293 cells were prepared similarly to those in panel A with PTx pre-treatment for 16 hours. Cells were then treated with 10 M isoproterenol for 3 minutes, 100 ng/ml EGF for 5 minutes and 50 M clonidine for 5 minutes. Cells were lysed and endogenous ERK1/2 were immunoprecipitated from equivalent amounts of protein using agarose-coupled ERK antibodies (as in Fig. 1B). A representative immune complex kinase assay with the location and phosphorylation of the MBP substrate is shown (upper panel). The lower panel represents a western blot identifying the levels of ERK2 to control for protein loading.

ERK activation by β_2AR requires Rap1- Recent studies have identified a role for Rap1 in signaling via G proteins (Jordan et al., 1999; Mochizuki et al., 1999; Vossler et al., 1997). Therefore, we sought to determine whether endogenous β_2AR stimulation by isoproterenol could activate Rap1. To determine whether Rap1 was activated in response to isoproterenol treatment we performed a time course of Rap1 activation. Endogenous Rap1 was activated at the earliest time point examined with maximal activation observed from 3 to 5 minutes, and a return to baseline by 20 minutes (Fig. 2.4A). As previously demonstrated, thrombin was also able to induce endogenous Rap1 activity in these cells (Seidel et al., 1999). To investigate the requirement for PKA in activating Rap1, cells were pretreated with H89. Pretreatment of Hek293 cells with 10 μM H89 blocked the ability of either forskolin or isoproterenol to activate Rap1 at 3 minutes (Fig. 2.4B). Taken together, these results would suggest that β_2AR activates Rap1 in a PKA-dependent manner. Recent studies have suggested that the guanine-nucleotide exchange factor, C3G, may play a role in activating Rap1 (Gotoh et al., 1995). C3G is constitutively associated with a member of the Crk adaptor family and is stabilized by its association with Crk-L (York et al., 1998). As can be seen in Fig. 2.4C, cotransfection of Flag-Rap1 along with Crk-L and C3G results in Rap1 activation in Hek293 cells as in other cell types (York et al., 1998). To determine whether C3G is playing a role in activating Rap1 in response to isoproterenol we used a truncated mutant of C3G containing the CRK-binding region, CBR, which interferes with CRK function (Gotoh et al., 1995; York et al., 1998). Transfection of CBR along with Flag-Rap1 blocked the ability of isoproterenol to activate Rap1 (Fig. 2.4C). To further confirm the role for PKA in activating Rap1 in response to isoproterenol we co-transfected the PKA-specific

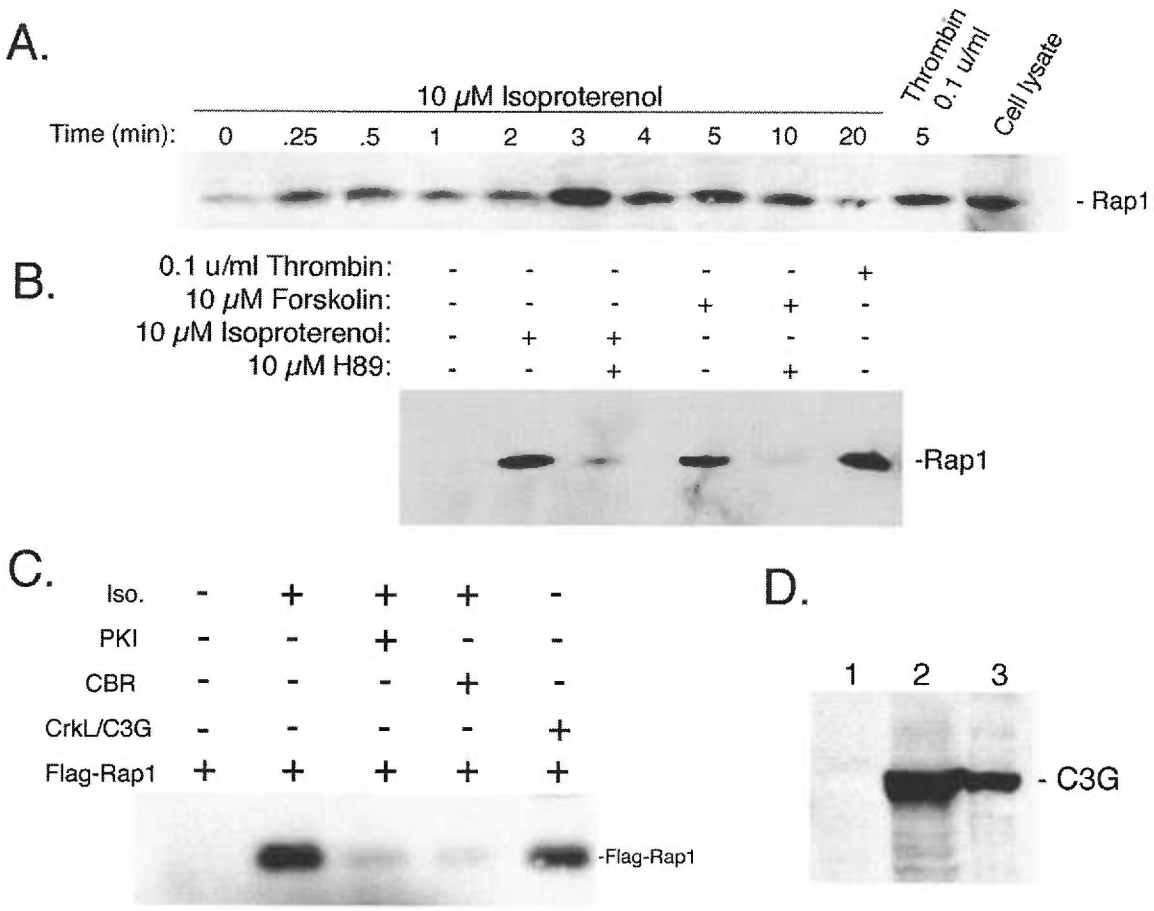

FIG. 2.4. **Isoproterenol activation of Rap1.** A, Time course of activation of Rap1 by isoproterenol. Serum-starved Hek293 cells were treated with 10 μM isoproterenol or 0.1u/ml thrombin for the indicated times. Equal amounts of cell lysate were incubated with precoupled GST-RalGDS protein, and analyzed by western blot for GTP loaded Rap1. Hek293 cell lysate was used to indicate the position of Rap1. B, Isoproterenol activation of Rap1 is sensitive to H89. Cells were stimulated with 10 μM isoproterenol for 3 minutes and 10 μM forskolin for 5 minutes, following a pretreatment with H89 (10mM), equal amounts of cell lysate were used to assay for GTP loaded Rap1. Thrombin was used as a control for Rap1 activation. C, Isoproterenol activation of Rap is sensitive to PKI and CBR. Hek293 cells were co-transfected with Flag-Rap1 and the indicated cDNAs, serum-starved, and stimulated with 10 μM isoproterenol for 3 minutes. Cells transfected with Crk-L/C3G were not stimulated. Equal amounts of cell lysate were incubated to assay for GTP loaded Rap1 using GST-RalGDS and a Flag (M2) antibody to identify Flag-Rap1 protein. D, Hek293 cells express C3G. Western blotting of equal amounts of protein were used to represent cell lysates from various cell types: COS 7 (lane 1), PC12 (lane 2), and Hek293 (lane 3).

inhibitory protein, PKI, which abolished the ability of isoproterenol to activate Rap1 (Fig. 2.4C). These results would suggest that Rap activation in response to β_2AR stimulation is PKA-dependent and also utilizes the guanine-nucleotide exchange factor C3G. Indeed, Hek293 cells express endogenous levels of C3G (Fig. 2.4D) indicating that the β_2AR may utilize C3G to activate Rap1.

Recent data have suggested that the small G protein Ras may play a role in mediating ERK activation by β_2AR (Daaka et al., 1997; Zou et al., 1999). To examine the ability of β_2AR to activate Ras we examined a time course of Ras activation. Similar to Rap1 activation, Ras appeared to be activated very early following isoproterenol stimulation and was inactive by 5 to 10 minutes (Fig. 2.5A). Hek293 cells were treated with EGF as a positive control for Ras activation. To determine whether Ras activation was PKA-dependent Hek293 cells were pretreated with H89 and stimulated with isoproterenol. H89 pretreatment had no effect on Ras activation (Fig. 2.5B), suggesting that Ras is activated by isoproterenol in a PKA-independent fashion. Consistent with this result, forskolin, did not activate Ras. Moreover, EGF stimulation of Ras was not blocked by H89 suggesting that H89's effect was specific for PKA. These data would indicate that Ras activation by β_2AR did not require cAMP or PKA and suggests that Gsα stimulation of adenylate cyclase was not directly involved in Ras activation.

Based on the finding that both Rap1 and Ras were rapidly activated in response to isoproterenol treatment, we next examined the role of these small G proteins in mediating ERK activation. Hek293 cells were transiently transfected with cDNAs encoding an

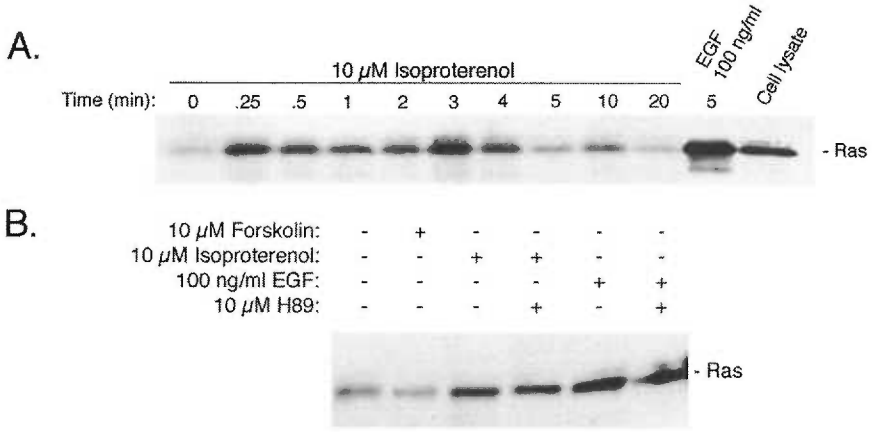

FIG. 2.5. **Isoproterenol activation of Ras.** A, Time course of activation of Ras by isoproterenol. Hek293 cells were serum-starved and treated with 10 μM isoproterenol or 100 ng/ml EGF for the indicated times. Equal quantities of cell lysate were incubated with GST-Raf1RBD, and analyzed by western blot for GTP loaded Ras. Hek293 cell lysate was used to indicate the position of Ras. B, Isoproterenol activation of Ras is insensitive to H89. Serum-starved Hek293 cells were treated with isoproterenol for 3 minutes, 10 μM forskolin for 5 minutes, or 100 ng/ml EGF for 5 minutes following a pretreatment with H89 (10mM), equal amounts of cell lysate were used to assay for GTP loaded Ras. EGF was used as a control for Ras activation.

interfering mutant of Rap1, RapN17, the Rap1 antagonist Rap1GAP1, and the interfering mutant of Ras, RasN17. These mutants have previously been characterized by our laboratory and others and function as selective blockers of Rap1 or Ras signaling (Carey et al., 2000; Feig and Cooper, 1988; Tsukamoto et al., 1999; Vossler et al., 1997). Cells transfected with myc-Erk and stimulated with 10 μM isoproterenol for 3 minutes displayed robust ERK kinase activity (Fig. 2.6A). Isoproterenol-induced ERK activation was significantly reduced when cells were co-transfected with either RapN17 or Rap1GAP1. RasN17 did not appear to have a significant effect (Fig. 2.6A). The differences in kinase activity were not attributed to varying levels of myc-ERK expression (Fig. 2.6A, lower panel). Quantification of three independent experiments revealed that ERK kinase activity, induced by isoproterenol for 3 minutes, was significantly reduced by either RapN17 or Rap1GAP1 (Fig. 2.6B). These data indicate that endogenous Rap1, but not endogenous Ras, is required for β_2AR to activate MAP kinase at this time point.

Isoproterenol induces Rap1/B-Raf association and B-Raf kinase activity - To further investigate the function of active Rap1 in mediating MAP kinase activation in Hek293 cells we examined the downstream target of Rap1, B-Raf. Prior studies from our laboratory have demonstrated in PC12 cells, which express high levels of B-Raf, that cAMP is able to activate ERKs through a PKA/Rap1/B-Raf pathway (Vossler et al., 1997). Hek293 cells also express high levels of endogenous B-Raf protein (Fig. 2.7A). Hek293 cells were left untransfected or transfected with His-Rap or a constitutively active mutant of His-Rap, His-RapV12 (Cook et al., 1993; Vossler et al., 1997), serum-

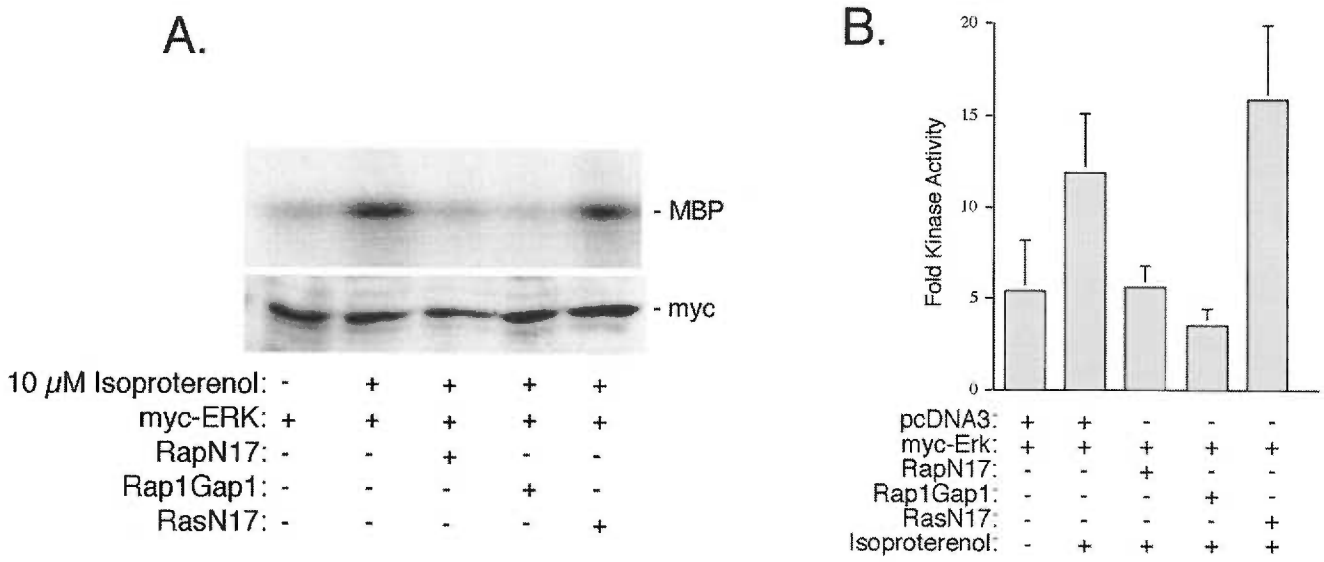

FIG. 2.6. **β2AR-mediated activation of ERKs requires Rap1.** A, Rap is required for ERK activation by isoproterenol. Hek293 cells were transfected with the indicated cDNAs and treated with 10 μM isoproterenol for 3 minutes. Equivalent amounts of cell lysate were immunoprecipitated using an agarose-coupled myc antibody followed by an immune complex kinase assay with the location and phosphorylation of MBP shown by autoradiography. A representative experiment can be seen in the upper panel (n=3). Lower panel: equal amounts of myc-tagged protein were loaded as evidenced by the western blot probed with myc antibody. B, Data representing multiple myc-ERK immune complex kinase assays (from part A) are shown as fold activation over basal (untreated cells) (n=3 +/- S.D.).

starved, and treated with isoproterenol for 3 minutes in the absence or presence of H89. Isoproterenol stimulation induced Rap1/B-Raf association and B-Raf kinase activity (Fig. 2.7B). Both the association and kinase activity was blocked by the PKA inhibitor H89. Results from three independent experiments are shown in Fig. 2.7C.

Isoproterenol stimulation of Hek293 cells induced the activation of Ras (Fig. 2.5A). To determine whether active Ras could couple to relevant downstream effectors, we investigated its ability to associate with the Raf isoforms B-Raf and Raf-1. Previous studies have suggested that recruitment of Raf to Ras is necessary for its activation (Marais et al., 1995; Marais et al., 1997; Mineo et al., 1997; Morrison and R. E. Cutler, 1997). Hek293 cells were transfected with His-tagged Ras cDNA (His-Ras) and treated with either isoproterenol or EGF, or pretreated with isoproterenol and then treated with EGF. Results presented in Fig. 2.7D suggest that isoproterenol stimulation did not induce the association of endogenous Raf-1 with Ras. More importantly, pretreatment with isoproterenol inhibited the ability of EGF to induce the association of endogenous Raf-1 with Ras (Fig. 2.7D). Parallel experiments examining the association of B-Raf with Ras indicated that isoproterenol alone inhibited basal as well as EGF-induced association of B-Raf with Ras (Fig. 2.7E). These results suggest that, while Ras is activated by β_2AR, it is unable to couple to either Raf-1 or B-Raf kinases.

ERK activation by β_2AR occurs independently of EGF receptor phosphorylation - A recent study has suggested a role for the EGF receptor in mediating β_2AR-induced ERK activation (Maudsley et al., 2000). To address the requirement for the EGF receptor in

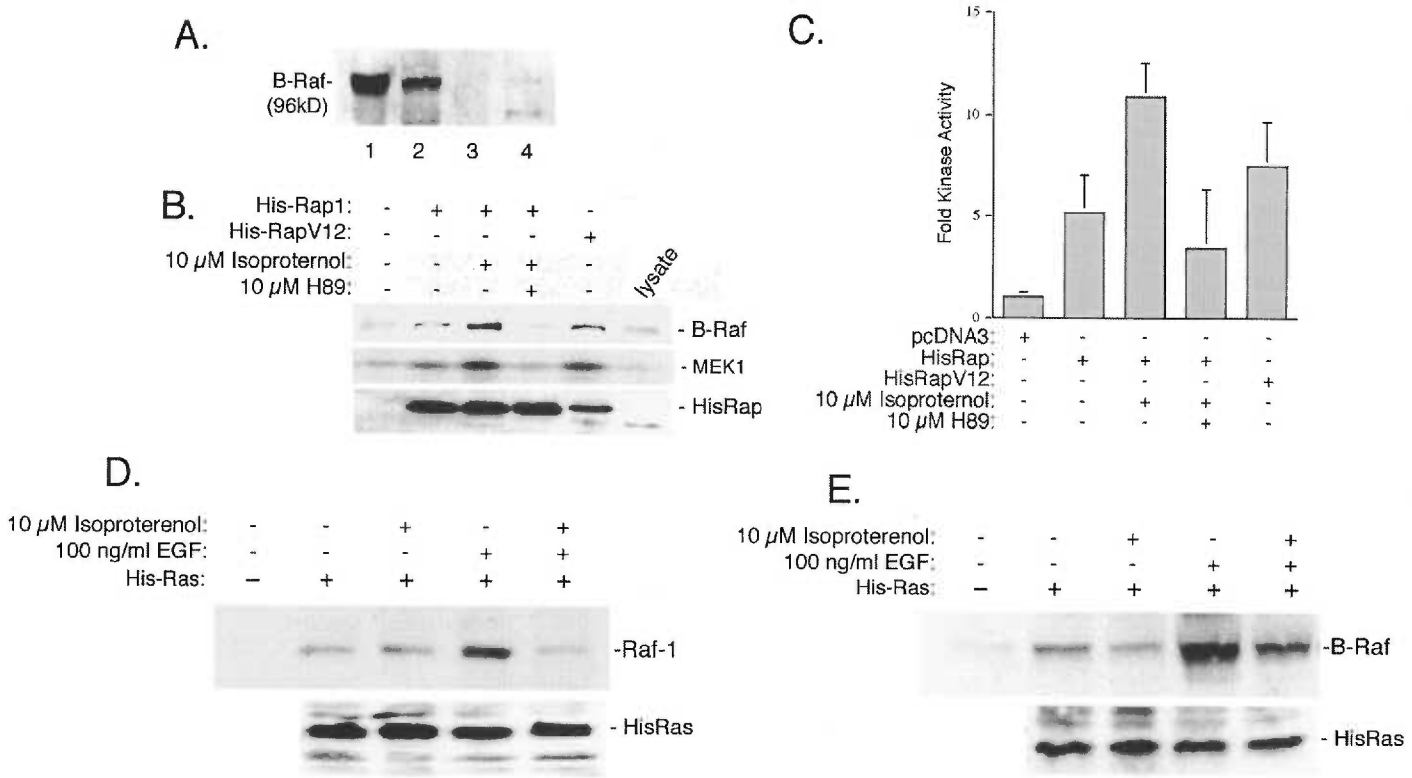

FIG. 2.7. **β2AR activates B-Raf, but not Raf-1, via Rap1.** A, Hek293 cells express B-Raf. Western blotting of equal amounts of protein were used to represent cell lysates from various cell types: PC12 (1), Hek293 (2), Rat1 fibroblast (3), and PC3 (4). B, Isoproterenol induces Rap1/B-Raf association and B-Raf kinase activity. Hek293 cells were transfected with His-tagged Rap1 (His-Rap) or His-RapV12 cDNAs, serum-starved, and treated with 10 μM isoproterenol for 3 minutes or left untreated, in the absence or presence of the PKA inhibitor, H89 as indicated. Equal amounts of protein were passed over a nickel column and eluates were probed by western blotting for B-Raf (upper panel) and kinase activity using MEK-1 as a substrate (middle panel). Representative results are shown (n=3). The bottom panel indicates similar protein amounts of His-Rap per treatment as assayed by western blot. C, Data representing multiple B-Raf kinase assays. Bars indicate fold activation over basal (n=3, +/- S.D.). D, Isoproterenol inhibits Ras/Raf-1 association. Hek293 cells were transfected with His-Ras, serum-starved, and treated with either 10 μM isoproterenol for 3 minutes, 100 ng/ml EGF for 5 minutes, or pretreated with 10 μM isoproterenol for 5 minutes and then EGF. Equal amounts of protein lysate underwent nickel affinity purification and eluates were probed by western blotting for Raf-1 (upper panel). The lower panel demonstrates similar levels of His-Ras protein. E, Isoproterenol inhibits Ras/B-Raf association. Hek293 cells were transfected with His-Ras, serum-starved, and stimulated identically to Fig. 6D. Following nickel affinity purification, eluates were probed by western blotting for B-Raf (upper panel). The lower panel indicates similar levels of His-Ras.

β_2AR signaling we treated cells with the EGF receptor kinase inhibitor AG1478, which specifically inhibits kinase activity of the receptor. Pretreatment of cells with AG1478 did not block isoproterenol-induced activation of endogenous ERKs (Fig. 2.8A). The above results would suggest that Rap-1-dependent activation of ERKs by β_2AR does not require EGF receptor transactivation.

Recent studies have also suggested that the activation of Ras by β_2AR may also utilize the EGF receptor, via non-classical coupling to Giα (Maudsley et al., 2000). To further elucidate the mechanism by which Ras is activated by β_2AR, we determined whether endogenous Ras activation by isoproterenol was dependent on EGF receptor activation. Pretreatment of Hek293 cells with AG1478 did not block Ras activation by isoproterenol at 3 minutes (Fig. 2.8B). To investigate the possibility that Giα may signal to Ras we pretreated Hek293 cells with PTx and stimulated cells with either isoproterenol or carbachol for 3 and 5 minutes, respectively. Representative data presented in Fig. 8C demonstrate that Ras activation by isoproterenol, but not by carbachol, was insensitive to PTx. As a positive control, we show that Ras activation by carbachol was sensitive to PTx (Fig. 2.8C). The above data as well as that presented in Fig. 2.5A indicate that Ras is activated by the endogenous β_2AR independently of either the EGF receptor or Giα.

DISCUSSION

The second messenger cAMP is the best studied intracellular signal. Its major action, the activation of the cAMP-dependent protein kinase, PKA (Beavo et al., 1975; Butcher et al., 1968) allows hormonal signals to couple to intracellular phosphorylation events.

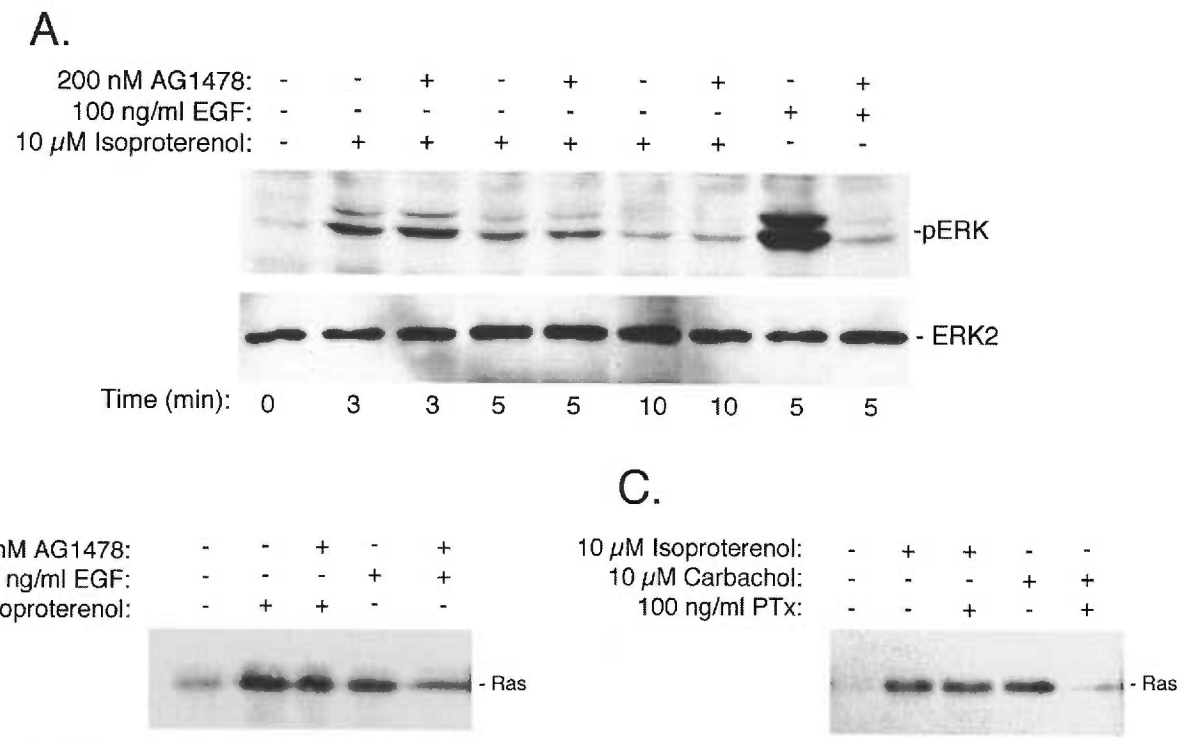

FIG. 2.8. **ERK/Ras activation by β2AR does not require EGF receptor phosphorylation.** A, Isoproterenol-mediated activation of ERKs is EGF receptor-independent. Serum-starved Hek293 cells were pretreated with 200 nM AG1478 for 20 minutes followed by 10 μM isoproterenol stimulation for the indicated times. As a control, cells were also treated with 100 ng/ml EGF for 5 minutes. Lysates were subjected to western blotting using pERK antibodies (upper panel) or ERK2 antibody (lower panel) to confirm equal protein amounts of cell lysate were utilized. B, Isoproterenol stimulation of Ras is EGF receptor-independent. Hek293 cells were serum-starved and pretreated with 200 nM AG1478 for 20 minutes followed by a 3 minute stimulation with 10 μM isoproterenol. Stimulation with 100 ng/ml EGF for 5 minutes was used as a control for Ras activation. Equal amounts of cell lysate were incubated with pre-coupled GST-Raf1RBD and analyzed by western blot for GTP loaded Ras. C, Isoproterenol-mediated activation of Ras is insensitive to PTx. Hek293 cells were pretreated with 100 ng/ml PTx for 16 hours and then stimulated with either 10 μM isoproterenol for 3 minutes or 10 μM carbachol for 5 minutes, as indicated. Equal amounts of cell lysate were incubated with pre-coupled GST-Raf1RBD and analyzed by western blot for GTP loaded Ras.

Hormonal elevation of cAMP levels is triggered by the specific heterotrimeric G protein subunit Gαs. The range of extracellular ligands that couple to Gαs is extensive and includes moderately sized peptides, including vaso-active intestinal peptide like (VIP), members of the glucagon/secretin superfamily, adrenocorticotropic hormone (ACTH), parathyroid stimulating hormone (PTH), and a large family of hypothalamic releasing factors, as well as the family of large glycoproteins thyroid stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Small molecules can also activate Gs to stimulate adenylate cyclases, including dopamine (via the D1 receptor), adenosine (via the A_{2A}-receptor), prostaglandin E, and the family of adrenergic molecules, including epinephrine and nor-epinephrine (Iismaa and Shine, 1992; Ji et al., 1998; Spiegel et al., 1992). The cognate receptors for all these ligands are heptahelical transmembrane proteins (also called serpentine receptors) that associate with Gsα.

In the unliganded, resting state, these receptors bind inactive GDP-bound Gsα subunits that are associated with specific βγ subunits. Upon ligand binding, exchange of GTP for GDP converts α into its active GTP-bound state, causing it to be released from the receptor, where it is free to bind to, and activate, membrane-associated adenylate cyclases. At the same time that Gsα dissociates from the receptor, βγ is released from Gsα and can activate effectors independently of Gsα. βγ signaling from Gs-coupled receptors has not been reported. However, βγ release from Gi and Gq is well known to activate a number of intracellular kinases, including phosphoinositol-3 kinase (PI3-K) (Hawes et al., 1996; Lopez-Llasaca et al., 1997), phospholipase C (Birnbaumer, 1992), src (Luttrell et al., 1996) and ERK (Florio et al., 1999; Luttrell et al., 1995).

The ability of Gs-coupled receptors to modulate the MAP kinase (or ERK) cascade provides a mechanism for cAMP-coupled signaling pathways to regulate cell growth (Dhanasekaran et al., 1995). The best studied actions of cAMP on ERK signaling are inhibitory and lead to a decrease in cellular proliferation (Graves et al., 1993; Sevetson et al., 1993; Wu et al., 1993). This is achieved, in part, by a PKA-dependent phosphorylation of the MAP kinase kinase kinase Raf-1 on serine 43 which uncouples Raf-1 from its upstream activator Ras (Wu et al., 1993). In cells that express the Raf isoform B-Raf (which does not contain a PKA site corresponding to serine 43), cAMP can activate ERKs (Dugan et al., 1999; Vossler et al., 1997; Wan and Huang, 1998). While this has been shown in multiple cell types, additional factors may influence cAMPs ability to activate B-Raf. Indeed, cAMP has also been reported to inhibit the activation of B-Raf through a PKA phosphorylation near the kinase domain itself. However, this effect is only seen in truncated proteins lacking the N-terminus of B-Raf (MacNicol and MacNicol, 1999). In cells which express a truncated splice variant of B-Raf that also lacks the N-terminus, cAMPs inhibitory effects may predominate (Vaillancourt et al., 1994). However, cAMP robustly activates the full length B-Raf protein which is achieved via the activation of the small G protein Rap1 (Ohtsuka et al., 1996; Okada et al., 1999; Vossler et al., 1997). Interestingly, Rap1 is also an antagonist of Ras-dependent signaling (Cook et al., 1993; Kitayama et al., 1990; Palsson et al., 2000) and blocks Ras-dependent activation of Raf-1 (Boussiotis et al., 1997; Cook et al., 1993; Hu et al., 1997; Hu et al., 1999). Unlike Ras, Rap1 is activated by increased cAMP levels via PKA. Recently, Rap1 activators have been identified that can be directly

activated by cAMP, suggesting that cAMP can activate Rap1 via both PKA-dependent and PKA-independent mechanisms (de Rooij et al., 1998; Kawasaki et al., 1998a). The ability of β_2AR to inhibit ERK signals has been demonstrated in adipocytes (Sevetson et al., 1993) and smooth muscle cells (Graves et al., 1993). Recently, β_2AR has been shown to activate ERKs in Hek293 cells (Daaka et al., 1998; Daaka et al., 1997; Della Rocca et al., 1997). In this study, we show that β_2AR can activate ERKs in Hek293 cells by activating a Rap1/B-Raf pathway, while simultaneously blocking Ras-dependent signals.

Hek293 cells are commonly used to examine signaling pathways downstream of transfected receptors (Daaka et al., 1997; Della Rocca et al., 1997; Schramm and Limbird, 1999; Seidel et al., 1999). We show that these cells express endogenous β_2AR and upon isoproterenol stimulation utilize β_2AR to activate ERKs. This activation shows an EC_{50} of roughly 1-3 μM, consistent with other actions of isoproterenol, and is rapid and transient (Crespo et al., 1995). Its actions on ERKs are mimicked by forskolin and require PKA, suggesting the involvement of Gsα and cAMP. Although signaling via Gsα is classically thought to be insensitive to PTx, recent reports have demonstrated that β_2AR can couple to ERKs via PTx-sensitive pathways (Daaka et al., 1997). These studies, which utilized transiently transfected cDNAs encoding β_2AR in Hek293 cells, proposed a PKA-dependent switch in β_2AR affinity from Gs to Gi. In our hands, PTx did not block β_2AR's activation of ERKs, while blocking the action of known Gi-coupled agents, including carbachol, LPA, and clonidine. It is possible that the ability of β_2AR to couple to PTx-sensitive pathways is dependent on elevated levels of β_2AR expression.

Both Ras-dependent and Rap1-dependent mechanisms of β_2AR's activation of ERKs have been proposed (Della Rocca et al., 1997; Wan and Huang, 1998). Indeed, we show that both Ras and Rap1 were activated by isoproterenol. Ras is activated rapidly and transiently, whereas Rap1 activation is slower and is sustained. This is similar to the kinetics seen in other cell types, including PC12 cells (York et al., 1998) and in platelets (Franke et al., 2000). Interestingly, the activation of Rap1, but not Ras, required PKA. Forskolin, which acts downstream of Gsα to elevate cAMP, also activated Rap1 but did not activate Ras. These data suggest that β_2AR utilized distinct pathways to activate Ras and Rap1. We propose that Rap1 is activated by Gsα (via cAMP and PKA), and that Ras is activated independently of Gsα, possibly by a $\beta\gamma$-dependent pathway. For Rap1, PKA appears to act upstream of Rap1 itself, possibly through a mechanism involving the Rap1 guanine-nucleotide exchanger C3G (York et al., 1998). C3G is expressed in Hek293 cells and is distinct from recently proposed exchangers like cAMP-GEF's (Epacs) that appear to be activated by cAMP in a PKA-independent manner (de Rooij et al., 1998; Kawasaki et al., 1998a).

Surprisingly, only Rap1, but not Ras, was required for β_2AR's activation of ERKs. Two agents that interfere with Rap1 signaling, RapN17 and Rap1GAP1 were used. Overexpression of RapN17 is thought to sequester endogenous activators of Rap1, whereas Rap1GAP1 stimulates the GTPase activity of endogenous Rap1 to terminate Rap1 signaling (Jordan et al., 1999; Reedquist et al., 2000; Vossler et al., 1997). RasN17 is a well characterized selective interfering mutant of Ras (Feig and Cooper, 1988; Stacey et al., 1991). These data suggest that while both Ras and Rap1 are activated by β_2AR,

only Rap1 is capable of transmitting a signal to ERKs. The signal to ERKs is likely to be B-Raf, since B-Raf is the only known MAP kinase kinase kinase that can be activated by Rap1. Indeed, Hek293 cells express the 96 kD isoform of B-Raf that is activated by cAMP (Vossler et al., 1997), and endogenous B-Raf is recruited to Rap1 upon isoproterenol stimulation, in a PKA-dependent manner. Both Raf-1 and B-Raf have been shown to be efficiently recruited to Ras under the appropriate conditions (Hallberg et al., 1994; Marais et al., 1995; Okada et al., 1999; Vojitek et al., 1993). However, neither Raf-1 nor B-Raf were recruited to Ras by isoproterenol treatment, although Ras was GTP-loaded (activated) at the time point used for this study. The inability of Ras to couple to Raf explains why β_2AR's activation of ERK was independent of Ras.

Isoproterenol not only did not induce Ras association with effectors, it reversed the ability of Ras to recruit both Raf-1 and B-Raf following EGF stimulation. For Raf-1, this may be due to the phosphorylation of Raf-1 at serine 43 by PKA, which dissociates Raf-1 from activated Ras. However, the ability of isoproterenol to block the recruitment of B-Raf to Ras cannot be explained by this mechanism and suggests that an additional action of PKA is antagonizing Ras function, in general. Indeed, cAMP can also block recruitment of B-Raf to Ras (data not shown). A potential mediator of this effect is Rap1 itself. We propose a model in which Rap1 activation by PKA has two opposing functions in B-Raf/Raf-1 expressing cells; the activation of B-Raf and the antagonism of Ras. The net effect of these two actions will depend on the relative levels of Rap1 as well as B-Raf and Raf-1 in each cell type.

Although we show that activated Ras cannot activate ERKs in these cells, the mechanism by which Ras was activated by β_2AR in these cells is not known. Recently, the ability of β_2AR to activate Ras-dependent signaling has been suggested by Lefkowitz and colleagues. In their model, transiently transfected β_2AR utilized a PTx-sensitive pathway to transactivate the endogenous EGF receptor (EGFR). However, using cells expressing endogenous β_2AR, we show that isoproterenol's ability to activate either ERK or Ras did not require EGFR kinase activity. In addition, Ras activation by isoproterenol was not blocked by PTx. Since Ras activation by isoproterenol was not sensitive to H89, we propose that Ras activation by β_2AR is not mediated by either PKA, Gi, or EGFR. We suggest that $\beta\gamma$ subunits, which have been shown to activate Ras in many systems, may contribute to β_2AR's actions. The ability of both α and $\beta\gamma$ to regulate ERK signaling following receptor binding may be a common mechanism of coordinating signals to ERKs. For example, hormones that are able to activate Gi-coupled pathways have been shown to modulate ERKs via both $\beta\gamma$ and α subunits. $\beta\gamma$ activates Ras via PI3-Kγ (Lopez-Llasaca et al., 1997) and Giα activates a Rap1GAPII to inactivate Rap1 (Mochizuki et al., 1999). Here, we show a second mechanism of Rap1 regulation by α subunits, the activation of Rap1 via elevation of intracellular cAMP levels. Although PKA-independent regulation of Rap1 by cAMP has been proposed (de Rooij et al., 1998; Kawasaki et al., 1998a), the data shown here demonstrate that cAMP requires PKA to activate Rap1 in Hek293 cells, as well as other cell types (Seidel et al., 1999; Vossler et al., 1997; Wan and Huang, 1998).

The Rap1/B-Raf pathway identified here may be an important mechanism by which β_2AR stimulates ERKs in multiple systems. This may be especially true in neurons and in prostate cells that express high levels of B-Raf and where cAMP signaling to ERKs has been shown to require Rap1 (Chen et al., 1999; Dugan et al., 1999; Vossler et al., 1997). For example, β_2AR-dependent models of long term potentiation (LTP) in hippocampal neurons has recently been shown to require ERKs (Winder et al., 1999) and deficits in this form of LTP have been identified in transgenic mice deficient in hippocampal Rap1 signaling (Morozov et al., 1999). Taken together, these studies suggest that the ability of Gs-coupled receptors to activate or inhibit ERKs may depend, in part, on the expression of B-Raf (Schaeffer and Weber, 1999). While the activation of Rap1 may have a significant positive effect on ERK signaling in B-Raf-expressing cells, one can speculate that the activation of Rap1 by Gs-coupled receptors may antagonize Ras-dependent signaling to ERKs in cells that do not express B-Raf.

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CHAPTER THREE

Cyclic AMP-mediated Inhibition of Cell Growth Requires the Small G Protein Rap1

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ABSTRACT

In many normal and transformed cell types, the intracellular second messenger cyclic adenosine monophosphate (cAMP) blocks the effects of growth factors and serum on mitogenesis, proliferation, and cell cycle progression. cAMP exerts these growth inhibitory effects via inhibition of the MAP kinase cascade. Here, using Hek293 cells and NIH3T3 cells, we show that cAMP's inhibition of the MAP kinase cascade is mediated by the small G protein Rap1. Activation of Rap1 by cAMP induces the association of Rap1 with Raf-1 and limits Ras-dependent activation of ERK. In NIH3T3 cells, Rap1 is required not only for cAMP's inhibition of ERK activation, but inhibition of cell proliferation and mitogenesis as well.

INTRODUCTION

Hormones that elevate intracellular cyclic adenosine monophosphate (cAMP) have a wide range of cell type-specific effects on cell growth and differentiation (Dhanasekaran et al., 1995; Pastan et al., 1975). In many cell types, cAMP inhibits the physiological actions of growth factors (Indolfi et al., 1997) and blocks the transformation phenotype in selected malignant cells (Chen and Iyengar, 1994). For example, in fibroblasts and smooth muscle cells, cAMP inhibits MAP kinase activation by growth factors (Cook and McCormick, 1993; Graves et al., 1993) and during anchorage-independent cell growth (Howe and Juliano, 2000). These growth inhibitory actions are thought to be mediated by G protein pathways that regulate cAMP (Chen and Iyengar, 1994; Kim et al., 1997) and the cAMP-dependent protein kinase PKA (Indolfi et al., 1997).

One of the ways that PKA can oppose the actions of growth factors is to block growth factor activation of the mitogen-activated protein (MAP) kinase cascade. The mechanism of cAMP action has been proposed in a number of model systems including adipocytes (Sevetson et al., 1993), smooth muscle cells (Graves et al., 1993) and fibroblast cell lines (Cook and McCormick, 1993). MAP kinases (or ERKs, extracellular signal-regulated kinases) are required for a broad array of biological processes, including two that are tightly linked to cellular transformation; mitogenesis (Kolch et al., 1991; Thomas et al., 1992) and anchorage-independent cell growth (Howe and Juliano, 2000). ERKs are required for the proliferative actions of growth factors in many cell types (Blenis, 1993; Cowley et al., 1994; Lewis et al., 1998; Mansour et al., 1994) through multiple mechanisms (Graves et al., 2000) including the regulation of the levels of critical cell cycle proteins (Wilkinson and Millar, 2000) including cyclin D1 (Kerkhoff

and Rapp, 1997; Lavoie et al., 1996) and p27kip1 (Woods et al., 1997). ERKs are activated by growth factors through consecutive cascades of tyrosine and serine/threonine phosphorylations (Crews and Erikson, 1993). The activation of the small G protein Ras serves as a link between this phosphorylation cascade and the receptors to which growth factors bind (Crews and Erikson, 1993; Leevers and Marshall, 1992). This cascade is initiated by Ras' association with (Vojitek et al., 1993; Zhang et al., 1993) and activation of Raf-1, a ubiquitously expressed protein kinase (Alest et al., 1993; Moodie et al., 1993). Raf-1, in turn, activates MAP kinase kinase (mitogen and extracellular signal-regulated kinase or MEK), which then activates ERK (Crews and Erikson, 1993).

It has previously been established that cAMP's inhibition of ERKs maps to a site downstream of Ras and upstream of Raf-1 (Cook and McCormick, 1993). cAMP-dependent inhibition of the MAP kinase cascade requires PKA (Graves et al., 1993; Sevetson et al., 1993). MEK and ERK are not targets of PKA, nor is Ras activation by growth factors itself a target of PKA inhibition (Burgering et al., 1993; Cook and McCormick, 1993; Graves et al., 1993; Wu et al., 1993). In contrast, Raf-1 is both phosphorylated and inhibited by PKA *in vivo*. Thus, direct phosphorylation of Raf-1 by PKA has been proposed to be the site of action of PKA's antimitogenic effects (Wu et al., 1993). Two potential sites for PKA phosphorylation on Raf-1 have received significant attention: serine 43 (Morrison et al., 1993; Wu et al., 1993) and serine 621 (Hafner et al., 1994; Mischak et al., 1996). Phosphorylation of serine 43 has been shown to interfere with Raf-1's interactions with Ras *in vitro* (Wu et al., 1993). Although the serine 43 site may participate in the inhibitory actions of other kinases, for example PKG (Suhasini et al., 1998), recent studies have shown that phosphorylation of this site is not required for

PKA's inhibition of Raf-1 signaling (Sidovar et al., 2000). Serine 621 phosphorylation has also been proposed to be a site of PKA-dependent inhibition of Raf-1 activity (Hafner et al., 1994; Mischak et al., 1996). However, the ability of PKA to phosphorylate serine 621 *in vivo* (Mischak et al., 1996) has recently been challenged (Sidovar et al., 2000). Therefore, it is likely that additional mechanisms of PKA's inhibition of Raf-1 may play a role in regulating the action of this kinase.

Another candidate protein that may function to antagonize Ras-dependent activation of Raf-1 is the small G protein Rap1. Rap1 was first cloned based on its ability to revert Ras-dependent transformation of fibroblasts and was initially named Krev-1(Kirsten Ras Revertant) (Kitayama et al., 1989). It shares 50% amino acid sequence homology with Ras, which is greatest in the effector and the GTPase domains. Two human proteins, Rap1a and Rap1b, share 97% homology within their amino acid sequence (Pizon et al., 1990) and both antagonize Ras-induced activation of mitogenesis and MAP kinase in multiple cell types (Campa et al., 1991; Cook et al., 1993). For example, the constitutively active mutant of Rap1b, RapV12, has been shown to block Ras-dependent activation of ERK-2 in Rat-1 fibroblasts (Cook et al., 1993), and to potentiate the transformation-reverting effect of Rap1 (Kitayama et al., 1989). Although Rap1 can be activated by cAMP (de Rooij et al., 1998; Kawasaki et al., 1998a) and is phosphorylated by PKA (Altschuler and Lapetina, 1993; Altschuler et al., 1995), the role of Rap1 in cAMP-dependent growth inhibition has not been examined. The ability of Rap1 to block Ras-dependent signals to ERK has generally been examined in studies using transfected Rap1 proteins (Cook et al., 1993; Hu et al., 1997; Kitayama et al., 1989; Lin et al., 2000). Indeed, it has been suggested that endogenous Rap1 does not

antagonize Ras signaling (Bos, 1998; Zwartkruis and Bos, 1999; Zwartkruis et al., 1998). In this study we asked whether endogenous Rap1 is required for cAMP's inhibitory actions on the MAP kinase cascade and cellular proliferation.

EXPERIMENTAL PROCEDURES

Materials. Antibodies to Rap1, Raf-1, ERK2 (c-14), c-myc (9E10), and agarose-conjugated antibodies to HA (F-7) and myc-ERK were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibodies to HA (12CA5) were purchased from Boehringer Mannheim (Indianapolis, IN). Anti-Ras antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Phosphorylation-specific AKT antibodies (pAKT) that recognize phosphorylated AKT/PKB at threonine 308 was purchased from New England Biolabs (Beverly, MA). Phosphorylation-specific ERK antibodies (pERK) that recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2), at residues threonine 183 and tyrosine 185 were purchased from New England Biolabs (Beverly, MA). Isoproterenol, Flag (M2) antibody, and MTT were purchased from Sigma (St. Louis, MO). Forskolin, PD98059, epidermal growth factor (EGF), and N- [2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89) were purchased from Cal Biochem (Riverside, CA). Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatswoth, CA.). [^{3}H]-thymidine was purchased from N.E.N. Life Science Products (Boston, MA.).

Cell culturing conditions and treatments. Hek293 and NIH3T3 cells were cultured in Dulbecco-Modified Eagle Medium (DMEM) plus 10% fetal calf serum at 37° C. in 5%

CO_2. Cells were maintained in serum-free DMEM for 16 hours at 37° C in 5% CO_2 prior to treatment with various reagents for co-immunoprecipitation assays, western blotting, MTT, and [^{3}H]-thymidine labeling. In all experiments, cells were treated with isoproterenol (10 μM), EGF (100 ng/ml) or Forskolin (10 μM), for 5 minutes unless otherwise indicated. Where indicated, cells were pretreated with isoproterenol or Forskolin for 5 minutes and then stimulated with EGF for 5 minutes. H89 (10 μM) and PD98059 (10 μM),were added to cells 20 minutes prior to treatment.

Western blotting. Cell lysates were prepared as described (Schmitt and Stork, 2000; Vossler et al., 1997). Cell lysate protein concentrations were quantified using Bradford protein assay. For detection of Raf-1, myc-Raf-1, ERK2, myc-ERK2, HA, Flag, Ras, Rap1, pAKT, and phospho-ERK1/2 (pERK), equal protein amounts of cell lysate per treatment condition were resolved by SDS-PAGE, blotted onto PVDF (Millipore Corporation, Bedford, MA) membranes and probed with the corresponding antibodies according to the manufacturers guidelines.

Plasmids and transfections. Seventy to eighty percent confluent Hek293 or NIH3T3 cells were co-transfected with the indicated cDNAs using a Lipofectamine kit (Gibco BRL) according to the manufacturer's instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the same amount of DNA. The control vector pMACS 14.1 (Miltenyi Biotec.) was used for MACS selection. Following transfection, cells were allowed to recover in

serum containing media for 24 hr. Cells were then starved overnight in serum free DMEM before treatment and lysis.

Nickel affinity chromatography. NIH3T3 and Hek293 cells were transfected using Lipofectamine reagent with polyhistidine-tagged Rap1 (His-Rap1 and His-RapV12) and Ras (His-Ras) as previously described (Schmitt and Stork, 2000). Briefly, cells were lysed in ice-cold buffer containing 1% NP40, 10mM Tris, pH 8.0, 20 mM NaCl, 30 mM $MgCl_2$, 1mM PMSF, and 0.5mg/ml aprotinin and supernatants were prepared by low speed centrifugation. Transfected His-tagged proteins were precipitated from supernatants containing equal amounts of protein using Ni-NTA Agarose and washed with 20mM imidazole in lysis buffer and eluted with 500 mM imidazole and 5mM EDTA in phosphate-buffered saline. The eluates containing His-tagged proteins were separated on SDS-PAGE and Raf-1 proteins were detected by western blotting (Schmitt and Stork, 2000; Vossler et al., 1997). Western blots for Raf-1 were scanned and analyzed using NIH Image (Version 1.57) to quantitate the amount of Raf-1 protein. Equal amounts of His-Rap1 and His-Ras was confirmed by western blotting.

Affinity assay for Rap1 activation in NIH3T3 cells. A GST fusion protein of the Rap1-binding domain of RalGDS was expressed in *Escherichia coli* following induction by isopropyl-1thio-β-D-galactopyranoside (GST-RalGDS was a gift from Dr. Bos, Utrecht University, The Netherlands to P. J. S. S.). NIH3T3 cells were grown as described, and were stimulated at 37° C. for the indicated times and immediately lysed in ice-cold lysis buffer (50 mM Tris-HCL (pH 8.0), 10% glycerol, 1% nonidet P-40, 200

mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Active Rap1 was isolated using methods as described by Franke *et al.* (Franke et al., 1997a). Briefly, cell lysates were cleared by centrifugation, and equivalent amounts of supernatants (500 μg) were incubated with GST-RalGDS-Rap1 binding domain pre-coupled to glutathione beads. Following a 1 hour incubation at 4° C., beads were pelleted and rinsed threes times with ice-cold lysis buffer, protein was eluted from the beads using 2X Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane, blocked in 5% milk for 1 hour and, probed with either α-Rap1/Krev-1 or Flag antibody overnight at 4° C., followed by an HRP-conjugated anti-rabbit secondary antibody (or anti-mouse secondary for anti-Flag blots). Proteins were detected using enhanced chemiluminescence.

Affinity assay for Ras activation in NIH3T3 cells. NIH3T3 cells were grown as described, and were stimulated at 37° C for the indicated times and immediately lysed in ice-cold lysis buffer (50 mM Tris-HCL (pH 8.0), 10% glycerol, 1% nonidet P-40, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 mM NaF, 0.1 μM aprotinin, and 1 mM $NaVO_4$). Following the manufacturer's recommended protocol, activated Ras was isolated from stimulated lysates using agarose coupled GST-Raf1-RBD provided in the Ras Activation Assay Kit (Upstate Biotechnology, Lake Placid, N.Y.). Proteins were eluted from the beads using 2X Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane, blocked in 5% milk for 1 hour and, probed with

either α-Ras or Flag antibody as indicated overnight at 4° C., followed by an HRP-conjugated anti-mouse secondary antibody. Proteins were detected using enhanced chemiluminescence.

Magnetic selection of transfected NIH3T3 cells. NIH3T3 cells were transfected with either Flag-Rap1 orRap1Gap1 or the pMACS 14.1 control vector along with the pMACS K^k.II positive selection plasmid according to the manufacture's guidelines (Miltenyi Biotec, Auburn, CA) (Innocente et al., 1999; Tetsu and McCormick, 1999). Cells were also transfected with GFP to monitor both selection and protein expression. Briefly, cells were transfected using Lipofectamine kit (Gibco BRL) for 5 hours and then allowed to recover for 24 hours. Cells were prepared according to the manufacturer's guidelines and incubated with MACSelect K^k microbeads for 20 minutes with gentle rocking. Cells were then passed over sterile-prepared MACS separation columns (type BS) while using the Vario MACS separation magnet. Columns were washed 4 times with PBE wash buffer (phosphate buffered saline supplemented with 0.5% bovine serum albumin and 5 mM EDTA). Columns were then removed from the Vario MACS separation magnet and cells were eluted using Dulbecco-Modified Eagle Medium (DMEM) plus 10% fetal calf serum and recovered on 10 cm plates. Selected cells were then utilized for Rap1 assay, the MTT assay, and [^{3}H]-thymidine incorporation (described below) at the indicated times.

MTT Assay for cell proliferation in NIH3T3 cells. NIH3T3 cells were grown as described, serum starved overnight and plated onto 96 well plates. Cells were then

treated and incubated as indicated. Two and one-half hours prior to lysis, 20 μl of sterile 2.5 μg/ml MTT was added to the cells and allowed to incubate at 37° C. At the appropriate time, cells were lysed and proteins solubilized in 50% volume/volume H_2O and N,N,-dimethylformamide containing 20% SDS, 0.5% of 80% acetic acid, and 0.4% 1M HCL. Plates were read using a microplate reader and presented as the difference between optical densities at 570 and 650nm.

Thymidine uptake assay for DNA synthesis and proliferation in NIH3T3 cells. Serum-starved NIH3T3 cells were plated at 2 X 10^4 cells/well on 96-well plates. Cells were then treated and incubated with serum or EGF in the presence or absence of Forskolin pre-treatment, as indicated. The cells were labeled with 1 μci/well [^{3}H]-thymidine (NEN, Boston, MA) 12 hours prior to lysis. Cells were then lysed in 50% volume/volume H_2O and N,N,-dimethylformamide containing 20% SDS, 0.5% of 80% acetic acid, and 0.4% 1M HCL. Unincorporated counts were aspirated off with an automated harvester and incorporated counts were harvested onto filters. The amount of [^{3}H]-thymidine incorporated was determined with an automated TopCount NXT v2.13 scintillation and luminescence counter and software (Packard Instr. Co., Meriden, CT).

RESULTS

Rap1 inhibition of the association of Ras and Raf-1 in Hek293 cells. Epidermal growth factor (EGF) stimulates cell growth in a variety of cell types, via activation of a Ras-dependent signaling cascade to ERKs. Following stimulation by growth factors, Ras is activated and subsequently recruits Raf-1 to initiate the MAP kinase cascade.

Association with Ras can be utilized as an assay for Raf-1 activation. The association of endogenous Raf-1 with polyhistidine-tagged Ras (His-Ras) was measured within lysates harvested from transfected cells using Nickel affinity chromatography, elution, and western blotting for Raf-1. The Raf-1 antibody is specific for Raf-1 and does not cross react with B-Raf isoforms (Schmitt and Stork, 2000). In Hek293 cells, EGF, but not isoproterenol, stimulated the recruitment of endogenous Raf-1 to Ras. Importantly, EGF's recruitment of Raf-1 to Ras was inhibited by isoproterenol (Fig. 3.1A), demonstrating that isoproterenol's inhibition of Raf-1 activation is occurring at the level of Ras recruitment of Raf-1, consistent with previous reports in other cell types (Cook and McCormick, 1993; Graves et al., 1993; Wu et al., 1993). In Hek293 cells, isoproterenol can signal through endogenous β_2-adrenergic receptors to activate PKA via Gs-coupled signaling pathways (Daaka et al., 1997; Schmitt and Stork, 2000). In these cells, isoproterenol can activate Ras, but this action does not require PKA (Schmitt and Stork, 2000). In contrast, isoproterenol's inhibition of Raf-1/Ras association was completely blocked by pretreatment with H89, an inhibitor of the cAMP-dependent protein kinase PKA (Chijiwa et al., 1990) (Fig. 3.1A), suggesting the involvement of PKA via the intracellular second messenger cAMP.

Previously, it has been shown that isoproterenol and cAMP could activate the small G protein Rap1 (Schmitt and Stork, 2000; Wan and Huang, 1998). Endogenous Rap1 activity can be selectively inhibited by the expression of Rap1GAP1 (Polakis et al., 1991), a Rap1-specific GTPase-activating protein that can block Rap1 pathways when expressed ectopically within cells (Anneren et al., 2000; Reedquist et al., 2000; York et al., 2000). To test the hypothesis that Rap1 was involved in the inhibition of Raf-1, we

examined Raf-1 recruitment to Ras in cells transfected with Rap1GAP1. EGF stimulated Raf-1 recruitment to Ras which was unaffected by Rap1 inhibition (Fig. 3.1B, lanes 5, and 6), suggesting that endogenous Rap1 does not participate in EGF signaling to Ras/Raf-1 in these cells. However, isoproterenol's ability to inhibit EGF's actions was completely blocked following Rap1 inhibition (Fig. 3.1B, lanes 7, 8), demonstrating that endogenous Rap1 mediates isoproterenol's inhibitory effects in these cells. Since Rap1 is activated by isoproterenol through the actions of cAMP and PKA (Schmitt and Stork, 2000), these results show that cAMP/PKA's inhibition of Raf-1 recruitment to Ras required Rap1. Interestingly, although isoproterenol by itself was incapable of stimulating the recruitment of Raf-1 to Ras, blocking Rap1 action with Rap1GAP1 revealed isoproterenol's ability to recruit Raf-1 to Ras (Fig. 3.1B, lanes 3, 4). We have previously shown that despite isoproterenol's ability to activate Ras in these cells, isoproterenol does not permit signaling from activated Ras to Raf-1/ERK (Schmitt and Stork, 2000). The data presented here demonstrate that isoproterenol's activation of Rap1 uncouples isoproterenol's activation of Ras signaling from Raf-1, and provides an explanation for isoproterenol's inability to activate Raf-1 while activating Ras.

The mechanism of Rap1 antagonism of Ras-dependent signaling pathways is not well understood. To determine whether or not Rap1 antagonism of Ras function could be generalizable to other Ras effectors, we examined another Ras effector in these cells; phosphoinositide 3-kinase (PI3-K) (Downward, 1998b). PI3-K activation can be monitored by the phosphorylation of the kinase AKT (PKB) at threonine 308, through the action of PDK1 (Franke et al., 1997b; Peterson and Schreiber, 1999). Isoproterenol activated AKT in a PKA-independent manner (Fig. 3.1C). This suggests that the

activation of at least one Ras effector (Raf-1) is blocked by PKA while another (PI3-K) is not. We have shown that isoproterenol's activation of Ras in these cells is PKA-independent (Schmitt and Stork, 2000), and others have shown that this requires the action of the βγ subunits of the heterotrimeric G proteins that couple to the β-adrenergic receptor (Koch et al., 1994). To determine whether the βγ subunits of G proteins are necessary for AKT phosphorylation in response to isoproterenol, a peptide derived from the carboxyl terminus of the β-adrenergic receptor kinase 1 (β-ARKct) that sequesters Gβγ subunits (Koch et al., 1993) was transfected into cells. β-ARKct has previously been shown to block βγ-dependent signaling downstream of G protein-coupled receptors (Lopez-Llasaca et al., 1997). AKT phosphorylation was blocked by β-ARKct expression (Fig. 3.1D) which suggests that, like isoproterenol's activation of Ras, isoproterenol's activation of AKT is not mediated by Gsα signaling to PKA, but utilizes the βγ subunits instead. The Ras-dependence of isoproterenol's activation of AKT was confirmed by the ability of RasN17, an interfering mutant of Ras (Stacey et al., 1991), to completely block the activation of AKT. In contrast, Rap1GAP1 (Fig. 3.1D) did not block this activation. The inability of Rap1 to interfere with other Ras-dependent signaling pathways suggests that Rap1 antagonism is selective for Raf-1.

One model for Rap1's action is that it binds to Raf-1, sequestering it from Ras (Okada et al., 1999). Indeed, as isoproterenol blocked Raf-1's recruitment to Ras, it increased Raf-1 binding to Rap1 (Fig. 3.1E, lane 3). Activation of Rap1 was necessary and sufficient for this effect, since Rap1GAP1 blocked this effect (Fig. 3.1E, lane 4) and RapV12, a constitutively active mutant of Rap1 (Vossler et al., 1997) (but not wild type Rap1) mimicked this effect. (Fig. 3.1E, lane 2, 7). EGF did not trigger Raf-1 recruitment

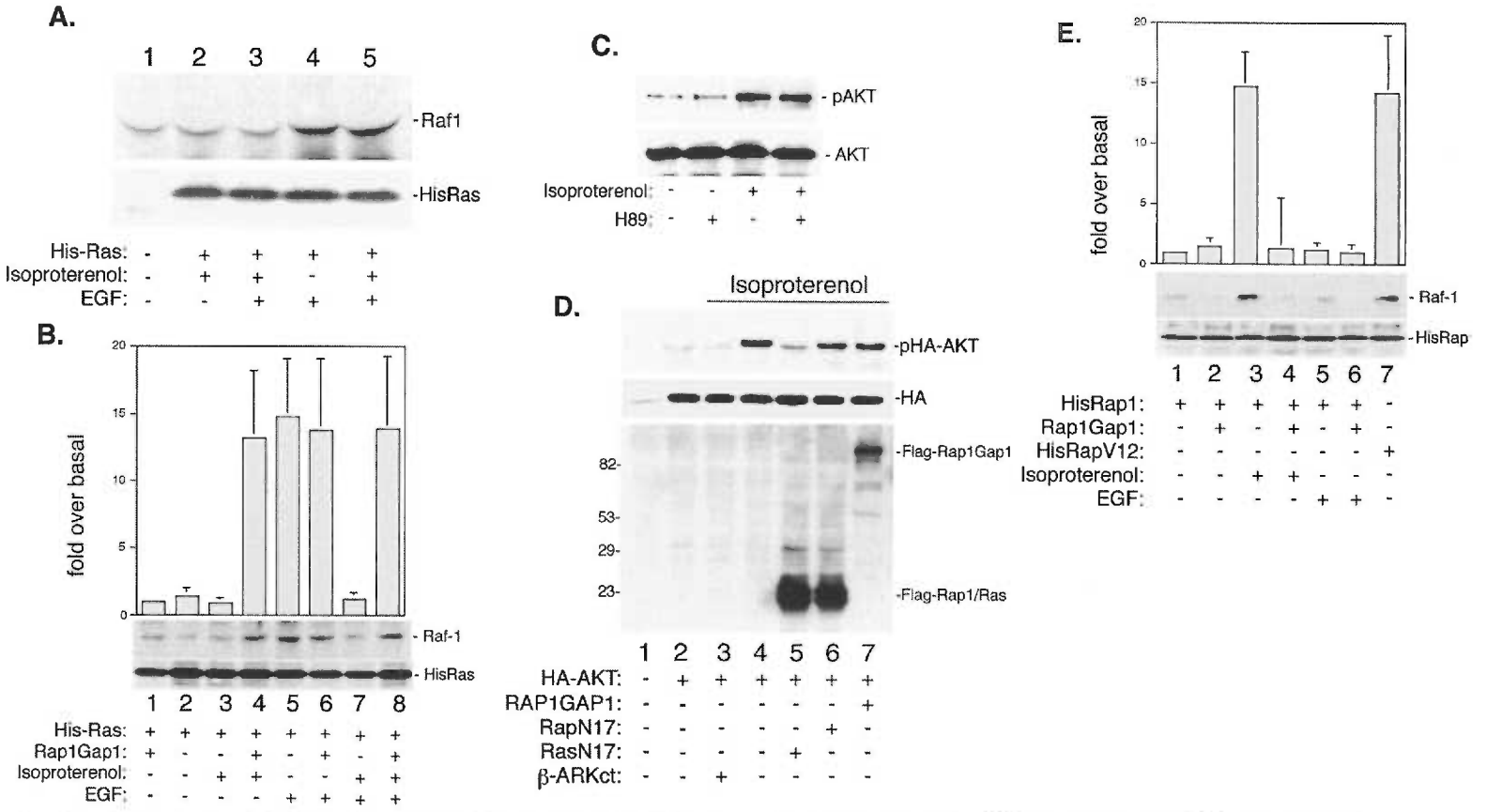

FIG. 3.1. **cAMP's inhibition of Ras and Raf-1 association by Rap1 in Hek293 cells.** (A) Isoproterenol-mediated inhibition of Ras/Raf-1 association requires PKA. Cells were serum starved overnight and treated with isoproterenol, EGF, or pretreated with isoproterenol and/or H89 and then treated with EGF. Upper panel: western blot of Raf-1 within purified eluates, prepared from equal amounts of polyhistidine-tagged Ras (His-Ras)-transfected Hek293 cell lysates. Lower panel: western blot of Ras within purified eluates, demonstrating equal amounts of His-Ras proteins within Hek293 cell lysates. (B) Rap1 mediates the ability of isoproterenol to block Ras' association with endogenous Raf-1. Hek293 cells were transfected with cDNAs encoding His-Ras and Rap1GAP1 as indicated, serum starved, and stimulated with isoproterenol, EGF or pretreated with isoproterenol and then treated with EGF. Lysates were then purified using a Nickel column and eluates were examined for the presence of either Raf-1 or His-Ras by western blot. The bar graph represents data from multiple pulldown experiments (n=3 + S.D.). Bars indicate fold Raf-1 binding over basal (fold over basal). (C) Isoproterenol induced phosphorylation of AKT does not require PKA. Hek293 cells were stimulated with isoproterenol in the presence or absence of H89 pretreatment, as indicated. Lysates containing equivalent amounts of protein were probed by western blotting for pAKT (upper panel) or AKT (lower panel). (D) Rap1 does not regulate AKT activation by Ras and isoproterenol. Hek293 cells were co-transfected with the indicated cDNAs, serum starved, and stimulated with isoproterenol. Lysates containing equivalent amounts of protein were immunoprecipitated using HA-conjugated antibody and purified proteins were probed by western blotting for either pAKT (pHA-AKT; upper panel) or HA as a loading control for AKT (middle panel). Lysates were also probed by western blot for Flag-tagged proteins, Ras, Rap1 and Rap1GAP1, as indicated (lower panel). (E) Active Rap1 binds to endogenous Raf-1. Hek293 cells were transfected with cDNAs encoding polyhistidine-tagged Rap1 (His-Rap1), HisRapV12, and Rap1Gap1 as indicated, serum starved, and stimulated with isoproterenol or EGF. Lysates were then purified using a Nickel column and eluates were examined for the presence of either Raf-1 or His-Rap-containing proteins (Rap1 or RapV12) by western blot. The bar graph represents data from multiple pulldown experiments (n=3 + S.D.). Bars indicate fold Raf-1 binding over basal (fold over basal).

to Rap1 (Fig. 3.1E) nor did it stimulate GTP loading (data not shown), confirming that EGF did not activate Rap1 in these cells.

Rap1 inhibition of the association of Ras and Raf-1 in NIH3T3 cells. The best studied consequence of Raf-1's recruitment to Ras is the subsequent activation of the MAP kinase kinase MEK and ERK (Ahn, 1993; Avruch et al., 1994; Cobb and Goldsmith, 1995) to stimulate cell proliferation (Mansour et al., 1994; Pagés et al., 1993). However, the ability of Rap1 to antagonize the activation of MEK and ERK and cell growth is cell type-specific (Altschuler and Ribeiro-Neto, 1998; Dugan et al., 1999; Vossler et al., 1997; Xing et al., 2000; Yoshida et al., 1992), and depends on the specific Raf isoform(s) expressed in a given cell (Okada et al., 1999). For example, both cAMP (Vossler et al., 1997) and Rap1 can activate B-Raf (Ohtsuka et al., 1996), a potent activator of MEK, and both cAMP and Rap1 are activators of ERK signaling in B-Raf-expressing cells (Vossler et al., 1997). Hek293 cells express the 95 kD isoform of B-Raf which confers regulation by PKA (MacNicol and MacNicol, 1999; Qiu et al., 2000). In these cells, both isoproterenol and cAMP/PKA can activate ERK through Rap1 (Schmitt and Stork, 2000). To examine cAMP's inhibition of ERK in a B-Raf-negative cell type, we chose NIH3T3 cells. These cells do not express B-Raf (Vossler et al., 1997), and they have been a model system for the study of cAMP on ERKs and cell growth (Chen and Iyengar, 1994; Wu et al., 1993).

Forskolin is a potent activator of adenylyl cyclase, and rapidly increases cAMP levels and PKA activity in all cell types. In NIH3T3 cells, Forskolin rapidly activated Rap1 (Fig. 3.2A and B), as measured by a GST-RalGDS assay (Franke et al., 1997a). In

contrast, EGF did not activate Rap1 (Fig, 3.2B and C), nor did it stimulate the association between Rap1 and Raf-1 (Fig. 3.2D). As an independent test for the requirement of PKA in Forskolin's actions, we transfected the cDNA encoding PKI, a specific inhibitor of PKA (Day et al., 1989). Briefly, cells were transfected with Flag-Rap1, Rap1GAP1, PKI, cPKA (catalytic subunit of PKA), or vector DNA along with cDNA encoding the truncated mouse MHC class I molecule H-2K^k, and transfected cells were separated using anti-H-2K^k coupled to MACSelect K^k microbeads (Miltenyi Biotec.), as described in Materials and Methods. Importantly, either PKI or Rap1GAP1 blocked the actions of both transfected and endogenous Rap1 (Fig. 3.2C). These results demonstrate that cAMP's activation of endogenous Rap1 in these cells required PKA and was blocked by Rap1GAP1. Binding of GTP-Ras to the Ras-binding domain (RBD) of Raf-1 *in vitro* can be used to monitor EGF's stimulation of Ras GTP loading (Fig. 3.2E). While EGF potently activated Ras, Forskolin did not, nor did Forskolin alter EGF's activation of Ras (Fig. 3.2E). This suggests that Forskolin inhibits Ras-dependent activation of Raf-1, without affecting Ras activation (GTP loading) itself. Moreover, Rap1GAP1 did not block Ras activation (Fig. 2F), suggesting that its actions on Rap1 (Fig. 3.2C and D) were selective (Rubinfeld et al., 1991).

Forskolin inhibited Ras recruitment of endogenous Raf-1 in NIH3T3 cells, as shown for Hek293 cells (Fig. 3.3A and C). The action of Forskolin was blocked by pretreatment with H89 or transfection of PKI, and was mimicked by RapV12 (Fig. 3.3A). Examining endogenous Ras association with myc-tagged Raf-1 showed the same results; Forskolin blocked EGF's action, and this was blocked by H89 (Fig. 3.3B). The requirement of Rap1 activation for Forskolin's inhibition of Ras/Raf-1 association is

shown in Fig. 3.3C. Inactivating endogenous Rap1 by expressing Rap1GAP1 in these cells prevented Forskolin's inhibitory actions (Fig. 3.3C, lanes 7 and 8). Not only did the activation of Rap1 by Forskolin uncouple Raf-1 from Ras activation, but it also triggered the association of Rap1 with endogenous Raf-1 (Fig. 3.3D and F). This association required PKA, since it was blocked by either H89 or PKI (Fig. 3.3D). In addition, Forskolin stimulated the association of endogenous Rap1 with myc-tagged Raf-1 in the presence or absence of EGF, and this was blocked by H89 (Fig. 3.3E). Moreover, it required activated Rap1, as it was blocked by the ectopic expression of Rap1GAP1 (Fig. 3.3F), and could be mimicked by RapV12 (Fig. 3.3D and F). Thus, in both Hek293 and NIH3T3 cells, cAMP's inhibition of Raf-1 binding to Ras required Rap1 and PKA.

Rap1 and cAMP's inhibition of ERKs and cell proliferation in NIH3T3 cells. ERK activation can be measured by western blotting using phospho-specific antibodies that recognize the activating phosphates within the ERK activation loop (Yung et al., 1997). Using this assay, Forskolin inhibited activation of endogenous ERK by EGF in NIH3T3 cells (Fig. 3.4A), confirming previous results (Wu et al., 1993), and this action of Forskolin, but not EGF, was blocked by H89, demonstrating the specificity of H89 and confirming a role for PKA in Forskolin's inhibition of ERKs. An extended time course of Forskolin's action is shown in Fig. 3.4B. Activated Rap1 (RapV12) did not stimulate ERKs in NIH3T3 cells; rather it blocked the activation of ERKs by activated Ras (RasV12) (Fig. 3.4C), suggesting that overexpression of Rap1 was sufficient to antagonize Ras-dependent signals in NIH3T3 cells, as in other cell types (Cook et al., 1993). The proteins appeared to be expressed to equivalent levels, as judged by Flag

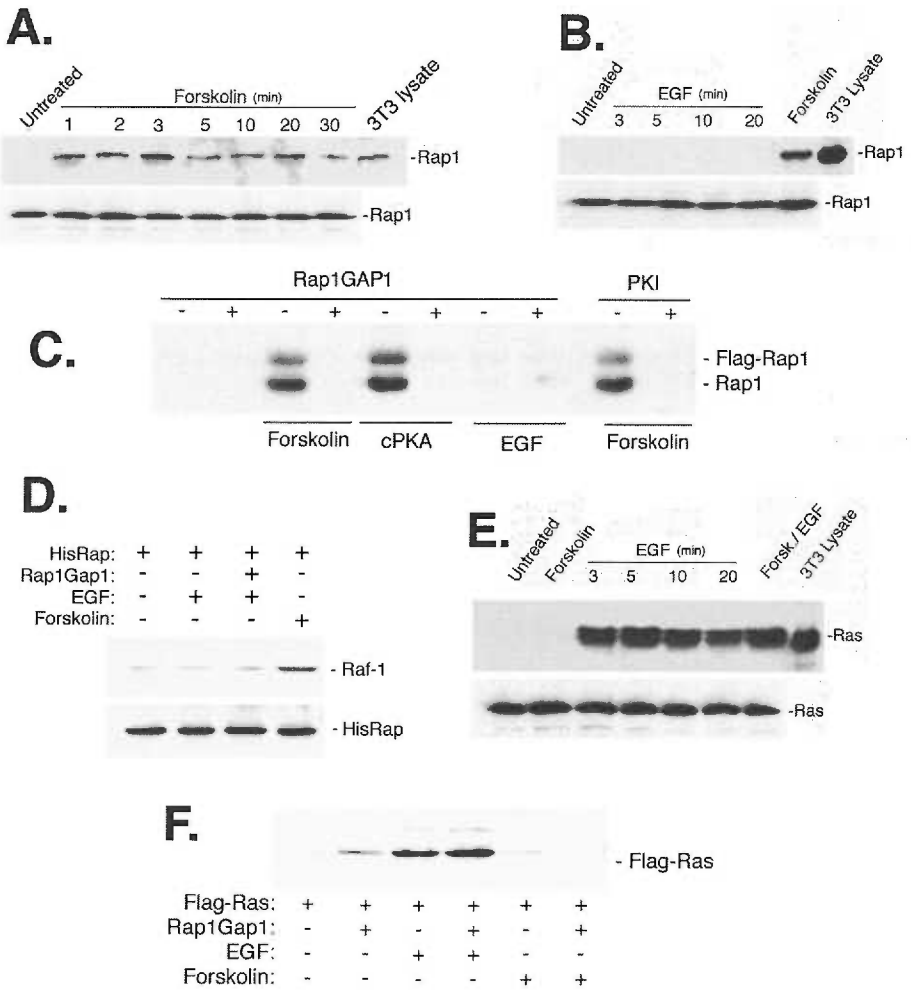

FIG. 3.2. **Rap1 activation by cAMP in NIH3T3 cells.** (A) cAMP stimulation activates Rap1. NIH3T3 cells were serum starved and stimulated with Forskolin for the indicated times. Lysates containing equivalent amounts of protein (500 μg) were incubated with GST-RalGDS and probed by western blotting for active Rap1. NIH3T3 lysate (3T3 lysate; 10 mg) was used as a control to indicate the position of Rap1 (upper panel). Total lysates were probed for Rap1 as a control for protein loading (lower panel). (B) EGF does not activate Rap1 in NIH3T3 cells. Serum starved NIH3T3 cells were treated with EGF for the indicated times or with Forskolin (5 min). Lysates containing equivalent amounts of protein were incubated with GST-RalGDS and probed by western blotting for active Rap1. NIH3T3 lysate (3T3 lysate; 50 mg) was used as a control to indicate the position of Rap1 (upper panel). Total lysates were probed for Rap1 to control for protein loading (lower panel). (C) Rap1GAP1 and PKI block Forskolin's activation of both transfected and endogenous Rap1. NIH3T3 cells were transfected with Rap1GAP1, PKI, cPKA, or vector along with pMACS Kk.II, and transfected cells were positively selected (as described in methods). Transfected cells were treated with Forskolin or EGF, or left untreated, as indicated, and Rap1 assays performed using Gst-RalGDS. Western blotting with anti-Rap1 antibodies identified activation of endogenous Rap1 (lower band) and transfected Flag-Rap1 (upper band). (D) EGF does not induce the association of Rap1 with endogenous Raf-1. NIH3T3 cells were transfected with HisRap1 and Rap1Gap1, serum starved, and stimulated with EGF, or Forskolin as a positive control. Lysates containing equivalent amounts of protein were then purified using a Nickel column and eluates were examined for the presence of Raf-1 (upper panel) or His-Rap1 (lower panel) by western blot using Raf-1 and Rap1 antisera, respectively. (E) EGF, but not cAMP, activates Ras in NIH3T3 cells. Cells were serum starved, and stimulated with Forskolin, EGF for the indicated times, or pretreated with Forskolin and then stimulated with EGF. Lysates containing equivalent amounts of protein were incubated with GST-Raf1-RBD and probed by western blotting for active Ras (upper panel). Total lysates were probed for Ras to control for protein loading (lower panel). NIH3T3 lysate (3T3 lysate; 50 mg) was used as a control to indicate the position of Ras. (F) Rap1GAP1 does not interfere with Ras activation. NIH3T3 cells were co-transfected with Flag-Ras and Rap1GAP1 or Flag-Ras alone. Transfected cells were left untreated, treated with Forskolin or EGF as indicated, and Ras assays were performed using GST-Raf1-RBD. Western blotting with Flag antibody was performed to identify activated Flag-Ras.

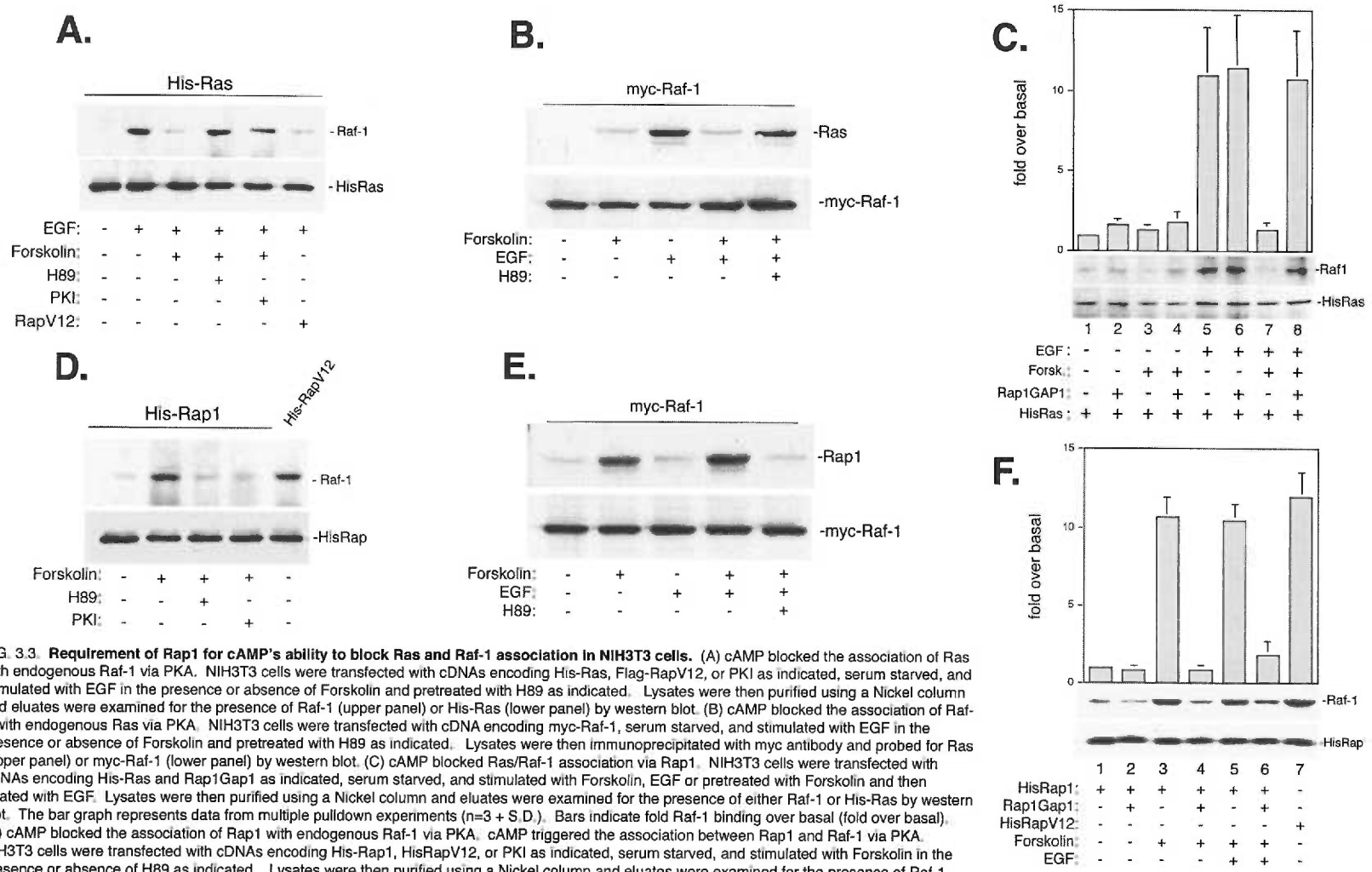

FIG. 3.3. **Requirement of Rap1 for cAMP's ability to block Ras and Raf-1 association in NIH3T3 cells.** (A) cAMP blocked the association of Ras with endogenous Raf-1 via PKA. NIH3T3 cells were transfected with cDNAs encoding His-Ras, Flag-RapV12, or PKI as indicated, serum starved, and stimulated with EGF in the presence or absence of Forskolin and pretreated with H89 as indicated. Lysates were then purified using a Nickel column and eluates were examined for the presence of Raf-1 (upper panel) or His-Ras (lower panel) by western blot. (B) cAMP blocked the association of Raf-1 with endogenous Ras via PKA. NIH3T3 cells were transfected with cDNA encoding myc-Raf-1, serum starved, and stimulated with EGF in the presence or absence of Forskolin and pretreated with H89 as indicated. Lysates were then immunoprecipitated with myc antibody and probed for Ras (upper panel) or myc-Raf-1 (lower panel) by western blot. (C) cAMP blocked Ras/Raf-1 association via Rap1. NIH3T3 cells were transfected with cDNAs encoding His-Ras and Rap1Gap1 as indicated, serum starved, and stimulated with Forskolin, EGF or pretreated with Forskolin and then treated with EGF. Lysates were then purified using a Nickel column and eluates were examined for the presence of either Raf-1 or His-Ras by western blot. The bar graph represents data from multiple pulldown experiments (n=3 + S.D.). Bars indicate fold Raf-1 binding over basal (fold over basal). (D) cAMP blocked the association of Rap1 with endogenous Raf-1 via PKA. cAMP triggered the association between Rap1 and Raf-1 via PKA. NIH3T3 cells were transfected with cDNAs encoding His-Rap1, HisRapV12, or PKI as indicated, serum starved, and stimulated with Forskolin in the presence or absence of H89 as indicated. Lysates were then purified using a Nickel column and eluates were examined for the presence of Raf-1 (upper panel) or His-Rap-containing proteins (lower panel) by western blot. (E) cAMP blocked the association of Raf-1 with endogenous Rap1 via PKA. NIH3T3 cells were transfected with cDNA encoding myc-Raf-1, serum starved, and stimulated with EGF in the presence or absence of Forskolin and pretreated with H89 as indicated. Lysates were then immunoprecipitated with myc antibody and probed for Rap1 (upper panel) or myc-Raf-1 (lower panel) by western blot. (F) Active Rap1 binds to Raf-1. NIH3T3 cells were transfected with cDNAs encoding His-Rap1, HisRapV12, and Rap1Gap1 as indicated, serum starved, and stimulated with Forskolin, or pretreated with Forskolin prior to stimulation by EGF. Lysates were then purified using a Nickel column and eluates were examined for the presence of either Raf-1 or His-Rap-containing proteins (Rap1 or RapV12) by western blot. The bar graph represents data from multiple pulldown experiments (n=3 + S.D.). Bars indicate fold Raf-1 binding over basal (fold over basal).

western blotting (data not shown). To determine whether endogenous Rap1 was necessary for Forskolin's inhibition of ERK activation, we examined ERK activation in the presence and absence of Rap1GAP1. The expression of Rap1GAP1 blocked the action of Forskolin to inhibit EGF-dependent activation of ERK (Fig. 3.4D). These data demonstrate that cAMP's activation of Rap1 mediated cAMP's block of Ras-dependent signaling to Raf-1, MEK and ERK in NIH3T3 cells. To compare the relative effectiveness of Forskolin to activate Rap1 and inhibit ERK, we examined the dose dependency of these actions of Forskolin. For both actions, Forskolin showed a similar EC50 (roughly 1.0 μM) (Fig. 3.4E). H89 blocked both actions of Forskolin with an IC50 of roughly 0.4 μM (Fig. 3.4F), a dose that is selective for PKA (Chijiwa et al., 1990). At the highest dose used, H89 had no effect on the PKA-independent activation of Rap1 by thrombin (data not shown) (Franke et al., 2000; Schmitt and Stork, 2000). These results and those using PKI (Fig. 3.3) demonstrate that, in NIH3T3 cells, PKA is required for Rap1's activation by cAMP. To determine whether Rap1 mediates cAMP's inhibition of cell growth in NIH3T3 cells, we first confirmed that Forskolin blocked EGF's proliferative actions in these cells. Both EGF and serum (10%) stimulated cell proliferation, as measured 96 hrs later. Similar results were seen at 24 and 48 hrs (data not shown). All these proliferative responses were blocked by inhibiting MEK with PD98059, and by pretreatment with Forskolin (Fig. 3.5A). Forskolin's actions were blocked by H89, demonstrating that PKA was required. Forskolin did not induce apoptotic changes in these cells during the course of the experiment (data not shown). Taken together, the data confirm that EGF stimulates proliferation via ERK, and that cAMP/PKA inhibition of ERK activation blocks EGF's proliferative effects (Fig. 3.5A).

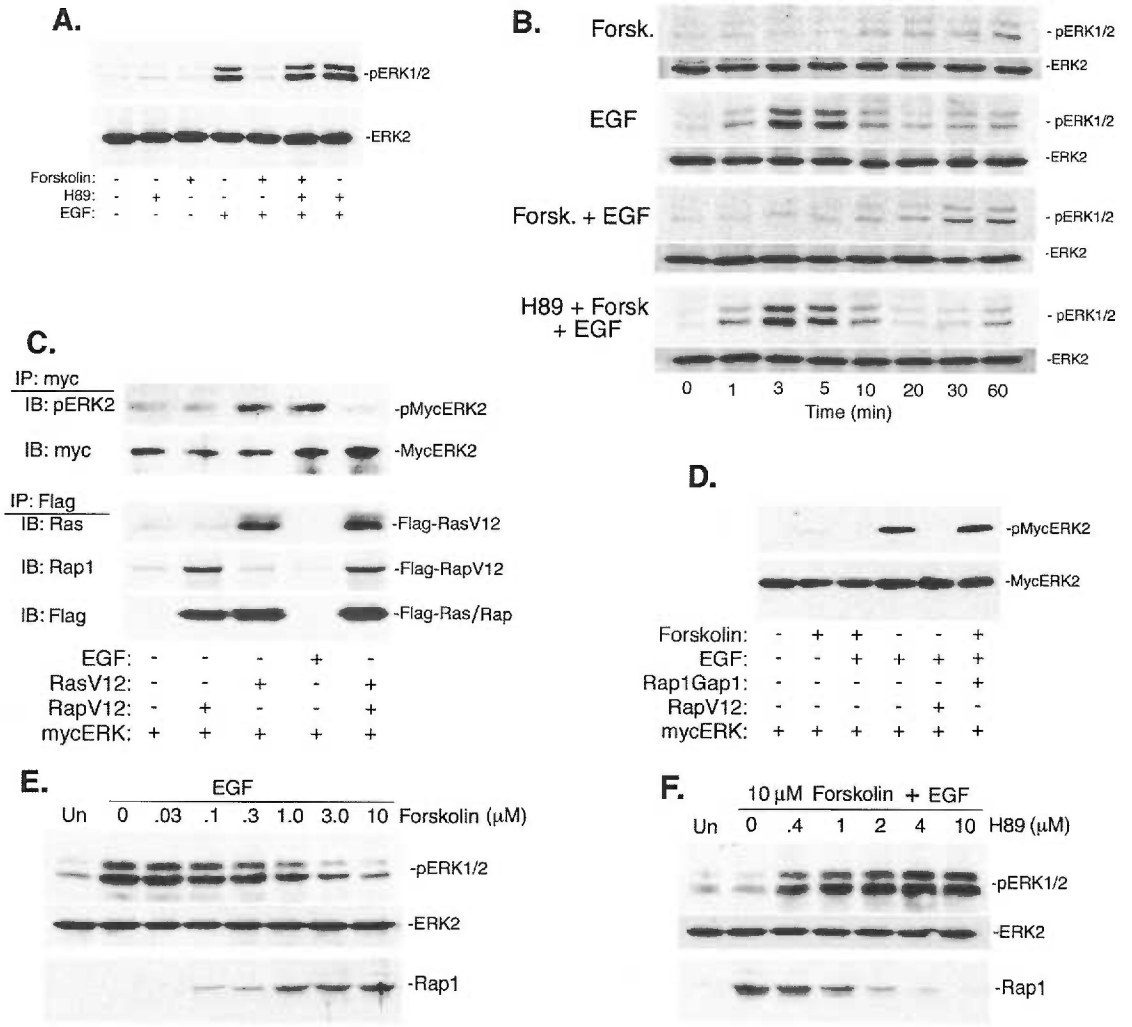

FIG. 3.4. **cAMP blockade of EGF's activation of ERKs via Rap1.** (A) cAMP's inhibition of ERK phosphorylation by EGF requires PKA. NIH3T3 cells were serum-starved and treated with Forskolin, H89, or EGF, or pretreated with Forskolin and/or H89 and then treated with EGF, as indicated. Lysates containing equivalent amounts of protein were probed for phosphorylation of endogenous ERK (pERK1/2) as well as total ERK (ERK2) to control for loading. (B) An extended time course of Forskolin's inhibition of EGF's activation of ERK. Cells were treated with EGF, Forskolin, or H89 for the times indicated and analyzed as in 4A. (C) Rap1 antagonizes Ras' activation of ERKs. NIH3T3 cells were transfected with cDNAs encoding mycERK2, Flag-RasV12, and Flag-RapV12, serum starved and treated with EGF as indicated. Lysates containing equivalent amounts of protein were immunoprecipitated (IP) with conjugated-myc antibody and immunoblotted (IB) by western blotting for phospho-ERK (p-mycERK2) or myc (mycERK2) to control for protein loading. The levels of both transfected proteins RasV12 and RapV12 are also shown, following Flag immunopreciptation (IP) and Ras or Rap1 immunoblot (IB), as indicated (lower panels). (D) Rap1 mediates cAMP's ability to inhibit EGF stimulation of ERK phosphorylation. NIH3T3 cells were transfected with cDNAs encoding mycERK2, RapV12, and Rap1Gap1, serum starved and treated with Forskolin, EGF or pretreated with Forskolin and then treated with EGF, as indicated. Lysates containing equivalent amounts of protein were immunoprecipitated with conjugated-myc antibody and probed by western blotting for phospho-ERK (p-mycERK2) or myc (mycERK2) to control for protein loading. (E) Forskolin activates Rap1 and inhibits ERKs in a dose-dependent manner. NIH3T3 cells were serum-starved and pretreated with the indicated concentrations of Forskolin and then treated with EGF, as indicated. Lysates containing equivalent amounts of protein were either probed for phosphorylation of endogenous ERK (pERK1/2) (top panel), total ERK (ERK2) to control for protein loading (middle panel), or used for Rap1 activation assay (lower panel). (F) H89 blocks Forskolin's activation of Rap1 and its inhibition of ERKs in a dose-dependent fashion. NIH3T3 cells were serum-starved, pretreated with the indicated concentration of H89, treated with Forskolin, and then stimulated with EGF, as indicated. Lysates containing equivalent amounts of protein were either probed for phosphorylation of endogenous ERK (pERK1/2) (top panel), total ERK (ERK2) to control for protein loading (middle panel), or used for Rap1 activation assay (lower panel).

To test whether Rap1 activation was required for Forskolin's antiproliferative actions, we examined proliferation in cells transfected with Rap1GAP1 and positively selected as described in the previous section. In all conditions where Forskolin inhibited the proliferative effects of EGF or serum, these effects were completely blocked by the expression of Rap1GAP1 (Fig. 3.5B). Similar results were seen examining mitogenesis, as measured by [^{3}H]-thymidine uptake (Fig. 3.6). The ability of EGF or serum to stimulate [^{3}H]-thymidine uptake was blocked by PD98059, demonstrating the requirement of MEK for these effects. Forskolin also blocked the mitogenic actions of both EGF and serum. This inhibition required PKA, as Forskolin's antimitogenic actions were blocked by H89 (Fig. 3.6A). H89 had no effect on the proliferation of untreated cells. As for cell proliferation, in all conditions where Forskolin inhibited the mitogenic effects of EGF or serum, these effects were completely blocked by the expression of Rap1GAP1 (Fig. 3.6B). These data demonstrate that the activation of endogenous Rap1 is required to mediate cAMP's inhibition of cell growth and proliferation.

DISCUSSION

Most studies examining cAMP's inhibition of ERK have focused on cAMP's regulation of either Ras (Burgering et al., 1993) or Raf-1 signaling (Cook and McCormick, 1993; Hafner et al., 1994; Mischak et al., 1996; Sidovar et al., 2000; Wu et al., 1993). This is largely because of the critical role of Ras signaling in proliferation (Feig and Cooper, 1988), oncogenesis and metastasis (Webb et al., 1998). Ras is thought to stimulate cell cycle progression by multiple mechanisms (Gille and Downward, 1999). One of the earliest consequences of Ras action on cell proliferation is the stimulation of G_1/S

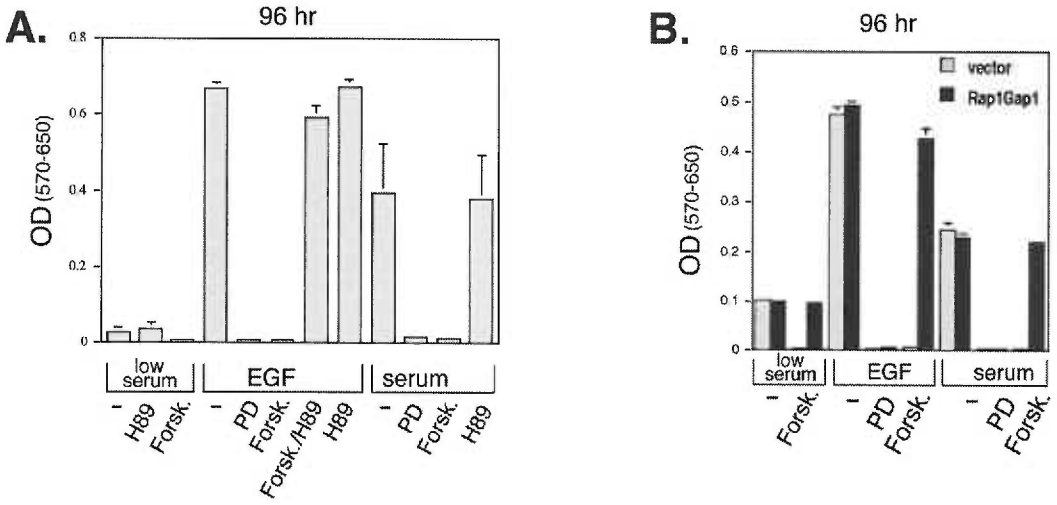

FIG. 3.5. **Requirement of Rap1 for cAMP's ability to block EGF-induced cell proliferation.** (A) Forskolin blocked cell proliferation induced by either serum or EGF. NIH3T3 cells were serum starved, plated into 96 well plates, and placed into either 0.5% serum (low serum), EGF, or 10% serum (serum) plus Forskolin, PD98059, or H89, as indicated. Forskolin was applied 5 minutes prior to either EGF or serum addition, as indicated. MTT assay buffer was added to the cells 2.5 hours prior to the indicated time of lysis and plates were read using a microplate reader, and the data presented as the difference between optical densities (OD) at 570 and 650nm (570-650). The bar graph represents data from multiple independent experiments (n=5 + S.E.). (B) Rap1Gap1 reverses cAMP's inhibitory effects on NIH3T3 cell proliferation. NIH3T3 cells were transfected with cDNAs encoding pMACS 14.1 or Rap1Gap1 and pMACS Kk.II, and positively selected (as described in methods). Selected cells were serum starved, plated into 96 well plates, and placed into either 0.5% serum (low serum), 100 ng/ml EGF, or 10% serum (serum) plus Forskolin, PD98059, or H89, as indicated. MTT assay buffer was added to the cells 2.5 hours prior to the indicated time of lysis and plates were read using a microplate reader, and the data presented as the difference between optical densities (OD) at 570 and 650nm (570-650). The bar graph represents data from multiple independent experiments (n=5 + S.E.).

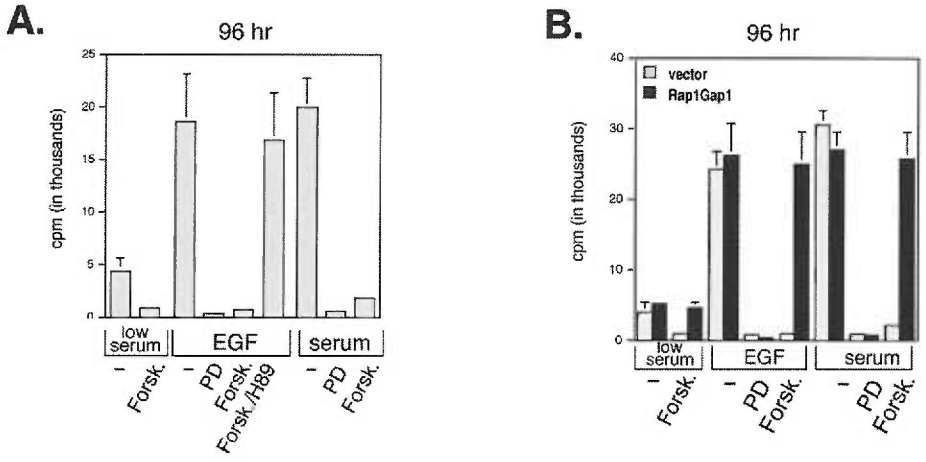

FIG. 3.6. **Requirement of Rap1 for cAMP's ability to block EGF-induced DNA synthesis.** (A) Forskolin blocked DNA synthesis induced by either serum or EGF. NIH3T3 cells were serum starved, plated into 96 well plates, and placed into either 0.5% serum (low serum), EGF, or 10% serum (serum) plus Forskolin, PD98059, or H89, as indicated. Forskolin was applied 5 minutes prior to either EGF or serum addition, as indicated. [3H]-Thymidine uptake assays were performed as described in Methods and the data presented as counts per minute (cpm) (n=5 + S.E.). (B) Rap1Gap1 reverses cAMP's inhibitory effects on NIH3T3 cell mitogenesis. NIH3T3 cells were transfected with cDNAs encoding pMACS 14.1 or Rap1GAP1 and pMACS Kk.II, and positively selected (as described in methods). Selected cells were serum starved, plated into 96 well plates, and placed into either 0.5% serum (low serum), EGF, or 10% serum (serum) plus Forskolin, PD98059, or H89, as indicated. Forskolin was applied 5 minutes prior to either EGF or serum addition, as indicated and [3H]-thymidine uptake was measured as described in Methods. The bar graph represents data presented as counts per minute (cpm) from multiple independent experiments (n=5 + S.E.).

transition (Gille and Downward, 1999; Takuwa and Takuwa, 1997; Taylor and Shalloway, 1996). Although Raf-1/ERK is the best studied stimulator of cellular proliferation in NIH3T3 cells (Cowley et al., 1994; Pagés et al., 1993) as well as other fibroblast cells (Stang et al., 1997), oncogenic Ras activation of non-Raf-1/ERK pathways is sufficient to cause malignant transformation (Khosravi-Far et al., 1996). Specific Ras effectors distinct from Raf-1 have been implicated in Ras' actions on cell growth and proliferation in selected cell types, including Rho (Khosravi-Far et al., 1996; Qiu et al., 1995), Rac-1 (Cobellis et al., 1998; Joyce et al., 1999; Moore et al., 1997; Olson et al., 1995), PI-3K (Downward, 1995b; Takuwa et al., 1999; Treinies et al., 1999) and RalGDS (de Ruiter et al., 2000; Hernandez-Munoz et al., 2000; Miller et al., 1997; Wolthuis et al., 1996). Although the stimulation of cell growth clearly involves multiple Ras effectors, Raf-1/ERK appear to mediate the initial events in this transition, by stimulating the expression of cyclin D1 (Gille and Downward, 1999) and possibly by decreasing the levels of the cell cycle inhibitor p27 (Kawada et al., 1997; Kerkhoff and Rapp, 1997; Woods et al., 1997). Therefore, cAMP's inhibition of Ras/ERK signaling may inhibit cell growth in a variety of cell types where cell cycle proteins are tightly regulated by ERK. In NIH3T3 cells, growth factor stimulation of cell growth requires Ras and MEK (Cowley et al., 1994; Mansour et al., 1994), suggesting that Raf-1 is a critical effector of Ras' proliferative actions in these cells.

The ability of cAMP to block Ras-dependent signals was examined in both Hek293 and NIH3T3 cells. In Hek293 cells, both Raf-1 and PI3-K are downstream of Ras (Fig. 3.1). Although cAMP inhibited Ras recruitment of Raf-1, it did not block the Ras-dependent activation of PI3-K, ruling out the general inhibition of Ras function as a

mechanism for cAMP's actions. Indeed, recent reports suggest that under certain circumstances, cAMP can actually stimulate Ras (Busca et al., 2000; Pham et al., 2000; Tsygankova et al., 2000). The studies presented here using Hek293 cells demonstrate that cAMP can selectively block Raf-1-dependent processes without affecting Ras activation and function.

A number of potential models by which cAMP can inhibit Raf-1 signaling have been proposed, some of which postulate a direct action of PKA on Raf-1 itself (Hafner et al., 1994; Mischak et al., 1996; Wu et al., 1993). Most studies have examined the phosphorylation of Raf-1 at serine 43 within the amino-terminus (Wu et al., 1993). In one study, recombinant Raf-1 phosphorylated by PKA at this site *in vitro* bound less well to GTP-loaded Ras than did unphosphorylated Raf-1, although this difference was not greater than 50%, and was absent when higher concentrations of unphosphorylated and phosphorylated Raf-1 proteins were examined (Kikuchi and Williams, 1996). Indeed, contrary conclusions have been drawn from studies performed *in vivo*. Studies in both Hek293 and NIH3T3 cells have demonstrated that phosphorylation at serine 43 in Raf-1 is dispensable for cAMP's inhibitory effects (Sidovar et al., 2000), and the role of serine 43 phosphorylation in Raf-1 regulation remains unknown.

Support for additional mechanisms of PKA's ability to regulate Raf-1 comes from examination of the Raf isoform B-Raf. It has been previously shown that the association of B-Raf with Ras, like that of Raf-1, is also blocked following cAMP stimulation (Peraldi et al., 1995; Schmitt and Stork, 2000; Sidovar et al., 2000; Vaillancourt et al., 1994). B-Raf lacks a PKA consensus site at the residue analogous to serine 43. Therefore, the ability of cAMP to block B-Raf/Ras association suggests that the direct

phosphorylation of Raf cannot fully account for PKA's inhibition of the Raf family of kinases (Sidovar et al., 2000). We propose that the same protein or proteins upstream of both Raf-1 and B-Raf may mediate these effects. Interestingly, like Ras/Raf-1, this block of Ras/B-Raf association by cAMP is also inhibited by the expression of Rap1GAP1 (data not shown). However, this action does not block ERK activation, since ERKs are activated by Rap1 in B-Raf-expressing cells (Schmitt and Stork, 2000; Vossler et al., 1997; Xing et al., 2000), via the activation of B-Raf by Rap1 itself (Ohtsuka et al., 1996).

Our data suggest that PKA's actions do not require direct phosphorylation of Raf-1. It is possible that cAMP's activation of Rap1 prevents Ras from associating with Raf-1 by direct binding as suggested by other studies (Okada et al., 1999; Zhang et al., 1993), although additional studies may be necessary to establish this model *in vivo*. For example, biochemical experiments have shown that Ras has a higher affinity for Raf-1 than does Rap1 (Herrmann et al., 1996), suggesting that sequestration of Raf-1 by Rap1 *in vivo* may only occur when the level of activated Rap1 protein exceeds that of Ras. Given that the transfection of Rap1 and Ras result in similar levels of expression (Fig. 3.4C), it is possible that the ability of Rap1 to block Ras signals to ERKs *in vivo* may proceed by another mechanism. We propose that Rap1 activation by cAMP may block the activation of Raf-1 by Ras, thereby limiting signals downstream of Raf-1, including MEK and ERK, resulting in the inhibition of cell proliferation and mitogenesis. These studies implicating cAMP/PKA activation of Rap1 are distinct from those studies in other cell types identifying a PKA-independent activation of Rap1 (Dremier et al., 2000; Tsygankova et al., 2001; von Lintig et al., 2000), presumably via cAMP-GEFs (de Rooij et al., 1998; Kawasaki et al., 1998a).

Studies examining PKA actions on Rap1 have focused on the direct phosphorylation of Rap1 by PKA. PKA phosphorylates both Rap1a and Rap1b at a site within the carboxyl terminus (serine 179 in Rap1b). Rap1 is a target of phosphoryation following cAMP elevation *in vivo* (Quilliam et al., 1991; Vossler et al., 1997) and *in vitro* (Altschuler and Lapetina, 1993). PKA phosphorylation of Rap1 at this site has been proposed to enhance Rap1 activation (Hata et al., 1991), inhibit Rap1 coupling (Hu et al., 1999), or have only modest effects (Altschuler and Lapetina, 1993).

Although the regulation of Rap1 by direct phosphorylation remains controversial, direct phosphorylation of Rap1 has been proposed to influence its association with specific effectors (Hu et al., 1999). Hu *et al.* showed that PKA decreases the association of Rap1 and Raf-1. In contrast, using transfected Rap1b, we show that in both Hek293 cells and NIH3T3 cells PKA increases the association of Rap1b with Raf-1 (Figs. 3.1E and 3.3D, E, and F) via the activation of Rap1 itself. In that study, the decrease in affinity of Rap1 and Raf-1 was due to the PKA-dependent phosphorylation of serine 180 in Rap1a, which decreases Rap1's affinity for the cysteine-rich domain of Raf-1. Both Rap1a and Rap1b are targets of PKA phosphorylation *in vivo*. Therefore, it is unlikely that the differences in our finding with Hu *et al.* relate to differences between the two Rap1 isoforms. Possibly, these differences may be due to distinct biochemical properties of truncated Raf-1 fragments used in those studies, or may reflect cell type-specific effects. In any case, the precise mechanism of Rap1 stimulation by PKA remains unknown and the role of PKA's phosphorylation of Rap1 in this process is not well understood. Importantly, targets of PKA that lie upstream of Rap1 itself have not been ruled out. These targets will be subjects of future research.

Because Hek293 cells express B-Raf, examination of the inhibitory role of Rap1 in cAMP inhibition of ERKs is complicated by Rap1's simultaneous activation of B-Raf and ERKs in these cells (Schmitt and Stork, 2000; Xing et al., 2000). To enable us to examine the role of Rap1 in cAMP-mediated inhibition of ERKs we utilized NIH3T3 cells. These cells express little or no B-Raf (Vossler et al., 1997) and have been previously used as a model for cAMP-dependent inhibition of cell growth. One study showed that expression of constitutive activation of Gsα suppresses Ras-dependent proliferation and cellular transformation (Chen and Iyengar, 1994). In another study, PKA activation during anchorage-independent growth of these cells blocked ERK activation by growth factors, in part, via PKA's inhibition of PAK (Howe and Juliano, 2000). However, inhibition of PAK could not entirely account for PKA's inhibitory actions and additional targets of PKA were proposed (Howe and Juliano, 2000). Interestingly, in studies examining signaling pathways during anchorage-dependent cell growth, and cell adhesion, Rap1 regulation has been reported (Posern et al., 1998; Reedquist et al., 2000; Tsukamoto et al., 1999).

As with other members of the MAP kinase family (Gong et al., 2000), the kinetics of ERK activation are critical for determining the consequence of ERK signaling (McKenzie and Pouyssegur, 1996; Woods et al., 1997; York et al., 1998), and modest reductions in ERK activation may be physiologically significant. For example, in CCL39 fibroblast cells, PKA inhibition of ERK is transient (McKenzie and Pouyssegur, 1996). We show in NIH3T3 cells that sustained activation of cAMP blocks ERK activation completely and for an extended period of time, suggesting that the extent of cAMP's inhibition may be cell type-specific.

In NIH3T3 cells, EGF stimulated ERK phosphorylation, cell proliferation, and mitogenesis. EGF's stimulation of cell proliferation and mitogenesis was blocked by PD98059 consistent with the requirement for MEK and ERK for growth factor-induced cell growth in these cells (Cowley et al., 1994; Mansour et al., 1994; Pagés et al., 1993). cAMP blocked all three aspects of EGF's actions; stimulation of ERKs, cell proliferation, and mitogenesis, suggesting that cAMP's inhibition of mitogenesis was dictated, in part, by its inhibition of ERKs. We show here that Rap1 is required for cAMP's inhibitory actions of Raf-1, ERK activation, cellular proliferation, and mitogenesis. The ability of cAMP and PKA to block ERK activation by growth factors inhibits two central aspects of malignant transformation; cellular proliferation and anchorage-independent cell growth (Howe and Juliano, 2000). We show here that Rap1 is a critical component of PKA's inhibition of ERK-dependent mitogenesis.

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CHAPTER FOUR

PKA Phosphorylation of Src Mediates cAMP's Inhibition of Cell Growth via Rap1

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ABSTRACT

In fibroblast cells, cAMP antagonizes growth factor activation of ERKs and cell growth via PKA and the small G protein Rap1. We demonstrate here that PKA's activation of Rap1 was mediated by the Rap1 guanine nucleotide exchange factor C3G, the adaptor Crk-L, the scaffold protein Cbl, and the tyrosine kinase Src. Src was required for cAMP activation of Rap1, and the inhibition of ERKs and cell growth. PKA activated Src both *in vitro* and *in vivo* by phosphorylating Src on serine 17 within its amino-terminus. This phosphorylation was required for cAMP's activation of Src and Rap1, as well as cAMP's inhibition of ERKs and cell proliferation. This study identifies an anti-proliferative role for Src in the physiological regulation of cell growth by cAMP.

INTRODUCTION

Intracellular signaling by second messengers has played a central role in our understanding of cell growth and proliferation for many decades (Ryan and Heidrick, 1968). In particular, hormones linked to cAMP have displayed well-studied effects on cell growth and differentiation (Dhanasekaran et al., 1995). cAMP is synthesized by the action of adenylyl cyclases which are themselves targets of hormonal regulation. Positive and negative regulation is achieved via the stimulatory G protein Gs and the inhibitory G protein Gi, respectively, which activate and inhibit membrane-bound adenylyl cyclases (Gilman, 1984). The anti-proliferative action of cAMP has been well studied largely in conjunction with hormone receptors linked to Gs, adenylyl cyclase, and the activation of the cyclic AMP-dependent protein kinase PKA (Beavo et al., 1974). For example, hormones like epinephrine, norepinephrine, prostaglandins, adenosine, VIP, glycagon, and parathyroid hormone inhibit the proliferation of a diverse group of cells and tissues including myocytes (Graves et al., 1993; Indolfi et al., 1997), adipocytes (Sevetson et al., 1993), fibroblasts (Burgering et al., 1993; McKenzie and Pouyssegur, 1996; Wu et al., 1993), lung cells (Maruno et al., 1998), endothelial cells (Sexl et al., 1997), glial cells (Dugan et al., 1997; Wang et al., 2001), osteoblasts (Chaudhary and Avioli, 1998), chondrocytes (Zuscik et al., 1994), lymphocytes (Tamir et al., 1996), and hepatocytes (Dixon et al., 1999), via the stimulation of cAMP synthesis and PKA. Given these growth inhibitory effects, strategies to regulate cAMP and PKA have been proposed as anti-tumor therapies (Cho-Chung et al., 1999; Puck, 1977; Tortora et al., 1995).

One important feature of cAMP's growth effects is its cell-type specificity (Burgering et al., 1993). A dramatic example of this specificity takes advantage of the discovery of Gsp oncogenic mutations within the G protein subunit Gsα protein (Lyons et al., 1990). These mutations lead to constitutively activated Gsα, and hence adenylyl cyclase and elevated cAMP levels within the human pituitary adenomas in which they are found (Landis et al., 1989). Interestingly, these oncogenic mutations are anti-proliferative when introduced into NIH3T3 fibroblast cells (Chen and Okayama, 1987) but are mitogenic when introduced into Swiss 3T3 cells (Zachary et al., 1990).

This cell-type specificity can be explained by cAMP's cell-type specific regulation of the extracellular signal regulated kinase (ERK) (Altschuler and Ribeiro-Neto, 1998; Burgering et al., 1993; Faure and Bourne, 1995; Schmitt and Stork, 2001; Zachary et al., 1990). ERK is a critical regulator of cell growth and mediates the mitogenic effects of many growth factors (Graves et al., 2000). cAMP activates ERKs in many neuronal and endocrine cells (Busca et al., 2000; Crepieux et al., 2001; Dugan et al., 1999; Grewal et al., 1999; Seger et al., 2001; Vossler et al., 1997) and potentiates the action of growth factors (Chen et al., 1999; Yao et al., 1995). In contrast, cAMP inhibits ERK activation in a variety of non-neuronal cells including NIH3T3 cells (Schmitt and Stork, 2001), Rat-1 fibroblasts (Burgering et al., 1993; Cook and McCormick, 1993; Wu et al., 1993), hepatocytes (Dixon et al., 1999), cardiomyocytes, myocytes (Graves et al., 1993), adipocytes (Sevetson et al., 1993), and others. cAMP blocks growth factor activation of ERK by PDGF (Graves et al., 1993), FGF (D'Angelo et al., 1997), EGF (Cook and McCormick, 1993; Wu et al., 1993) and insulin (Sevetson et al., 1993), by blocking the ability of the small G protein Ras to activate the MAP kinase kinase kinase

Raf-1 (Burgering et al., 1993; Cook and McCormick, 1993; Graves et al., 1993). Early studies proposed that PKA phosphorylation of Raf-1 uncoupled Raf-1 from Ras (Wu et al., 1993), however this has been recently debated (Sidovar et al., 2000).

One candidate mediating cAMP's inhibition of Raf-1 and ERKs is the small G protein, Rap1 (Dugan et al., 1999; Schmitt and Stork, 2001; Tsygankova et al., 2001). Rap1 is a ubiquitously expressed small GTP-binding protein that is activated by cAMP (Altschuler et al., 1995). It was first identified in NIH3T3 cells for its ability to antagonize mitogenic signals (Kitayama et al., 1989) and was subsequently shown to block Ras-dependent activation of Raf-1 (Cook et al., 1993). Depending on the cell type, Rap1, like cAMP, can either activate or inhibit ERKs (Altschuler and Ribeiro-Neto, 1998; Dugan et al., 1999; Vossler et al., 1997). In cells where cAMP activates ERKs, Rap1 has been shown to be required for this activation (Dugan et al., 1999; Vossler et al., 1997; Wan and Huang, 1998). In NIH3T3 cells where cAMP inhibits ERKs, Rap1 mediates this effect as well (Schmitt and Stork, 2001). Despite abundant literature on cAMP's ability to activate Rap1 (Altschuler et al., 1995; Chen et al., 1999; de Rooij et al., 1998; Dugan et al., 1999; Seidel et al., 1999; Tsygankova et al., 2001; von Lintig et al., 2000; Vossler et al., 1997; Wan and Huang, 1998; Zanassi et al., 2001), the mechanism of cAMP's activation of Rap1 is unknown.

One direct link between cAMP and Rap1 came with the discovery of Rap1-specific guanine nucleotide exchange factors (GEFs) that are themselves activated by direct binding of cAMP (de Rooij et al., 1998; Kawasaki et al., 1998a). The hallmark of this route of Rap1 activation is its independence of PKA. These cAMP-activated GEFs (Epacs, cAMP-GEFs) are expressed in a wide variety of cell types (de Rooij et al., 1998;

Kawasaki et al., 1998a) and have been implicated in selected cells where cAMP activation of Rap1 does not require PKA (Dremier et al., 2000; Leech et al., 2000; Tsygankova et al., 2001). However, in many cell types, Rap1 activation by cAMP requires PKA (Chen et al., 1999; Dugan et al., 1999; Grewal et al., 2000a; Schmitt and Stork, 2000; Schmitt and Stork, 2001; Vossler et al., 1997; Wan and Huang, 1998; Zanassi et al., 2001), and it is possible that cAMP-GEF activates small G proteins other than Rap1. Recently, a role for cAMP-GEFs has been proposed in physiological processes that are not thought to involve Rap1 (Ozaki et al., 2000). Many other potential Rap1 exchangers have been identified including C3G (Gotoh et al., 1995), CalDag-GEF (Kawasaki et al., 1998b; Yamashita et al., 2000), GFR (Ichiba et al., 1999), MR GEF (Rebhun et al., 2000), RasGRP2 (Clyde-Smith et al., 2000), nRap GEP (Ohtsuka et al., 1999), AND-34 (Gotoh et al., 2000), RA-GEF (Liao et al., 1999), and PDZ-GEF (de Rooij et al., 1999) for which neither their regulation by cAMP nor their physiological roles have been examined fully.

In this study, we set out to examine the mechanism by which cAMP activates Rap1 in fibroblast cells. We show that cAMP's actions require the Rap1 exchanger C3G, which is recruited to the membrane by cAMP in a complex containing the adapter protein Crk and the scaffold protein Cbl. Surprisingly, cAMP's recruitment of this complex and its activation of Rap1 required the activation of the Src tyrosine kinase via the direct phosphorylation within the amino-terminus of Src by PKA. These studies define a novel cross talk between two well-studied kinases, PKA and Src and demonstrate a role for Src in mediating PKA's antiproliferative effects.

EXPERIMENTAL PROCEDURES

Materials

Phosphorylation-specific ERK antibodies (pERK) that recognize phosphorylated ERK1 (pERK1) and ERK2 (pERK2), at residues threonine 183 and tyrosine 185 were purchased from New England Biolabs (Beverly, MA). Phospho-Src (Tyr416) and phospho-(Ser/Thr) PKA substrate antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to Rap1, C3G, Cbl, Raf-1, ERK2, Src, SOS, c-myc (9E10), pTyr, and agarose-conjugated antibodies to myc were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Flag (M2) antibody, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), PGE_1, isoproterenol, and MTT were purchased from Sigma (St. Louis, MO). Forskolin, PD98059, PP2, and N- [2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89) were purchased from Cal Biochem (Riverside, CA). Nickel agarose (Ni-NTA-Agarose) was purchased from Qiagen Inc. (Chatswoth, CA.).

Cell Culturing Conditions and Treatments

NIH3T3, SYF, and Src^{++} cells were purchased from ATCC and cultured in Dulbecco-Modified Eagle Medium (DMEM) plus 10% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37° C. in 5% CO_2. Cells were maintained in serum-free DMEM for 16 hours at 37° C in 5% CO_2 prior to treatment with various reagents for immunoprecipitation assays, membrane preparations, western blotting, and MTT assay. In all experiments, cells were treated with PDGF (100ng/ml), EGF (100 ng/ml),

isoproterenol (10 μM), PGE_1 (10 μM), or Forskolin (10 μM), for 5 minutes unless otherwise indicated. Where indicated, cells were pretreated with Forskolin for 5 minutes and then stimulated with EGF or PDGF for 5 minutes. PP2 (10μM), H89 (10 μM), and PD98059 (10 μM) were added to cells 20 minutes prior to treatment, unless otherwise indicated.

Western Blotting and immunoprecipitation

Cell lysates and western blotting were prepared as described (Schmitt and Stork, 2001; Vossler et al., 1997). For detection of Raf-1, ERK2, myc-ERK2, C3G, Cbl, Crk-L, Flag, Src, Rap1, phospho-Src, phospho-PKA substrate, and phospho-ERK1/2 (pERK), equal protein amounts of cell lysate per treatment condition were resolved by SDS-PAGE, blotted onto PVDF (Millipore Corporation, Bedford, MA) membranes and probed with the corresponding antibodies according to the manufacturers guidelines. For immunoprecipitation of myc-ERK2, Src, Flag-Src, Cbl, and myc-Cbl equal amounts of cell lysate per condition were precipitated at 4° C for 4 to 6 hours in lysis buffer. Proteins were then resolved by SDS-PAGE, blotted onto PVDF membranes and probed with the indicated antibodies. All western blots and immunoprecipitations were performed at least three times, and representative gels shown.

Plasmids and Transfections

The wild type Src cDNA was purchased from Upstate Biotechnology (Lake Placid, NY). The wild type Flag-Src construct was generated following subcloning Src into Bluescript KS (Clonetech, Palo Alto, CA). The N-terminal half of Src was then cut with Hind III

and an internal BspHI site. The C-terminal half of Src was generated by PCR from wild type Src, using specific primers to the sequence (sense oligo:ATGTCCCCAGAGGCCTTCCTGCAGGAC and antisense oligo: TTAAATCCTAGGTTCTCCCCGGGCTCGTACTGTGGCTCAGTGGA) and then cut with BspHI and BamHI and subcloned with the N-terminal fragment of Src into pcDNA3 containing a 2X C-terminal Flag. SrcS17A and Src17S-D were generated by PCR directed mutagenesis. Coding regions of all plasmids were sequenced in both directions prior to transfection. Wild type Fyn, Lck, and Yes were provided by Andrey Shaw (Washington University, St. Louis, MI) and subcloned into pcDNA3. Cbl-ct was provided by Brian Druker (OHSU, Portland, OR). SrcK296R (d.n.Src) was provided by Karin Rodland (OHSU, Portland, OR). NIH3T3, SYF, or Src^{++} cells were co-transfected at seventy to eighty percent confluency with the indicated cDNAs using a Lipofectamine 2000 kit (Gibco BRL) according to the manufacturer's instructions. The control vector, pcDNA3 (Invitrogen Corp.), was included in each set of transfections to assure that each plate received the same amount of DNA. Following transfection, cells were allowed to recover in serum containing media for 24 hr. Cells were then starved overnight in serum-free DMEM before treatment and lysis.

Src kinase assay

SYF cells were transfected with the indicated cDNAs and left untreated or stimulated with Forskolin, as indicated. Following immunoprecipitation and washing of Flag proteins, samples were subjected to an *in vitro* protein tyrosine kinase assay according to the manufacturer's guidelines (Life Technologies-Invitrogen, Carlsbad, CA). The assay

utilizes a peptide substrate (RR-Src) specific for tyrosine kinases. Immunoprecipitated proteins were reconstituted in 10 μl of buffer and incubated with 10 μl of either 2X substrate solution or 2X control solution both containing 0.5 μCi [γ-^{32}P]ATP. The reaction mixture was incubated at 30° C for 30 minutes and the reaction was stopped by the addition of 20 μl of ice cold 10% trichloroacetic acid. Following a 10-minute spin at 4° C and 14,000 rpm, 20 μl of supernatant from each reaction was spotted onto individual phosphocellulose discs. Discs were washed 2X in 1% acetic acid, placed into scintillation vials, and counted using a scintillation counter. Specific activity incorporated into peptide was calculated according to the manufacturer's guidelines and presented as counts per minute (cpm).

Nickel Affinity Chromatography

NIH3T3 cells were transfected using Lipofectamine reagent with polyhistidine-tagged Rap1 (His-Rap1) as previously described (Schmitt and Stork, 2000; Schmitt and Stork, 2001). Briefly, cells were lysed and supernatants were prepared by low speed centrifugation. Transfected His-tagged proteins were precipitated from supernatants containing equal amounts of protein using Ni-NTA agarose and washed with 20mM imidazole in lysis buffer and eluted with 500 mM imidazole and 5mM EDTA in phosphate-buffered saline. The eluates containing His-tagged proteins were separated on SDS-PAGE and Raf-1 proteins were detected by western blotting (Schmitt and Stork, 2000; Schmitt and Stork, 2001).

Affinity Assay for Rap1 Activation

Active Rap1 was assayed as previously described by Franke *et al.* (Franke et al., 1997a). Equivalent amounts of supernatants (500 μg) were incubated with GST-RalGDS-Rap1 binding domain coupled to glutathione beads. Following a 1 hour incubation at 4° C, beads were pelleted and rinsed threes times with ice-cold lysis buffer, protein was eluted from the beads using 2X Laemmli buffer and applied to a 12% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane, blocked in 5% milk for 1 hour and, probed with either α-Rap1/Krev-1 or Flag antibody overnight at 4° C., followed by an HRP-conjugated anti-rabbit secondary antibody (or anti-mouse secondary for anti-Flag western blots). Proteins were detected using enhanced chemiluminescence.

Membrane Preparations and Rap1 Activation

Cells were starved and stimulated with Forskolin for 5 min or pretreated or post-treated with H89 and then stimulated Forskolin, as indicated. After treatment, cells were lysed in ice cold lysis buffer. Nuclei and cytoskeleton were removed from equivalent amounts of lysate by centrifugation at 5,000 rpm for 5 min. The supernatant was then spun at 100,000 X g for 1 hour at 4° C and, the resultant membrane was resuspended in cold lysis buffer. Aliquots of the membrane fraction were analyzed for the presence of C3G by western blotting. The cytosolic or membrane fractions of treated or untreated cells were mixed with equivalent amounts of lysates from untreated Flag-Rap1 transfected cells. The mixture was incubated at 37° C for 5 minutes and equal amounts of lysate were subjected to the Rap1 activation assay (as described above).

Immunodepletion

The membrane fractions of treated or untreated cells were resuspended in equal volumes of lysis buffer. 10 μl of either C3G, SOS, or ERK2 antibodies (200μg/ml stock) were added to the membrane fraction and immunoprecipitated 6 hours at 4°. Following the immunoprecipitation, the precipitates were cleared from the tubes and analyzed by western blotting for C3G. The remaining membrane components were either incubated at 37° C for 5 minutes with equal amounts of Flag-Rap1 lysate and subjected to the Rap1 activation assay (as described above) or analyzed by western blotting for C3G.

Phosphorylation of Src Peptides

A peptide identical to amino acids 9-25 within the amino-terminus of Src, which contains potential PKA phosphorylation sites, was generated using an Auto-Spot Robot ASP 222 (ABiMED, Langenfeld, Germany) and spotted onto membranes. Similar to previous protocols (Tegge et al., 1995), the membranes were pre-incubated at room temp. overnight in buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1mM EDTA, 1mM DTT, and 0.2 mg/ml BSA. Membranes were then blocked at 30° C for 1 hour in buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1mM EDTA, 1mM DTT, and 1 mg/ml BSA and 30 μM cold ATP. To perform the kinase assay, the membranes were incubated at 30° C for 30 minutes in buffer containing 20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1mM EDTA, 1mM DTT, and 0.2 mg/ml BSA, and 12.5 nM purified catalytic subunit of PKA (a gift of John Scott, Vollum Institute). Membranes were washed at room temp. 10 X 10 minutes in 1M NaCl, 3 X 5 minutes in ddH_2O, 3 X 10 minutes in 5% H_3PO_4, and 3 X 5 in ddH_2O. The membranes

were then blocked, probed, and analyzed using chemiluminescence according the manufacturers guidelines for the phospho-PKA substrate antibody.

Selection of Transfected Fibroblasts

SYF cells were transfected with either pcDNA3 vector, Flag-Src, Flag-SrcS17A, Flag-SrcS17D or the pMACS 14.1 control vector along with pMACS K^k.II positive selection plasmid as specified by the manufacturers guidelines (Miltenyi Biotec) (Tetsu and McCormick, 1999). Cells were transfected and selected similar to previously published methods (Schmitt and Stork, 2001). Following the selection of SYF cells, cells were eluted in DMEM plus 10% fetal calf serum and recovered for 24 hours on 10 cm plates. SYF cells were then used for the MTT assay (described below) at the indicated times.

MTT Assay for Cell Proliferation

SYF and Src^{++} cells were grown as described, and subjected to the MTT cell growth assay as previously described (Schmitt and Stork, 2001). Briefly, cells were serum-starved overnight and plated onto 96 well plates. Cells were then treated and incubated as indicated. Two and one-half hours prior to lysis, 20 μl of sterile 2.5 μg/ml MTT was added to the cells and allowed to incubate at 37° C. At the appropriate time, cells were lysed and proteins solubilized in 50% volume/volume H_2O and N,N,-dimethylformamide containing 20% SDS, 0.5% of 80% acetic acid, and 0.4% 1M HCL. Plates were read using a microplate reader and presented as the difference between optical densities at 570 and 650nm.

RESULTS

cAMP/PKA Activation of Rap1 Requires C3G

Forskolin is a potent activator of adenylyl cyclases and rapidly elevates intracellular cAMP levels (Seamon and Daly, 1986). Forskolin's activation of endogenous Rap1 in NIH3T3 cells is blocked by pretreatment of cells with H89, a selective inhibitor of PKA (Chijiwa et al., 1990), as well as the protein kinase inhibitor of PKA, PKI (Schmitt and Stork, 2001), demonstrating that cAMP's activation of Rap1 required PKA in these cells.

PKA phosphorylates Rap1 directly (Altschuler and Lapetina, 1993; Altschuler et al., 1995; Vossler et al., 1997), but the consequences of this phosphorylation are not clear (Hata et al., 1991; Hu et al., 1999; Tsygankova et al., 2001). To test the possibility that PKA activates Rap1 by phosphorylating proteins lying upstream of Rap1, we examined the ability of lysates from Forskolin-treated cells to activate Rap1 within lysates prepared from unstimulated cells. In Figure 4.1A, we show that lysates from Forskolin-treated cells retained the ability to activate Flag-Rap1 expressed in untreated cells, and this activation *in trans* was blocked by pretreatment with H89, confirming a role for PKA.

To ask whether PKA was required to activate Flag-Rap1 directly, we added H89 ten minutes after the addition of Forskolin to permit PKA-dependent phosphorylation of substrates to occur prior to mixing of Forskolin-treated and untreated Flag-Rap1-expressing lysates. Interestingly, lysates from cells in which H89 was applied after Forskolin were still able to activate Flag-Rap1 *in trans* (Figure 4.1A). Since this protocol ensures that PKA can not phosphorylate Flag-Rap1 directly, these results demonstrate that PKA can activate Rap1 via proteins upstream of Rap1.

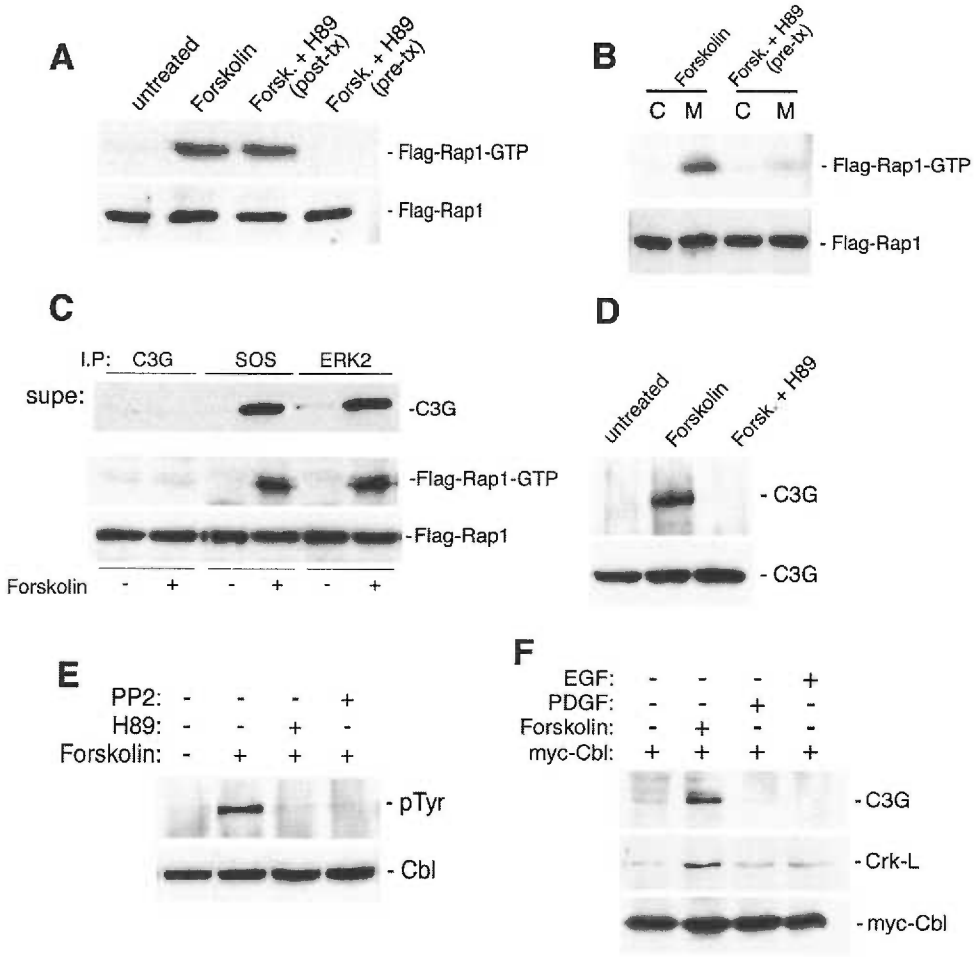

Figure 4.1. **PKA stimulation of Rap1 activity occurs via stimulation of membrane-associated C3G, Crk, and Cbl.** (A) PKA's activation of Rap1 is indirect. Separate plates of NIH3T3 cells were either transfected with Flag-Rap1 or stimulated with Forskolin. Forskolin treated cells received either a pretreatment (pre-tx) or post-treatment (post-tx) with H89. Flag-Rap1 lysates were incubated with treated lysates and analyzed for Flag-Rap1 activation (Flag-Rap1-GTP) by Gst-Ral GDS. Flag-Rap1 levels are shown in the lower panel.
(B) PKA activates Rap1 via a membrane-associated protein. Separate plates of NIH3T3 cells were treated as in Figure 1A. Forskolin treated cells received a pretreatment (pre-tx) with H89. The cytosolic (C) or membrane (M) fractions from treated lysates were prepared and incubated with Flag-Rap1 lysates and analyzed for Flag-Rap1 activation as in Figure 1A. Flag-Rap1 levels are shown in the lower panel.
(C) C3G is necessary for Rap1 activation by PKA. Membranes of lysates from untreated or Forskolin treated NIH3T3 cells were incubated with the immunoprecipitating antibodies C3G, SOS, or ERK2, as indicated and the remaining supernatant (supe) analyzed for the presence of C3G by western blot (top panel). The membrane supernatant was then incubated with lysates from Flag-Rap1 transfected cells and analyzed for Flag-Rap1 activation (Lower panels, Flag-Rap1-GTP). Flag-Rap1 levels are shown in the bottom panel.
(D) Forskolin stimulation recruits C3G into membranes. NIH3T3 cells were left untreated or stimulated with Forskolin in the presence or absence of H89. Membranes were then analyzed for C3G by western blot (upper panel). Total cell lysates were also examined for the presence of C3G (lower panel).
(E) Forskolin stimulates Cbl tyrosine phosphorylation in a PKA and Src-dependent manner. NIH3T3 cells were treated with Forskolin in the presence or absence of H89 or PP2 and endogenous Cbl was immunoprecipitated from cell lysates and analyzed by western blot for tyrosine phosphorylation (pTyr). Lower panel: Total cell lysates were also examined for endogenous Cbl by western blot (Cbl).
(F) C3G, Crk-L, and Cbl form a complex following Forskolin treatment of NIH3T3 cells. Cells were left untreated or stimulated with Forskolin, PDGF, or EGF. Myc-Cbl was immunoprecipitated from cells and the pellets analyzed by western blot using antibodies specific for Crk-L and C3G (upper and middle panels, respectively). Total cell lysates were examined for myc-Cbl by western blot using a myc antibody (lower panel).

We next investigated whether the proteins required to activate Rap1 reside within the membrane or cytosolic components of the treated cells. Only the membrane fraction of Forskolin-treated NIH3T3 cells could activate Rap1 *in trans* and this required PKA (Figure 4.1B). Previous studies have suggested that the Rap1-GEF, C3G, might be involved in cAMP activation of Rap1 (Schmitt and Stork, 2000). In the following experiments, we asked whether removing C3G from this membrane fraction would limit Forskolin's activation of Rap1. In Figure 4.1C, we show that immunodepletion with antibodies to C3G, but not control antibodies, eliminated C3G from the membrane (supe; upper panel). Membranes immunodepleted of C3G could no longer activate Rap1 *in trans* (Figure 4.1C, lower panels), whereas immunodepletion of the related Ras exchanger, SOS, did not block Forskolin's effects. Immunodepletion of ERK2 served a negative control.

It has been proposed that C3G may be recruited to membranes upon its activation (Tanaka et al., 1994). As shown in Figure 4.1D, C3G rapidly moved into the membranes following Forskolin stimulation, and this required PKA. Taken together, these data demonstrate that PKA activation of Rap1 was indirect and required a component of the membrane fraction of treated cells that we determined to be the Rap1 GEF, C3G.

cAMP/PKA Activation of Rap1 requires Crk-L, Cbl, and Src

C3G exists in the cytoplasm in a complex with a member of the Crk family of small adaptor molecules including Crk-L, Crk-I, and CrkII (Knudsen et al., 1994; Tanaka et al., 1994). Upon stimulation by growth factors, the Crk/C3G complex is thought to be recruited to the membrane where it binds to scaffolding molecules including FRS2 (Kao

et al., 2001), IRS-1 (Sorokin et al., 1998), and Cbl (Reedquist et al., 1996; Xing et al., 2000). All of these molecules are tyrosine phosphorylated following growth factor stimulation and bind Crk upon phosphorylation via Crk's SH2 domain. Multiple scaffold molecules are expressed in NIH3T3 cells including Cbl and IRS-1 (Broome et al., 1999). IRS-1 is not tyrosine phosphorylated by Forskolin in these cells (Calleja et al., 1997). Surprisingly, endogenous Cbl was tyrosine phosphorylated following Forskolin stimulation of NIH3T3 cells (Figure 4.1E). This phosphorylation was inhibited by either H89 or PP2, a selective inhibitor of Src family kinases (SFKs) (Hanke et al., 1996), suggesting that both PKA and SFKs are required for this effect (Figure 4.1E). NIH3T3 cells express Crk-L (Figure 4.1F), an isoform of Crk that is constitutively bound to C3G (Kiyokawa et al., 1997). Forskolin treatment of NIH3T3 cells stimulated the formation of a complex containing C3G, Crk-L, and Cbl (Figure 4.1F), which was also blocked by both H89 and PP2 (data not shown). Interestingly, neither PDGF nor EGF stimulated the formation of this complex (Figure 4.1F).

To investigate whether this Cbl/Crk/C3G complex was required for Rap1 activation by PKA, we transfected NIH3T3 cells with Flag-Rap1 and one of the following mutants: CBR, a truncated form of C3G which interferes with Crk function; Cbl-ct; a carboxyl-terminal fragment of Cbl that blocks Cbl function, or a kinase-dead Src mutant (SrcK296R, or d.n.Src). As can be seen in Figure 4.2A, Rap1 activation by Forskolin was blocked by each interfering mutant. Transfection of constitutively active Src (SrcY527F, or c.a.Src) is sufficient to activate Rap1, and this also required both C3G/Crk as well as Cbl (Figure 4.2B). Thus, cAMP's activation of Rap1 involves the recruitment of C3G/Crk to a membrane-associated complex with Cbl, and Src is both

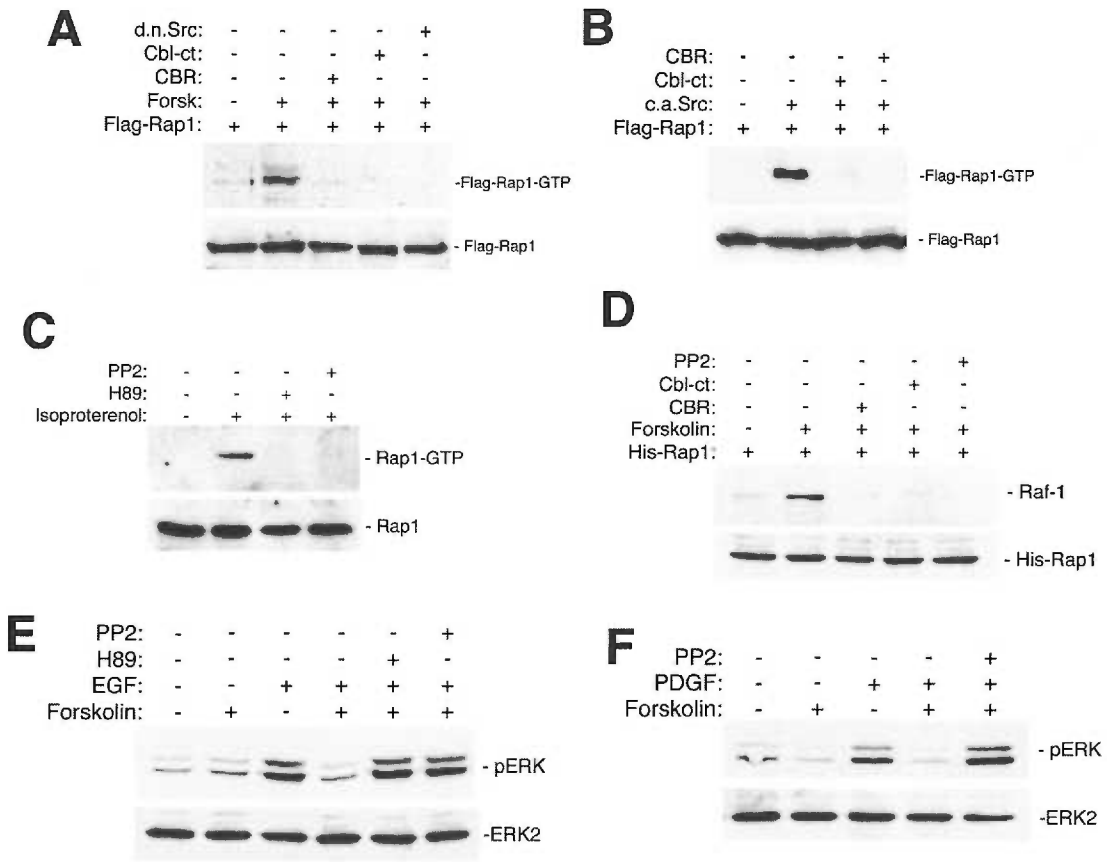

Figure 4.2. **cAMP activation of Rap1 and inhibition of ERKs occurs via C3G, Crk-L, Cbl, and Src family kinases in NIH3T3 cells**

(A) Crk, Cbl, and Src are required for Rap1 activation by cAMP. NIH3T3 cells were transfected with Flag-Rap1 along with d.n.Src, Cbl-ct, and CBR, and left untreated or stimulated with Forskolin. Lysates were then analyzed for Flag-Rap1 activation. The lower panel is a western blot control for levels of Flag-Rap1 expression.

(B) Constitutively active Src (c.a.Src) activates Rap1 via Crk and Cbl. Flag-Rap1 was co-transfected with c.a.Src into NIH3T3 cells, along with CBR and Cbl-ct. Cells lysates were then analyzed for Flag-Rap1 activation. The lower panel is a western blot control for levels of Flag-Rap1 expression.

(C) Isoproterenol activates endogenous Rap1 via PKA and SFKs. Cells were left untreated or treated with isoproterenol in the presence or absence of either H89 or PP2. Lysates were then analyzed for activation of endogenous Rap1 (Rap1-GTP). The lower panel is a western blot control for levels of Rap1 expression.

(D) C3G, Crk, Cbl, and SFKs mediate cAMP-stimulated Rap-1 association with Raf-1. NIH3T3 cells were transfected with His-Rap1 along with either CBR or Cbl-ct. Cells were left untreated or stimulated with Forskolin, as indicated. His-Rap1 was purified from lysates using a Nickel column and eluates were analyzed by western blot for the presence of Raf-1 (top panel) and His-Rap1 (lower panel).

(E) SFKs and PKA are necessary for cAMP's ability to inhibit EGF-mediated activation of ERKs in NIH3T3 cells. Cells were treated with Forskolin or EGF, in the presence or absence of either H89 or PP2. Cell lysates were analyzed by western blot for phosphorylation of endogenous ERK1/2 (pERK, top panel) or total ERK2 (bottom panel).

(F) SFKs and PKA are necessary for cAMP's ability to inhibit PDGF-mediated activation of ERKs in NIH3T3 cells. Cells were left untreated or treated with Forskolin and/or PDGF, as indicated, in the presence or absence of either H89 or PP2. Cell lysates were analyzed by western blot for phosphorylation of endogenous ERK1/2 (pERK, top panel) or total ERK2 (bottom panel).

necessary and sufficient for this action. Next, we determined whether this pathway was shared by hormones and agonists of G protein-coupled receptors (GPCRs) that are known to be linked to Gsα and cAMP. Isoproterenol, an agonist of the β2-adrenergic receptor has been shown to activate Rap1 (Schmitt and Stork, 2001). Both isoproterenol and prostaglandin PGE_1 activated Rap1 in NIH3T3 cells via PKA and SFKs (Figure 4.2C and data not shown). These data demonstrate that Forskolin and hormonal elevation of cAMP use similar mechanisms to activate Rap1.

PKA's Inhibition of ERKs and Cell Proliferation Requires Src

The antagonism of growth factor signaling by Rap1 is thought to be due to Rap1's ability to sequester Raf-1 away from Ras (Cook et al., 1993). In NIH3T3 cells, we have previously shown that cAMP/PKA triggers the association of Rap1 and Raf-1, and that this requires active Rap1 (Schmitt and Stork, 2001). In Figure 4.2D, we show that this association was blocked by CBR, Cbl-ct, and PP2 suggesting that sequestration of Raf-1 by Rap1, as well as Rap1 activation itself, required C3G, Crk, Cbl, and SFKs. Next, we investigated the requirement of SFKs in cAMP's antagonism of ERK activation. Forskolin blocked both EGF and PDGF-mediated ERK activation in NIH3T3 cells (Figures 4.2E and 4.2F), similar to what has been shown in other cells (Cook and McCormick, 1993; Graves et al., 1993). Although inhibition of SFKs had no effect on either EGF's or PDGF's activation of ERKs, it prevented Forskolin's inhibition of ERKs (Figures 4.2E and 4.2F).

To directly examine the role of Src in cAMP's activation of Rap1, we used mouse embryonic fibroblasts in which the genes encoding Src, Yes, and Fyn have been ablated

(SYF) (Klinghoffer et al., 1999). As a control, we used fibroblasts that were ablated for Yes and Fyn, but remained wild type at the Src locus (Src^{++}) (Klinghoffer et al., 1999). It has been previously shown that PDGF stimulation of ERKs and proliferation does not require Src family kinases in SYF cells (Klinghoffer et al., 1999). Therefore, this model system is well suited to examine Src's potential anti-proliferative role in cAMP signaling. As expected, cAMP was able to inhibit PDGF-mediated ERK activation in Src^{++} cells. However, cAMP was not able to do so in SYF cells (Figure 4.3A). Moreover, although cAMP robustly activated Rap1 in Src^{++} cells, cAMP did not activate Rap1 in SYF cells (Figure 4.3A, lower panel). Similar responses to cAMP (activation of Rap1, inhibition of ERKs) were seen in SYF cells that were transfected with wild type Flag-Src (Figures 4.3B and 4.3C). Taken together these results demonstrate that Src mediated cAMP's inhibition of ERK via Rap1 activation. The ability of Src to mediate cAMP's activation of Rap1 was not shared by related members of the SFK family including Yes, Fyn, and Lck (Figure 4.3D), suggesting that this action of Src is unique among SFKs.

Since the scaffolding protein Cbl appeared to be required for PKA's activation of Rap1 in NIH3T3 cells (Figure 4.2A), we next asked whether Src was required for the PKA-dependent phosphorylation of Cbl in mouse embryonic fibroblasts. As in NIH3T3 cells, Forskolin stimulated the tyrosine phosphorylation of Cbl in a PKA- and Src-dependent fashion in Src^{++} cells (Figure 4.3E), as well as SYF cells reconstituted with Flag-Src (Figure 4.3F), but did not stimulate Cbl phosphorylation in untransfected SYF cells (Figure 4.3E).

PDGF stimulation of proliferation was dependent on ERK signaling in both Src^{++} and SYF cells, as shown by experiments using the selective MEK inhibitor PD98059

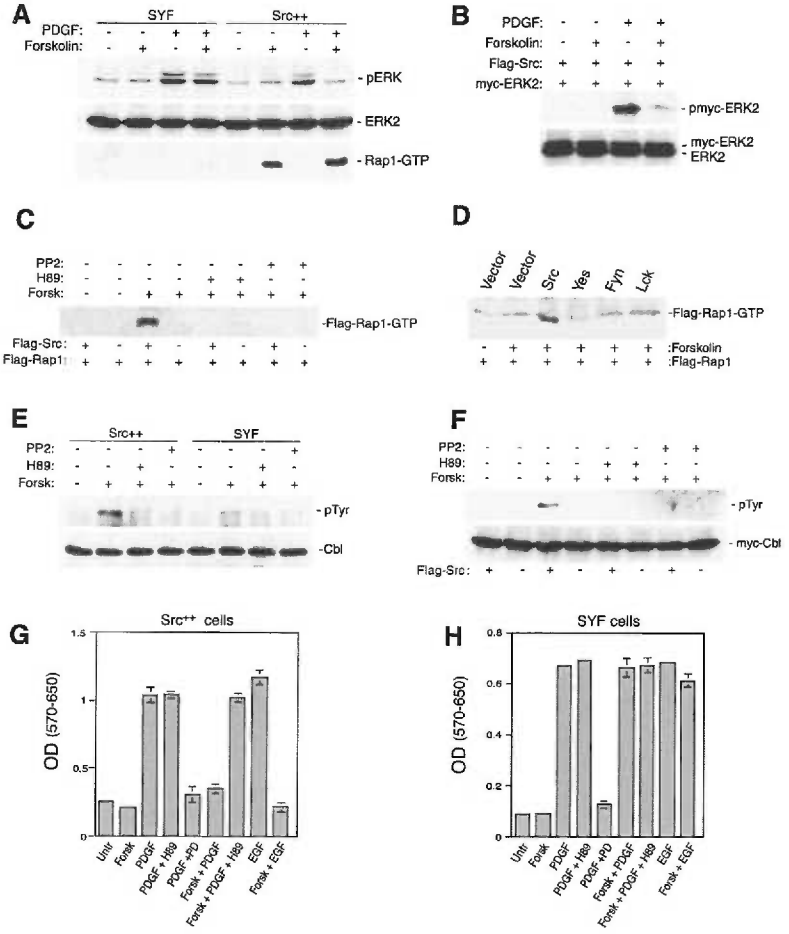

Figure 4.3. **Inhibition of growth factor-mediated activation of ERKs and cell proliferation by cAMP requires Src kinase**
(A) Src kinase is necessary for Forskolin's activation of Rap1 and inhibition of ERKs in Fibroblasts. Src++ or SYF cells were untreated or stimulated with Forskolin and/or PDGF, as indicated. Lysates were analyzed by western blot for activation of either endogenous ERK1/2 (pERK, top panel), or Rap1 (Rap1-GTP, bottom panel). Lysates were also examined for total ERKs to control for protein loading (ERK2, middle panel).
(B) Wild type Src restores the ability of cAMP to inhibit ERKs in SYF cells. Cells were transfected with the indicated cDNAs and stimulated as in panel A. Lysates were analyzed by western blot for phosphorylation of myc-ERK2 (pmyc-ERK2, top panel). The lysates were also probed with antibodies to ERK2 as a loading and transfection control. Note the detection of transfected myc-ERK2 migrating just above endogenous ERK2 (bottom panel).
(C) cAMP activates Rap1 in SYF cells that have been transfected with wild type Src. SYF cells were transfected with Flag-Rap1 and Flag-Src as indicated, and stimulated with Forskolin in the presence or absence of H89 or PP2. Cell lysates were examined for Flag-Rap1 activation.
(D) Only Src can reconstitute cAMP activation of Rap1 in SYF cells. SYF cells were transfected with Flag-Rap1 and cDNAs encoding wild type Src, Yes, Fyn, or Lck, and stimulated with Forskolin. Cell lysates were examined for Rap1 activation (Flag-Rap1-GTP).
(E) Tyrosine phosphorylation of endogenous Cbl by cAMP stimulation is rescued by Src. Src++ or SYF cells were treated with Forskolin in the presence or absence of H89 or PP2. Endogenous Cbl was immunoprecipitated from cell lysates and analyzed by western blot for tyrosine phosphorylation of Cbl (pTyr, top panel) or total Cbl protein (Cbl, bottom panel).
(F) Reconstitution of SYF cells with wild type Src restores Cbl tyrosine phosphorylation. Fibroblasts were transfected with myc-Cbl and Src, as indicated, and treated with Forskolin in the presence or absence of H89 or PP2. Myc-Cbl was immunoprecipitated from cell lysates and examined by western blot for either tyrosine phosphorylation (pTyr, upper panel), or myc-Cbl as a control for transfection and protein loading (bottom panel).
(G) cAMP stimulation blocks growth factor-stimulated cell growth in Src++ cells. Fibroblasts were treated with PDGF, EGF, Forskolin, H89, and/or PD98059 (PD), as indicated. Cells were analyzed 48 hours later by MTT assay (see experimental procedures) and data was quantified (n=4 ± S.E.).
(H) cAMP's inhibition of cell growth by PDGF and EGF is prevented in cells lacking Src kinase. Cells were treated with PDGF, EGF, Forskolin, H89, and/or PD98059 (PD), as indicated. Cells were analyzed 48 hours later by MTT assay (see experimental procedures) and data was quantified (n=4 ± S.E.).

(Dudley et al., 1995) (Figures 4.3G and 4.3H), which is similar to previous results examining EGF's action in NIH3T3 cells (Schmitt and Stork, 2001). Forskolin treatment of Src^{++} cells completely inhibited this response in a PKA-dependent manner (Figure 4.3G). However, in SYF cells, Forskolin was unable to inhibit growth factor stimulation of cell growth (Figure 4.3H). These data demonstrate for the first time a requirement for Src in cAMP's anti-proliferative actions.

PKA Phosphorylation of Src at Serine 17

To examine the mechanism of cAMP regulation of Src, Src kinase activity was assayed *in vivo* by monitoring the autophosphorylation of Src itself, on tyrosine 416 (Brown and Cooper, 1996; Martin, 2001). Forskolin stimulated phosphorylation of tyrosine 416 in both endogenous Src (Figure 4.4A) and transfected Flag-Src (Figure 4.4B). This required the kinase activities of both PKA and Src (Figures 4.4A and 4.4B). Activated Src associates with and directly phosphorylates Cbl *in vivo* (Thien and Langdon, 2001). Forskolin treatment induced an association between Src and Cbl in Flag-Src-transfected SYF cells which was also dependent on the kinase activities of both PKA and Src (Figure 4.4C). These data suggest that PKA stimulates Src kinase activity as measured by Src's autophosphorylation and association/phosphorylation with one of its endogenous substrates, Cbl.

Src contains one consensus PKA site at serine 17 (Ser17) within its amino-terminus that represents the major site of serine phosphorylation within Src (Brown and Cooper, 1996; Cross and Hanafusa, 1983; Roth et al., 1983). To provide additional evidence that PKA phosphorylates endogenous Src *in vivo*, we used an antibody designed

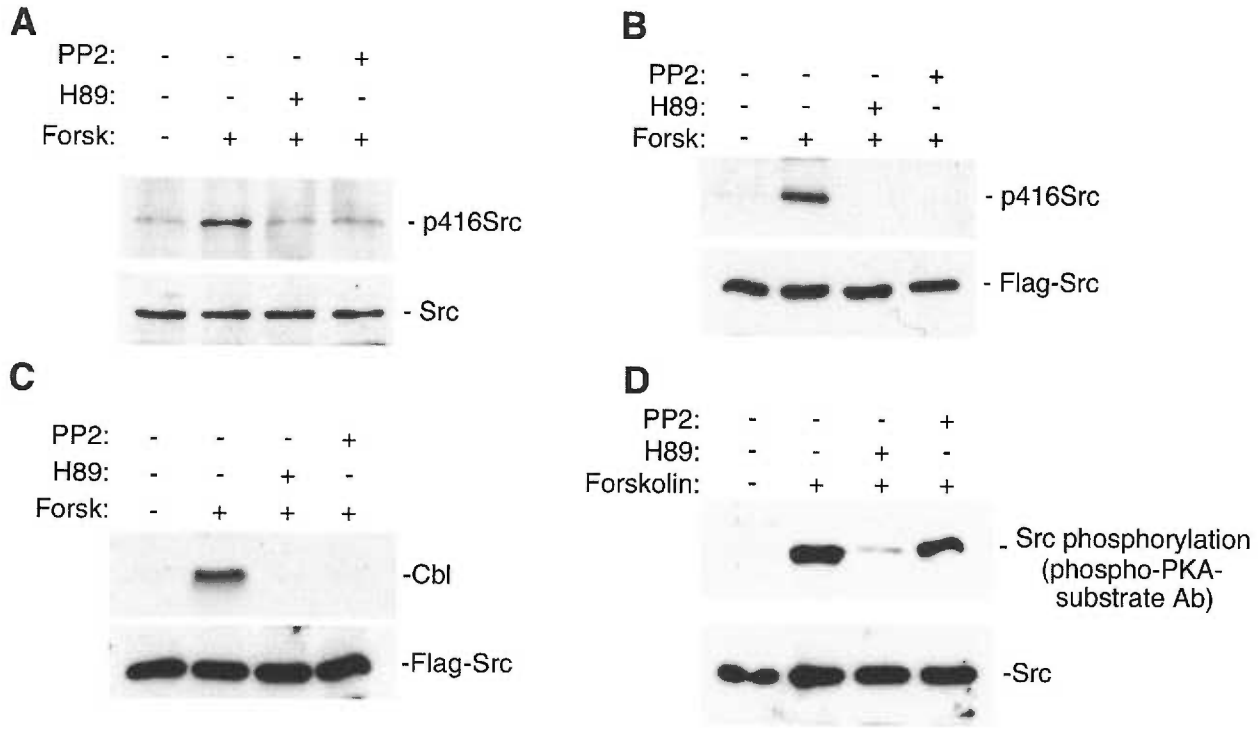

Figure 4.4. **Src is activated following phosphorylation of serine 17 (Ser17) by PKA in vivo**
(A) Endogenous Src is activated by cAMP/PKA in Src++ cells. Src++ cells were treated with Forskolin in the presence or absence of H89 or PP2, as indicated. Src was immunoprecipitated from cell lysates and analyzed by western blot for either tyrosine phosphorylation at site 416 (p416Src, upper panel) or Src (bottom panel).
(B) Exogenous Src is activated by cAMP/PKA in SYF cells. SYF cells were transfected with wild type Flag-Src and treated similarly to panel A. Flag-Src was immunoprecipitated from cell lysates and analyzed by western blot for either tyrosine phosphorylation at residue 416 (p416Src, upper panel) or Flag-Src (bottom panel).
(C) Forskolin stimulates Src/Cbl association via PKA. SYF cells were transfected with wild type Flag-Src and treated similarly to panel A. Flag-Src was immunoprecipitated from cell lysates and analyzed by western blot for either Cbl (upper panel) or Flag-Src (bottom panel).
(D) PKA stimulates phosphorylation of endogenous Src in vivo. Src++ cells were treated identically to panel A. Src was immunoprecipitated from cell lysates and analyzed by western blot for either PKA phosphorylation (Src phosphorylation, upper panel) or Src (bottom panel).

to recognize substrates phosphorylated by PKA (provided by Cell Signaling, Beverly, MA). This antibody recognizes phospho-serine/threonine residues that are preceded by arginine at the -3 position (RXXpS/T), comprising the recognition site of PKA as well as other arginine-directed kinases that are not activated by Forskolin (Kemp and Pearson, 1990). Indeed, the ability of this antibody to recognize Src protein was increased by Forskolin treatment, in a manner that required kinase activity of PKA (but not Src) (Figure 4.4D). Using this antibody, we examined the ability of PKA to phosphorylate Ser17 directly. Peptide fragments containing the N-terminal 5-25 amino acids of wild type Src (WT) were phosphorylated *in vitro* by the catalytic subunit of PKA. Control peptides with serines 12 and/or 17 replaced by alanine (S12A, S17A, and S12AS17A), serine 17 replaced by aspartate (S17D), and arginines at 14-16 replaced by alanine (R14A,R15A,R16A), as well as peptides designed to both a scrambled and an unrelated (FLAG) sequence, were also included (Figure 4.5A). Only the peptides containing Ser17 and adjacent arginines (WT and S12A) were recognized by the antibody, consistent with the consensus PKA recognition motif (Kemp and Pearson, 1990) (Figure 4.5A). These studies verify the utility of this antibody for these and subsequent studies.

Next, we examined the requirement of Ser17 for PKA phosphorylation of Src *in vivo*. For these experiments, we transfected a cDNA encoding a Src protein that had its serine at residue 17 substituted by an alanine (Flag-SrcS17A). Following transfection and Forskolin treatment, we detected phosphorylation of wild type Src but not Flag-SrcS17A (Figure 4.5B), confirming that Ser17 was the major PKA phosphorylation site located within Src that was recognized by this antibody, consistent with the findings of Erickson (Collett et al., 1979) and Hanafusa (Cross and Hanafusa, 1983). In summary,

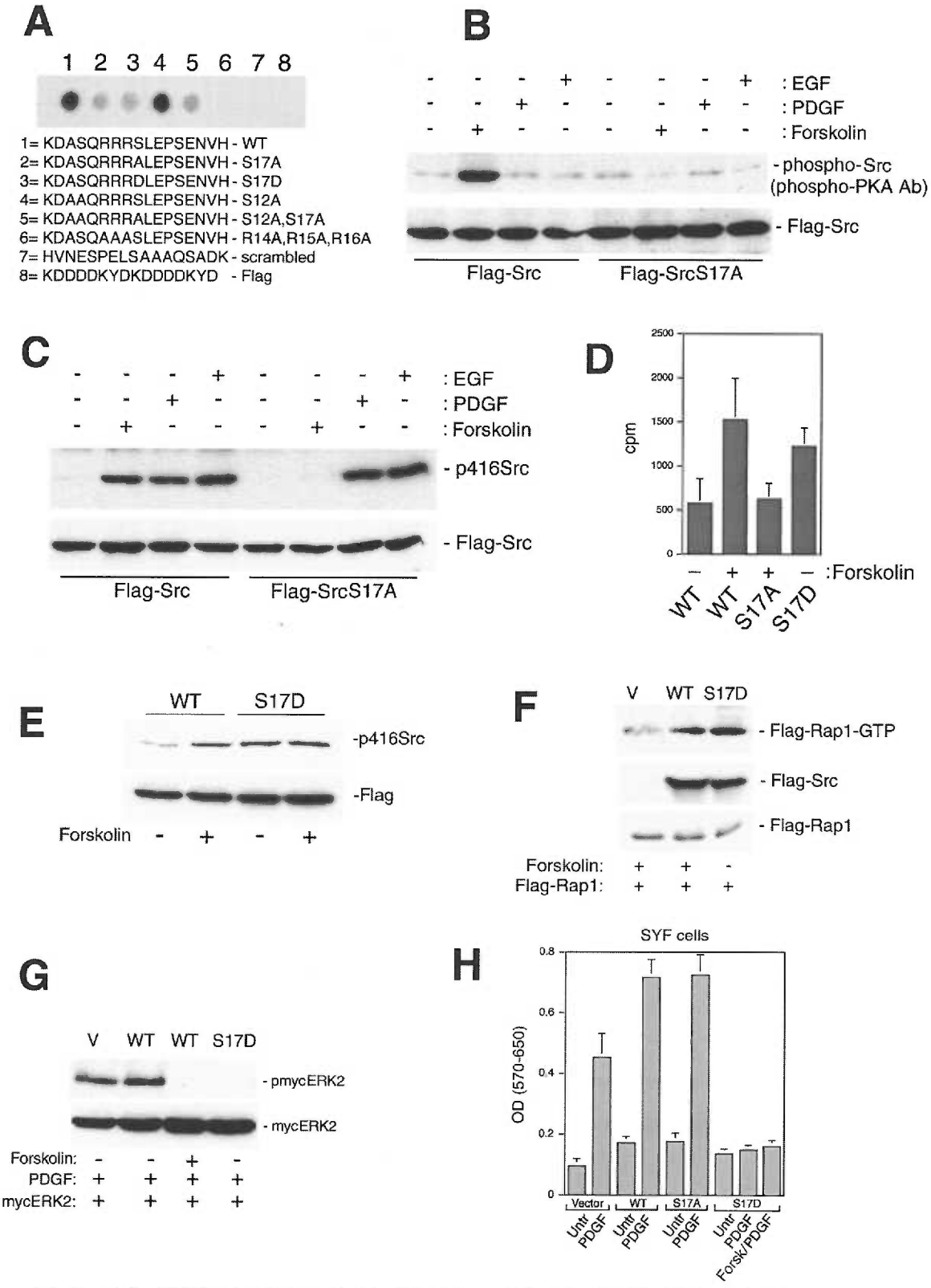

Figure 4.5. **PKA Phosphorylates Src on serine 17 *in vitro* and *in vivo***
See next page for figure legend.

Figure 4.5. **PKA phosphorylates Src on Ser17 *in vitro* and *in vivo***
(A) PKA directly phosphorylates Ser17 of Src *in vitro*. N-terminal Src peptides (whose sequences are indicated in the figure) were spotted onto membranes and incubated with the catalytic subunit of PKA. Membranes were then examined for PKA phosphorylation using the phospho-PKA substrate antibody.
(B) PKA phosphorylates wild type Src but not SrcS17A *in vivo*. SYF cells were transfected with either Flag-Src or Flag-SrcS17A and stimulated with Forskolin or PDGF or EGF, as indicated. Flag-Src was immunoprecipitated from cell lysates and analyzed by western blot for either PKA phosphorylation of Src [phospho-Src (phospho-PKA Ab), upper panel] or the presence of Flag-Src (bottom panel).
(C) Forskolin activates wild type Src, but not SrcS17A, in SYF cells. Cells were transfected with either Flag-Src or Flag-SrcS17A and treated with Forskolin and/or PDGF or EGF, as indicated. Flag-Src was immunoprecipitated from cell lysates and analyzed by western blot for either phosphorylation of Src on tyrosine 416 (pSrc416, upper panel) or the presence of Flag-Src (bottom panel).
(D) cAMP stimulates tyrosine kinase activity of wild type Src protein, but not the SrcS17A mutant. SYF cells were transfected with Flag Src, Flag-SrcS17A, or Flag-SrcS17D and treated with Forskolin as indicated. Kinase activity was assayed following Flag immunoprecipitation using RR-Src peptide as an *in vitro* substrate. Activity was measured as cpm incorporated into peptide and the data quantified (n=3 ± S.D.).
(E) SrcS17D displays constitutive phosphorylation of 416. SYF cells were transfected with either Flag-Src (WT) or Flag-SrcS17D(S17D) and treated with Forskolin, as indicated. Proteins were immunoprecipitated from cell lysates using the Flag antibody and analyzed by western blot for either phosphorylation of Src on tyrosine 416 (pSrc416, upper panel) or the presence of the Flag epitope (bottom panel).
(F) SrcS17D induces constitutive activation of Rap1. SYF cells were transfected with Flag-Rap1 and either pcDNA3 vector DNA (V) or Flag-Src (WT) and treated with Forskolin, or transfected with Flag-SrcS17D(S17D) as indicated. Lysates were then analyzed for Rap1 activation (Flag-Rap1-GTP). The lower panel is a western blot control for levels of Flag-containing proteins (Flag-Src and Flag-Rap1).
(G) SrcS17D inhibits PDGF activation of ERKs. SYF cells were transfected with mycERK2 and either vector DNA (V), Flag-Src (WT), or Flag-SrcS17D(S17D) and treated with PDGF plus or minus Forskolin, as indicated. ERK phosphorylation was assayed following myc immunoprecipitation and western blotting (pmycERK2). The lysates were also probed with antibodies to ERK2 (mycERK2) as a loading and transfection control.
(H) SrcS17D inhibits PDGF-mediated cell growth. SYF cells were transfected with either pcDNA3 (Vector), Flag-Src, Flag-SrcS17A, or Flag-SrcS17D and transfected cells were positively selected. Fibroblasts were treated with Forskolin and/or PDGF. Cells were analyzed 48 hrs later by MTT assay and data quantified (n=4 ± S.E.).

we have demonstrated the specificity of the phospho-PKA substrate antibody and have shown that Src is primarily phosphorylated both *in vitro* and *in vivo* at Ser17 by PKA, but not by EGF or PDGF (Figure 4.5B).

The activation of wild type Src by Forskolin was confirmed by two assays, autophosphorylation of Src at 416 (Figure 4.5C) and *in vitro* kinase (Figure 4.5D). Forskolin was unable to activate the SrcS17A mutant in either assay (Figures 4.5C and 4.5D). Interestingly, a Src mutant containing aspartate at position 17 (SrcS17D) showed elevated kinase activity (Figure 4.5D) and constitutive phosphorylation on tyrosine 416 that was not further increased by Forskolin treatment (Figure 4.5E). In addition, SYF cells expressing SrcS17D showed constitutive Rap1 activation (Figure 4.5F) and blocked ERK activation in response to PDGF (Figure 4.5G), and inhibited growth factor activation of cell proliferation (Figure 4.5H). This is consistent with the mutant aspartate residue mimicking serine phosphorylation at this site. These data suggest that PKA activation of Src *in vivo* requires Ser17, and this site specifically mediates cAMP's activation of Src. Importantly, EGF as well as PDGF activated both wild type Src and SrcS17A equally (Figure 4.5C). Moreover, both SrcS17A and wild type Src potentiated PDGF stimulation of cellular proliferation to the same degree (Figure 4.5H). These data demonstrate that the SrcS17A mutant retained proper protein folding for activation of catalytic activity by growth factors and was capable of participating in growth factor signaling pathways.

Ser17 Phosphorylation and Activation of Src is Required for Rap1 Activation and ERK Inhibition by PKA

To investigate the requirement of Ser17 phosphorylation of Src in cAMP's activation of Rap1, Rap1 activation was assayed in SYF cells expressing the mutant Src protein (Flag-SrcS17A). In contrast to wild type Flag-Src, Flag-SrcS17A was not able to reconstitute Forskolin's activation of Rap1 in these cells (Figure 4.6A), suggesting that PKA phosphorylation of Ser17 on Src was required for Rap1 activation. Interestingly, expression of SrcS17A inhibited the ability of Forskolin to stimulate Rap1 activation in Src^{++} cells as well, despite the presence of endogenous wild type Src in these cells (Figure 4.6B). This may suggest that overexpression of the mutant interfered with the function of endogenous Src to mediate cAMP's action. As seen in NIH3T3 cells, d.n.Src, Cbl-ct, and CBR all blocked Forskolin's actions in Src^{++} cells (Figure 4.6B). Next, we examined the role of Ser17 in Forskolin's ability to inhibit PDGF's activation of ERK. Again, only wild type Src, but not Src17A, restored Forskolin's inhibition of ERK in SYF cells (Figure 4.6C).

To determine whether Flag-SrcS17A could block cAMP's inhibition of cell growth, SYF cells were transfected with either Flag-Src or Flag-SrcS17A, selected, and assayed for cell proliferation. PDGF and EGF both stimulated SYF cell growth in an ERK-dependent manner that was blocked by PD98059 (Figure 4.6D). Expression of Flag-Src in SYF cells restored Forskolin's ability to inhibit PDGF- and EGF-mediated cell growth. In contrast, expression of Flag-SrcS17A in these cells was not able to restore Forskolin's ability to inhibit either PDGF or EGF-stimulated cell growth (Figure 4.6D). Taken together, we have shown that phosphorylation of Ser17 of Src is a primary target of PKA and that phosphorylation of this site is required for cAMP's ability to

activate both Src and Rap1, as well as to antagonize growth factor signaling to both ERKs and cell growth.

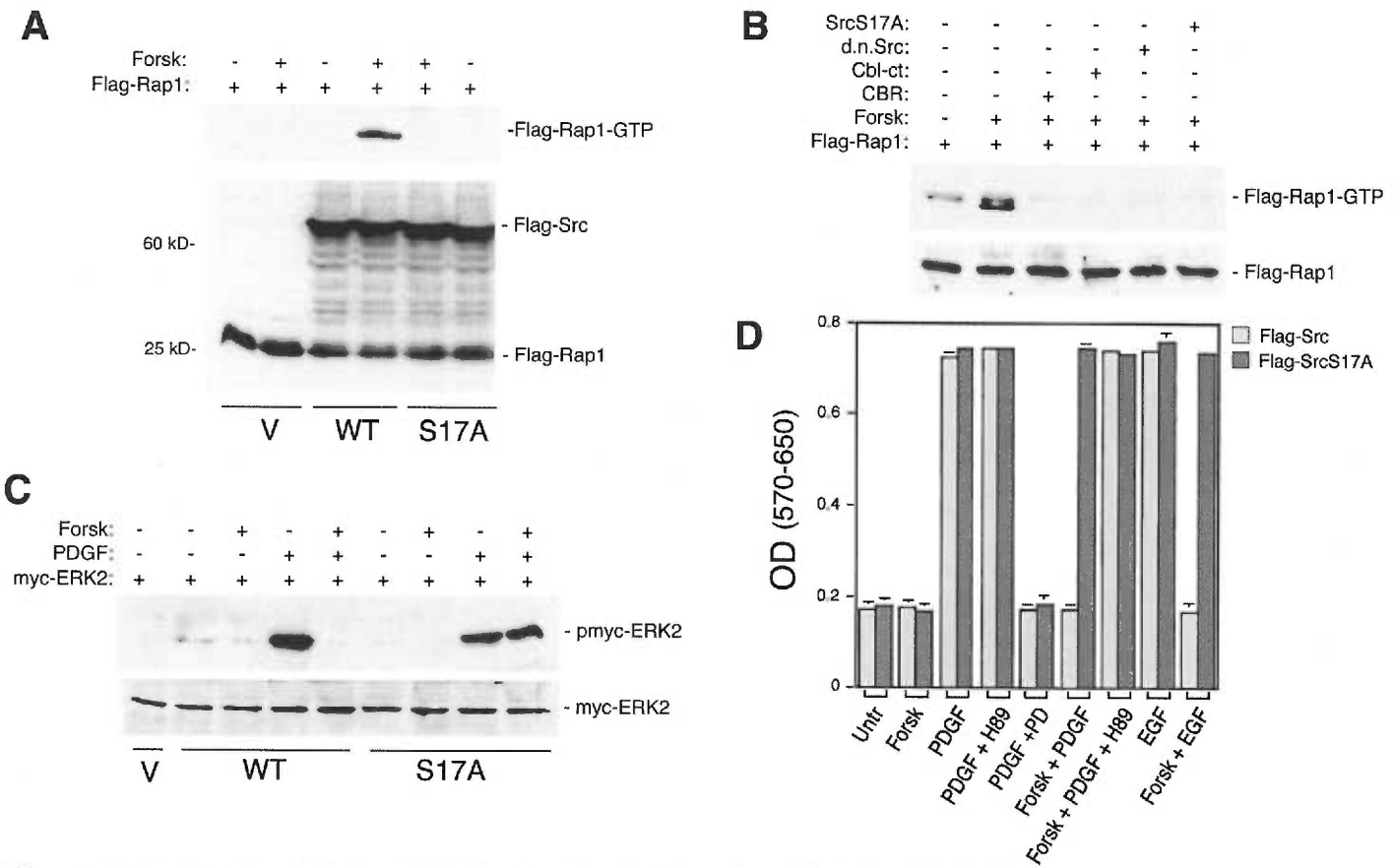

Figure 4.6. **PKA activation of Rap1 and inhibition of ERKs and cell growth require Ser17 of Src in fibroblasts**
(A) PKA activation of Rap1 in vivo requires Src phosphorylation at Ser17. SYF cells were transfected with either vector (V), Flag-Src (WT), or Flag-SrcS17A (S17A) along with Flag-Rap1 and stimulated with Forskolin. Cell lysates were examined for Rap1 activation (Flag-Rap1-GTP, upper panel) or Flag-containing proteins (lower panel).
(B) Flag-SrcS17A interferes with PKA's activation of Rap1 in Src++ cells. SYF cells were transfected with CBR, Cbl-ct, d.n.Src, and SrcS17A along with Flag-Rap1 and stimulated with Forskolin. Cell lysates were examined for activated Flag-Rap1 (upper panel) or total Flag-Rap1 (lower panel).
(C) PKA's inhibition of ERKs requires Src phosphorylation at Ser17. SYF cells were transfected with either vector (V), Flag-Src (WT), or Flag-SrcS17A (S17A) along with myc-ERK2 and stimulated with Forskolin and PDGF, alone or in combination. Myc-containing proteins were immunoprecipitated from cell lysates and analyzed by western blot for ERK phosphorylation (pmyc-ERK2, upper panel) or myc (myc-ERK2, lower panel).
(D) cAMP's inhibition of cell growth requires Src phosphorylation at Ser17 in SYF cells. SYF cells were transfected with either Flag-Src or Flag-SrcS17A and transfected cells were positively selected. Fibroblasts were treated with Forskolin, PDGF, and/or EGF, alone or following pretreatment with PD98059 (PD) or H89. Cells were analyzed 48 hrs later by MTT assay and data was quantified (n=4 ± S.E.)

DISCUSSION

PKA phosphorylation of Src

It was suggested over twenty years ago that Src was a direct substrate of PKA. In 1978, Erickson and colleagues demonstrated an increase in phospho-serine within Src's amino-terminus following treatment with cAMP (Collett et al., 1978; Collett et al., 1979) that increased the kinase activity of Src (Roth et al., 1983). A consensus PKA site at Ser17 of both v-Src and c-Src was proposed to be the major site of serine phosphorylation in both Src proteins (Takeya et al., 1982). Although deletion of amino acids 15-27 within v-Src reduced serine phosphorylation of v-Src, this did not interfere with constitutive kinase activity or oncogenicity of the mutant v-Src protein (Cross and Hanafusa, 1983), and no physiological role for phosphorylation at Ser17 has since been proposed (Brown and Cooper, 1996). Interestingly, we show here that the SrcS17A mutant also retained proper activation by growth factors, consistent with the lack of effect of this mutant on proliferative pathways. We suggest that the inability of previous studies to identify a role for this phosphorylation was due, in part, to the focus on Src's proliferative functions at that time. Here we show that the PKA site at Ser17 of Src regulated Src's anti-proliferative effects.

Src kinase in G protein signaling

Src has been implicated in hormonal signaling via other GPCR-linked pathways to activate the MAP kinase cascade. For example, mitogenic G protein signaling via thrombin, bradykinin, and lysophosphatidic acid signal through Src to couple G protein $\beta\gamma$ subunits and Src to activate Ras (Chen et al., 1994; Crespo et al., 1994; Della Rocca et

al., 1997; Luttrell et al., 1996). Similarly, other studies examining β-adrenergic receptor signaling to ERKs have identified a requirement for Src downstream of G βγ in the activation of Ras (Daaka et al., 1997).

β-adrenergic receptors can also activate Rap1 via PKA and Src (Figure 4.2C). The requirement for PKA in the activation of Src in this pathway is distinct from other models where adrenergic receptor or Gsα can activate Src directly (Cao et al., 2000; Ma et al., 2000). In cells expressing the Raf isoform B-Raf, this activation results in ERK activation (Schmitt and Stork, 2000). In many other cells that express B-Raf, cAMP activation of Rap1 can activate ERKs (Chen et al., 1999; Qiu et al., 2000; Vossler et al., 1997). It is possible that Src may be important for Rap1 activation in these and other cells where PKA and Rap1 can activate ERKs, as well as in fibroblast cells where PKA and Rap1 inhibit ERKs.

Cbl/C3G/Crk in Src signaling

The studies shown here demonstrate that the positive regulation of Rap1 by PKA is indirect, and requires the Rap1 activator C3G. C3G was the first Rap1 GEF cloned (Gotoh et al., 1995) and has recently been shown to be required for Rap1-mediated cell adhesion (Ohba et al., 2001), as well as activation of Rap1 by growth factors (Kao et al., 2001; York et al., 1998). We show here that cAMP's activation of PKA triggers the recruitment of Crk-C3G complexes to the scaffold protein Cbl (Thien and Langdon, 2001). Coupled with the previous studies that show that Crk/C3G is required for isoproterenol's activation of Rap1 (Schmitt and Stork, 2000), these data strongly suggest

that the Cbl/Crk/C3G complex is assembled following stimulation of cells by both cAMP-linked hormones and selected growth factors.

The Crk/C3G complex is recruited to a wide variety of membrane-targeted molecules following growth factor signaling. For example, Crk/C3G is recruited to the membrane by integrins (Buensuceso and O'Toole, 2000) and insulin via binding of the SH2-domain of Crk to phosphorylated membrane proteins (Okada et al., 1998; Okada and Pessin, 1997). In addition, NGF, HGF, and T cell receptors all recruit Crk/C3G to the membrane using three different docking proteins: NGF uses FRS2 (Kao et al., 2001), HGF uses Gab1 (Sakkab et al., 2000), and T cell receptors use Cbl (Reedquist et al., 1996). Interestingly, neither EGF not PDGF induced formation of this complex in NIH3T3 cells (Figure 4.1F) or Src^{++} cells (data not shown).

Src activation of Cbl/Crk/C3G can be triggered by pathways other that those initiated by cAMP/PKA. For example, Src-family kinases have been proposed to phosphorylate specific sites in Cbl to initiate binding of Crk. In lymphocytes, Cbl phosphorylation and Crk/C3G recruitment correlate with the activation of the SFK Fyn (Anderson et al., 1997; Boussiotis et al., 1997). In erythroid cells, erythropoietin induces the formation of a Cbl/Crk/C3G complex via the SFK Lyn (Arai et al., 2001a). Overexpression studies examining the activation of Src via the proteins Sin (Src interacting protein) (Alexandropoulos and Baltimore, 1996) and Cas (Crk-associated substrate) (Sakai et al., 1994) have also demonstrated a Src-dependent assembly of Crk/C3G that functions to activate Rap1 (Xing et al., 2000). Cbl has also been shown to act downstream of Src in osteoblast reabsorbtion (Tanaka et al., 1996) and upstream of Rap1 in the regulation of integrin-dependent cell adhesion of fibroblasts (Teckchandani

et al., 2001) and hematopoietic cells (Arai et al., 2001b). The studies presented here are consistent with previous reports defining Cbl as a negative regulator of ERK signaling (Rellahan et al., 1997) and cell growth (Broome et al., 1999; Murphy et al., 1998; Thien and Langdon, 2001). Although Cbl-mediated ubiquination and degradation of mitogenic signaling contributes to these actions (Lee et al., 1999; Miyake et al., 1999), we propose that Rap1 is an additional anti-proliferative target.

These studies demonstrate that Src is required for the ability of cAMP/PKA to 1) assemble a Cbl/Crk/C3G complex; 2) activate Rap1; 3) inhibit growth factor stimulation of ERKs; and 4) inhibit cell proliferation. The pathway utilized by Src is schematized in Figure 4.7.

Anti-proliferative actions of Src

By identifying an anti-proliferative action of Src coupled to the second messenger cAMP, we extend the possible physiological roles of this proto-oncogene (Thomas and Brugge, 1997). Following the discovery of c-Src as the cellular homologue of v-Src, it was clear that c-Src could not replicate the oncogenic potential of v-Src (Iba et al., 1984; Johnson et al., 1985). Physiological roles for c-Src in non-proliferative functions such as cell motility, differentiation (D'Arcangelo and Halegoua, 1993), and adhesion are now well established (Parsons and Parsons, 1997; Thomas and Brugge, 1997). However, defining its role in growth factor signaling has been more complex. SFKs have been shown to be important in mitogenesis by some (Boney et al., 2001; Broome and Hunter, 1996) but not all growth factors (Roche et al., 1995a). Src, Fyn and Yes are all activated by PDGF, yet all three SFKs appear dispensable for PDGF's activation of ERKs and stimulation of

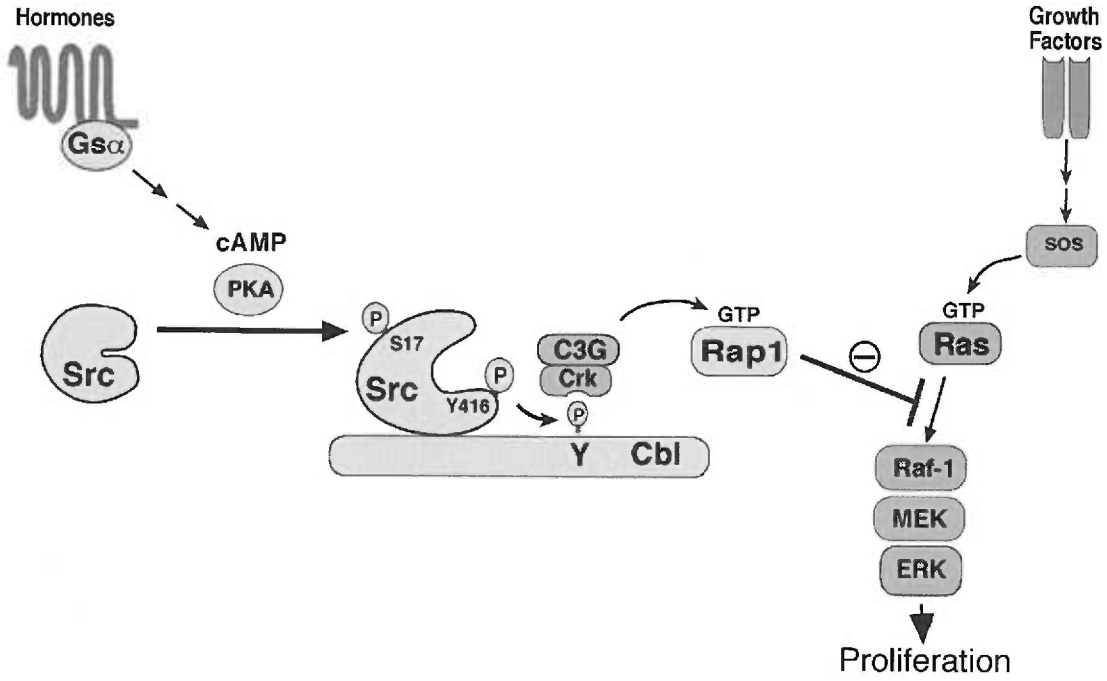

Figure 4.7. **Diagram of cAMP's inhibition of ERKs and cell growth**
Hormones binding to heptahelical GPCRs activate Gsα and adenylyl cyclase to elevate intracellular levels of cAMP. Upon cAMP activation of PKA, PKA phosphorylates Src at Ser17, triggering the activation of Src. This can be monitored by the autophosphorylation of tyrosine 416. This activation of Src results in the phosphorylation of downstream Src effectors, including Cbl. Cbl phosphorylation of tyrosines 700 and 774 by Src induces the binding of the Crk/C3G complex via the SH2 domains of Crk by bringing C3G to the membrane, where C3G activates Rap1. Upon activation, Rap1 binds to Raf-1, sequestering it from Ras, and preventing Ras activation of Raf-1, MEK, and ERK. Growth factors can potently activate Ras (by recruiting the Ras GEF, SOS, to the membrane). However, when Rap1 is activated, Rap1 prevents Ras from signaling to Raf-1, thereby inhibiting signals from Ras to ERKs and cell proliferation.

mitogenesis when examined in immortalized mouse embryo fibroblasts lacking all known SFKs (Klinghoffer et al., 1999). Consistent with this result, we show that although inhibition of SFKs blocked cAMP's inhibition of ERK, it did not block the activation of ERKs by either PDGF or EGF. This suggests that in these cells, Src activation of Rap1 and inhibition of ERKs may be more significant than Src's potentiation of ERK signaling. In other cell types, it is likely that Src can exert anti-proliferative effects downstream of cAMP, as well as proliferative effects downstream of growth factors within the same cell.

One of the surprising conclusions of this study is that specific modes of activation of Src can trigger distinct effector pathways of Src. In this regard, it is significant that SrcS17A selectively inhibited cAMP-dependent activation of Src, but retained the ability to be activated by growth factors and to potentiate growth factor-induced proliferation. We propose that hormonal activation of Src via PKA may utilize a mechanism of activation that is distinct from that used in growth factor activation of Src. Similar models have been proposed examining "ligand" activation of Src by both Sin and Cas, which results in Rap1, but not Ras, activation (Xing et al., 2000). This contrasts with the activation of both Ras and Rap1 by constitutively active v-Src (Xing et al., 2000). It has also been proposed that Ras mediates between distinct intracellular signals initiated by v-Src (Qureshi et al., 1992), suggesting that the consequences of Src activation can be regulated at multiple levels.

It is possible that the selective response of Src following activation by PKA reflects the restricted activation of downstream effectors. This restricted response may be due to phosphorylation-dependent changes in subcellular localization of Src, as

previously proposed (Walker et al., 1993) and/or the regulation of the binding of accessory proteins to Src's amino-terminus. Like other known binding proteins, this binding regulates the activity of the Src kinase domain (Sicheri and Kuriyan, 1997; Xu et al., 1999), as shown for other Src-binding proteins (Ma et al., 2000; Moarefi et al., 1997; Xing et al., 2000).

In summary, we have demonstrated a requirement for Src in cAMP's activation of Rap1, inhibition of ERKs, and inhibition of cell growth using molecular, pharmacological, and genetic tools. These actions require the direct phosphorylation of Src on Ser17 by PKA itself. This represents a novel example of a physiological regulation of Src function by PKA. More importantly, it identifies for the first time a physiological role of Src in the anti-proliferative actions of hormones linked to increased levels of intracellular cAMP.

Acknowledgments

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CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

cAMP and the regulation of Src, Rap1, ERKs and cell growth

The past 30 years has seen tremendous advances in our understanding of how hormones and cAMP regulate cellular physiology (Koyama et al., 2001). Over the last decade, it has also become well established that ERKs are important in the regulation of cellular proliferation and differentiation in many cell types (Kerkhoff and Rapp, 1998; Mazzucchelli and Brambilla, 2000). In particular, hormonal regulation of ERKs through G protein-coupled receptors (GPCRs) and cAMP has become an exciting area of cell biology. ERKs have been shown to mediate both hormonal and growth factor stimulation of proliferation and/or differentiation (Ariga et al., 2000; Schmitt and Stork, 2001; Vossler et al., 1997). One area of cell signaling that has developed in recent years is understanding how cAMP pathways may crosstalk with growth factor pathways to regulate ERKs and cell growth. In many cell types, cAMP stimulation inhibits growth factor activation of ERKs (Chen and Iyengar, 1994; Cook and McCormick, 1993) while in others cAMP may activate ERKs (Chen et al., 1999; Vossler et al., 1997). Work presented in this thesis has demonstrated the ability of cAMP to both activate (Schmitt and Stork, 2000) and inhibit (Schmitt and Stork, 2001) ERKs depending on the cell type. The small G protein Rap1 has also emerged as a critical player in cAMP's regulation of ERKs and cell growth (Schmitt and Stork, 2001). Similar to cAMP, results presented here suggest that Rap1 either activates or inhibits ERKs depending on the cell-type expression of B-Raf. More importantly, these data demonstrate for the first time that hormonal elevation of cAMP/PKA requires Rap1 as a mediator of ERK inhibition (Schmitt and Stork, 2001). Previous work from our laboratory, as well as data supplied here have also demonstrated the requirement for Rap1 in cells where cAMP activates

ERKs. Thus, cAMP signaling uses a similar signaling mechanism for both ERK inhibition and activation.

The ability of cAMP to activate ERKs was demonstrated here in Hek293 cells. This cell line was a valuable tool for examining GPCR signaling to Gs/cAMP/PKA and ERKs because they express both endogenous β-adrenergic receptors and B-Raf (Schmitt and Stork, 2000). These cells are responsive to stimulation with the β-adrenergic agonist, isoproterenol, which is crucial for examining the intrinsic signaling pathways downstream of GPCRs and Gs. The results presented in this thesis, demonstrate that hormones coupled to cAMP can activate a Rap1/B-Raf-dependent pathway to activate ERKs in Hek293 cells. The physiologic outcome of this pathway was not examined in these cells and may provide an area for future study in the laboratory. However, the activation of this pathway has been shown to have important physiologic outcomes in PC12 cells, where cAMP promotes neuronal differentiation (Vossler et al., 1997). Interestingly, isoproterenol has also been shown to induce hypertrophy of primary mouse cardiac myocytes (Xiao et al., 1999; Zou et al., 1999) and increase ERKs and long term potentiation in the CA1 area of the hippocampus (Vanhoose et al., 2002). Indeed, it was suggested that the Rap1/B-Raf module may play a role in mediating these cellular responses in the hippocampus (Vanhoose et al., 2002) .

Isoproterenol stimulation of Hek293 cells also activated the small G protein Ras. Rap1 and Ras activation proceeded nearly simultaneously, however active Ras was unable to bind to Raf-1 and contribute to ERK activation (See Figure 5.1). This is in contrast to previous work suggesting that Ras activation by the β-adrenergic receptor may activate ERKs (Daaka et al., 1997). One explanation for these differences may be that

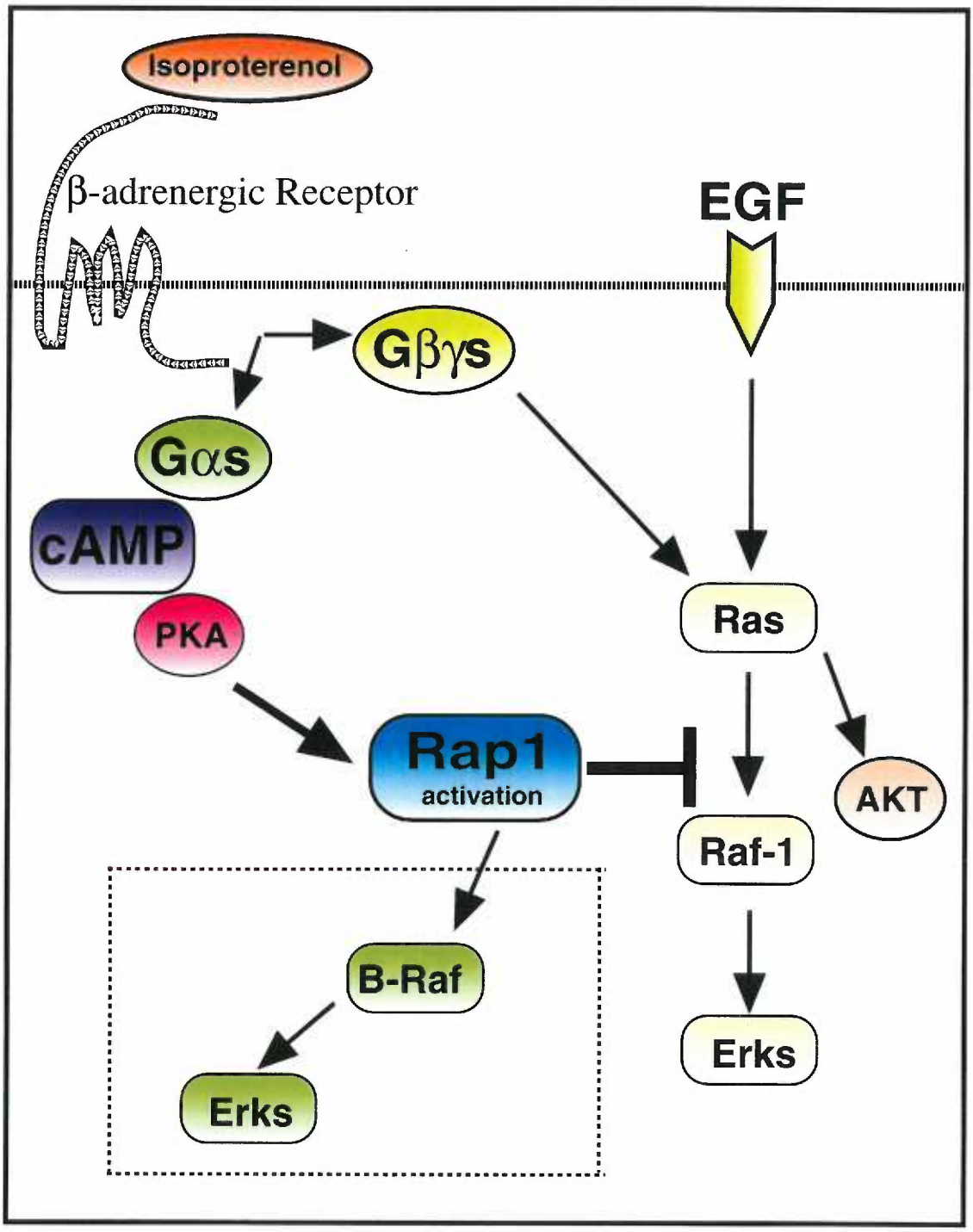

Figure 5.1. **Model for β-adrenergic receptor activation of ERKs.** Isoproterenol stimulation of the β-adrenergic receptor, in Hek293 cells, results in the activation of Rap1 through Gαs, cAMP, and PKA. Rap1 activation results in stimulation of B-Raf and ERKs. Ras is activated by isoproterenol through the Gβγs subunits or growth factors. Rap1 activation blocks Ras activation of Raf-1 and ERKs, but not AKT.

Ras signaling to ERKs was suggested by studies which over expressed the β-adrenergic receptor (Pierce et al., 2000). Over expression of GPCRs have been shown to cause several deleterious effects including augmentation of ERK activation (Wang et al., 2000) and receptor hetero-dimerization which may cause enhanced signaling activities (Liu et al., 2000; Rocheville et al., 2000). Furthermore, the precise role of Ras in ERK activation was not examined with interfering mutants of Ras, but rather with molecules believed to interfere with Ras activators (Daaka et al., 1997). Interestingly, data described in this thesis does demonstrate the first example of isoproterenol's ability to activate the serine/threonine kinase AKT through a βγ and Ras pathway. Subsequent studies have confirmed AKT activation through the βγ subunits following β-adrenergic stimulation (Schmidt et al., 2001b). AKT has previously been shown to be stimulated downstream of Ras activation (Datta et al., 1996; Downward, 1998b; Gille and Downward, 1999). AKT is a serine/threonine kinase known to participate in a variety of cellular effects including cell survival, adhesion, and growth (Downward, 1995a; Downward, 1995b; Downward, 1998a; Downward, 1998b). Interestingly, GPCR and Gβγ activation of CHO cell growth has been shown to be mediated by AKT (Sellers et al., 2000). Examining the mechanism of isoproterenol's activation of AKT and its physiological significance is a goal of future studies.

Isoproterenol simultaneously stimulated both a Rap1/B-Raf/ERK and Ras/AKT pathway (See Figure 5.1). Recent data has suggested that Ras activation by GPCRs may occur through a switching from Gs to Gi followed by transactivation of receptor tyrosine kinases (RTKs) (Daaka et al., 1997; Maudsley et al., 2000; Pierce et al., 2000). We have shown that the Rap1 pathway required a Gsα/cAMP/PKA while the Ras pathway did not.

Surprisingly, Ras activation proceeded primarily through a Gi and RTK-independent mechanism. This is the first data to suggest that the Gsβγ subunits mediate Ras activation in a signaling pathway downstream of GPCRs. This is consistent with the observation that the βγ subunits may mediate Ras activation (Luttrell et al., 1997) however, the precise mechanism of βγ activation of Ras has not been determined.

The inability of Ras to signal to Raf-1 and ERKs following isoproterenol stimulation was a surprising result. This data suggested that cAMP and/or Rap1 may be antagonizing Raf-1 activation in Hek293 cells. cAMP and PKA have been proposed to inhibit growth factor activation of Raf-1 and ERKs in several cell types (Burgering et al., 1993; Cook and McCormick, 1993; Wu et al., 1993). A number of mechanisms have been suggested for this inhibition including direct phosphorylation of Raf-1 by PKA *in vitro* (Wu et al., 1993). As mentioned previously, PKA is unable to directly inhibit Raf-1 *in vivo* (Sidovar et al., 2000). Work presented here and elsewhere suggests that Rap1 is a candidate protein to antagonize growth factor activation Raf-1 and ERKs (Cook et al., 1993). Rap1 has previously been shown to inhibit Ras activation of ERKs and cell growth in fibroblasts (Cook et al., 1993; Kitayama et al., 1989). In addition, work presented within this thesis clearly demonstrates the importance of cAMP/PKA to activate Rap1 (Schmitt and Stork, 2002; Schmitt and Stork, 2000; Schmitt and Stork, 2001). Therefore, we examined both the biochemical and physiological contribution of Rap1 in cAMP's inhibition of ERKs and cell growth in NIH3T3 cells. Importantly, NIH3T3 cells do not express B-Raf (Vossler et al., 1997). Consistent with previous studies, both epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulated Ras, Ras/Raf-1 association, ERK, and cell growth in NIH3T3 cells (Marais et

al., 1998; Marais et al., 1995; Marshall, 1999; Roche et al., 1995b). cAMP stimulation but not growth factors activated Rap1 in these cells however, previous work has suggested that growth factors may stimulate Rap1 in certain cell types (Garcia et al., 2001; Posern et al., 1998; Sakkab et al., 2000; Zwartkruis et al., 1998). For example, EGF and NGF have both been shown to activate Rap1 in neuronal cells (Kao et al., 2001; Vossler et al., 1997; York et al., 1998). The varying ability of specific growth factors to activate Rap1 may be due to cell-type specific effects or different timepoints of activation analysis. cAMP stimulation of NIH3T3 cells both dose- and time-dependently activated Rap1 and blocked ERK activation (Schmitt and Stork, 2001). Interestingly, the EC_{50} for Rap1 activation and IC_{50} or ERK inhibition displayed similar values. Moreover, cAMP's activation of Rap1 blocked Ras/Raf-1 association, and recruited endogenous Raf-1 onto Rap1 in NIH3T3 cells. Taken together, this data would suggest that Rap1 may be mediating cAMP's inhibition of ERKs. To examine the requirement for Rap1 in cAMP's inhibition of ERKs and cell proliferation we employed a molecular approach.

Several molecular tools have been generated to examine the signaling role of Rap1 and Ras proteins including, constitutively active mutants, interfering mutants, inhibitory GTPases (GAPs), and activators (GEFs). Expression of the constitutively active Rap1, RapV12, recruited Raf-1 association and inhibited both growth factor- and Ras-mediated activation of ERKs (Schmitt and Stork, 2001). Some studies have disputed the ability of the interfering mutant, RapN17 to block Rap1 activation (van den Berghe et al., 1999) however, our laboratory has made use of the Rap1 inhibitory protein, Rap1Gap1 (Schmitt and Stork, 2001; York et al., 2000). Activation of Rap1 but not Ras was completely inhibited by Rap1Gap1 moreover, cAMP's ability to inhibit growth factor stimulation of

ERKs and cell growth was absent in cells expressing Rap1Gap1 (Schmitt and Stork, 2001). These data are the first to demonstrate a requirement for Rap1 in mediating cAMP's inhibitory effects in fibroblasts (See Figure 5.2). Moreover, the actions of Rap1 are consistent with the original observation that Rap1 may antagonize ERKs and Ras-dependent cell transformation (Cook et al., 1993; Kitayama et al., 1989). Studies examining Rap1 activation in fibroblasts have also shown its ability to bind Raf-1(Okada et al., 1998; Okada and Pessin, 1997), but Rap1 is unable to activate Raf-1 (Okada et al., 1999). Taken together, these data argue that Rap1 antagonizes ERK activation and cell growth by binding and sequestering Raf-1 away from Ras. It is important to note that the requirement for Rap1's binding to Raf-1 to inhibit ERKs has not been directly tested. However, one recent study has also suggested that Rap1's ability to bind Raf-1 may indeed be important for inhibiting Raf-1 signaling (Li et al., 2002).

A major focus of this thesis is that Rap1 is an important regulator of cAMP signaling. The results from this thesis and elsewhere (Altschuler et al., 1995), have suggested that Rap1's activation by GPCRs and cAMP require PKA activation. In particular, the ability of cAMP to activate Rap1 and inhibit ERKs and cell growth was PKA-dependent (Schmitt and Stork, 2001). The mechanism for how PKA may activate Rap1 has been the focus of a number of studies including results presented here. The inability of PKA to directly activate Rap1 (Altschuler and Lapetina, 1993; Schmitt and Stork, 2002) suggested that PKA may activate Rap1 by phosphorylating an upstream target of Rap1. A number of GEFs for Rap1 have recently been identified including, Cal-DAG-GEFI, Cal-DAG-GEFII, PDZ-GEF, Epac1/2, C3G, and others (Grewal et al.,

1999). Neither the mechanism for how these activators of Rap1 are regulated nor their physiological relevance have been examined.

C3G was the first activator of Rap1 to be identified and it associates with the adaptor molecule Crk-L (Gotoh et al., 1995). Early results from our laboratory suggested that NGF's activation of Rap1 may require C3G (York et al., 1998). Work presented here extends these findings to include the requirement for C3G in GPCR-stimulated activation of Rap1 by PKA (Schmitt and Stork, 2002). Moreover, PKA stimulated the recruitment of C3G and Crk-L into the membrane fraction of cells, which was required for Rap1 activation. In contrast to previous studies (Sakkab et al., 2000), C3G and Crk-L were not recruited into the membrane fraction of fibroblasts following growth factor stimulation. This is consistent with our data which suggests that Rap1 is not activated by growth factors. C3G and Crk-L where also found to be associated with the large adapter molecule Cbl following its tyrosine phosphorylation. The C3G/Crk-L/Cbl molecules were each required for Rap1 activation by PKA. Recent work has also suggested that C3G and Crk-L may associate with the adaptor and scaffold molecules Sin and Cas, respectively to activate Rap1 (Kao et al., 2001; Xing et al., 2000). These proteins appear to be important for Rap1 activation in certain model systems however, their function downstream of PKA signaling to activate Rap1 has yet to be reported. Moreover, it will also be important to examine the ability of Cas and Sin to associate with Cbl following PKA stimulation.

One surprising finding from this thesis work was that PKA activation resulted in tyrosine phosphorylation of Cbl. Cbl contains several tyrosine phosphorylation sites (Thien and Langdon, 2001). We did not examine the exact residue(s) of Cbl

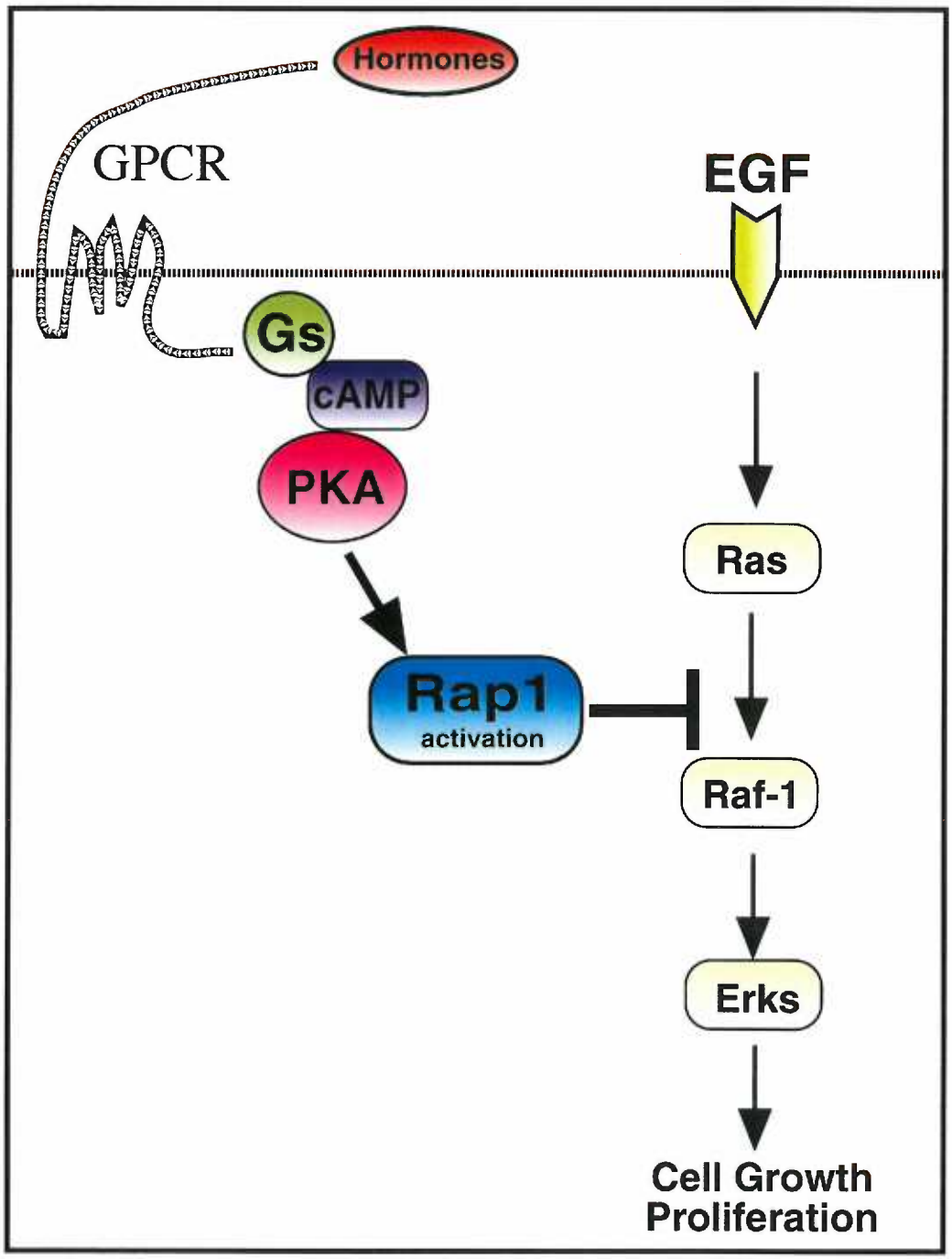

Figure 5.2. **Rap1 mediates cAMP/PKA's inhibition of ERKs and cell growth.** Stimulation of cAMP leads to Rap1 activation. Rap1 is able to bind to and antagonize Ras-dependent activation of Raf-1. Inhibition of Raf-1 by Rap1 blocks growth factor stimulation of ERKs and cell growth.

phosphorylation following cAMP stimulation, although Cbl phosphorylation could be blocked with pharmacological inhibitors of Src kinases. This would suggest that Cbl phosphorylation occurred on one or more Src-specific sites which are important for Crk-L binding to Cbl through its SH2 domain (Thien and Langdon, 2001). It will be important to determine which sites of phosphorylation are specific to PKA stimulation, and which Cbl domains are important for Rap1 activation in fibroblasts as well as other cell systems.

Our demonstration that Src is required for PKA's assembly of C3G/Crk-L/Cbl and activation of Rap1 and inhibition of ERKs raised a number of interesting questions. For example, is Src important for PKA's activation of Rap1 in other cell types? Definitive proof came with our observation that cAMP/PKA's ability to activate Rap1 and inhibit ERKs and cell growth was abolished in fibroblasts derived from mice lacking Src (Schmitt and Stork, 2002). ERK activation and proliferation by growth factors is not blocked in cells lacking Src (SYF), however cell migration is dramatically reduced (Klinghoffer et al., 1999). Therefore, these cells provide a good model system to examine the requirement of Src in cAMP's activation of Rap1 and inhibition of ERKs and cell growth. It is interesting to speculate as to whether under certain circumstances Rap1 may also play a role in Src's regulation of cell adhesion, as Rap1 has been proposed to play a role in cell adhesion (Bos et al., 2001; Ohba et al., 2001; Posern et al., 1998; Tsukamoto et al., 1999). In support of our model, recent work by Matsuda et al. demonstrated the requirement for Rap1 in C3G-dependent cell adhesion (Ohba et al., 2001). Interestingly, a role for the C3G/Crk-L/Cbl pathway to Rap1 was also suggested

recently in Src-mediated fibroblast adhesion (Li et al., 2002). It will be important to examine the role of the Src to Rap1 pathway using physiologic stimuli, such as integrins.

The ability of PKA to activate Src was observed in both fibroblasts expressing only endogenous Src (Src++) as well as the SYF cells reconstituted with wild type Src. These results are consistent with early studies which demonstrated the ability of PKA to activate Src kinase activity (Collett et al., 1979; Collett and Erikson, 1978; Roth et al., 1983). Src contains a consensus PKA phosphorylation site at serine 17, which was proposed to be the site of PKA's activation of Src (Brown and Cooper, 1996; Roth et al., 1983). Indeed, work presented within this thesis suggests that serine 17 is the only site within Src that is phosphorylated by PKA (Schmitt and Stork, 2002). This phosphorylation of Src is both necessary and sufficient for PKA to activate Rap1 and inhibit ERKs and cell growth in fibroblasts (See Figure 5.3). This work is the first demonstration for the novel ability of Src to inhibit cell growth. It will be important to examine the biochemical and physiological regulation of Src by PKA in other model systems. Previous work has suggested a role for a PKA/Src pathway to ERKs in cAMP-stimulated differentiation of PC12 cells (Minneman et al., 2000), adipocytes (Lindquist et al., 2000), and retinal pigment epithelial cells (Koh, 2000). Future studies in the laboratory will also examine whether Src plays a role in cAMP's activation of ERKs.

The structural regulation of Src kinase function by PKA phosphorylation has yet to be reported. However, it is possible to speculate that phosphorylation of Src may lead to its activation through several mechanisms including, recruitment of a cofactor to Src, steric opening of the Src molecule, redirecting Src's subcellular localization, or one or more of the above combinations. It is possible that the proximity of the serine 17

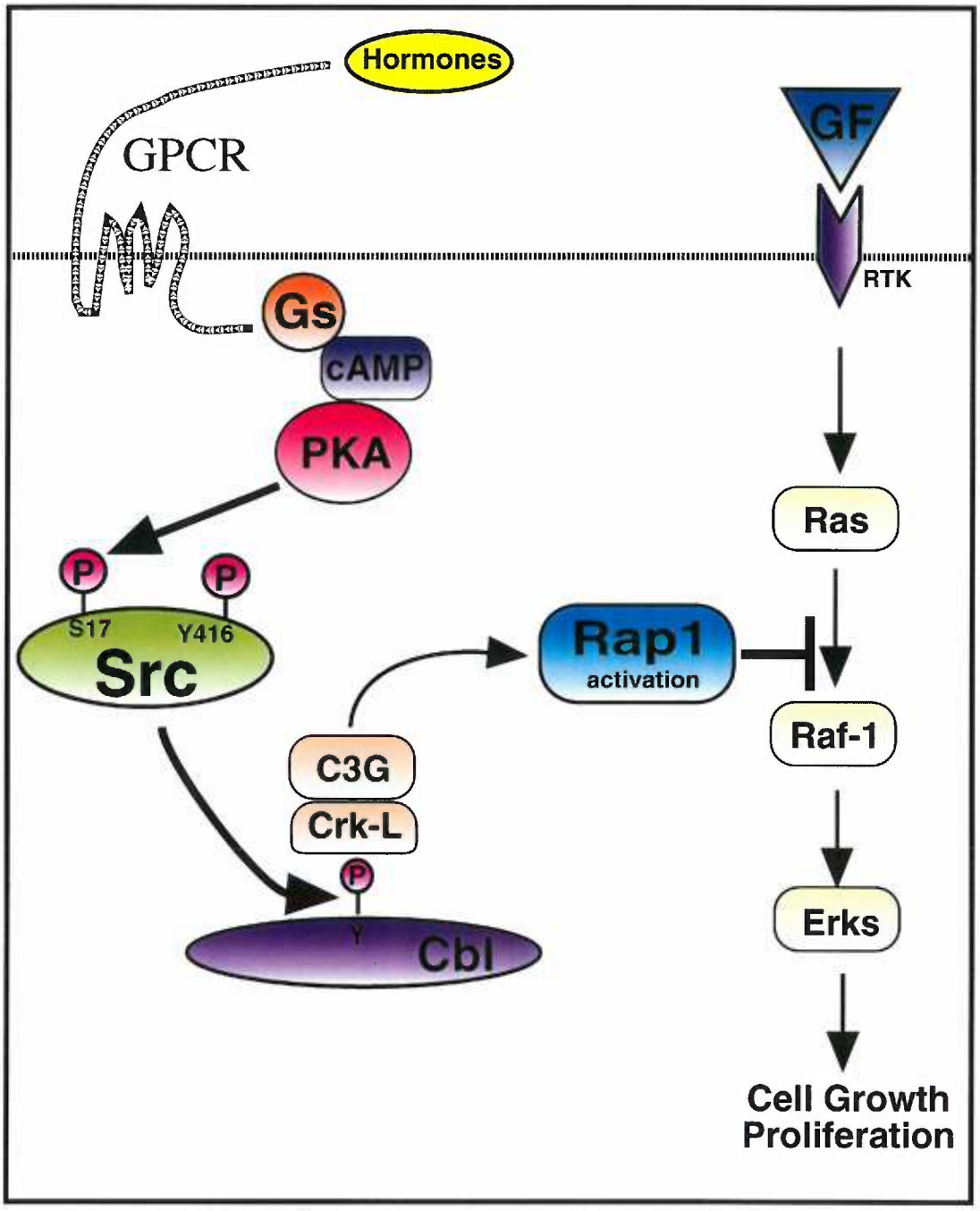

Figure 5.3. **PKA phosphorylation of Src mediates cAMP's activation of Rap1 and inhibition of ERKs and cell growth.** Stimulation of fibroblasts with hormones activates cAMP/PKA which leads to serine 17 (S17) phosphorylation of Src and Src activation (p416). Serine 17 phosphorylation of Src by PKA is both necessary and sufficient for Rap1 activation and inhibition of ERKs and cell growth, which are stimulated by growth factors (GF). Src activation by PKA also assembles a membrane associated complex of Cbl/Crk-L/C3G which are also required for Rap1 activation and ERK inhibition.

phosphorylation to the N-terminal myristoylation of Src may influence proper membrane targeting. Indeed, one previous study suggested that PKA's phosphorylation of Src may be sufficient to redirect Src's membrane localization (Walker et al., 1993). While these studies were primarily carried out in *in vitro* reconstituted membrane systems it is interesting to speculate that similar effects may be present *in vivo*. Interestingly, Src also contains two PKC phosphorylation sites near its amino-terminus which are sufficient to activate Src (Bjorge et al., 2000).

Recent studies have also suggested that Src association with the adaptor protein Cas is sufficient to activate Src leading to activation of Rap1 but not Ras (Xing et al., 2000). Whether this pathway is activated by PKA or capable of inhibiting cell growth was not examined however, this pathway was suggested to inhibit Raf-1 activation (Li et al., 2002). Taken together, the above studies reveal that several mechanisms exist for Src activation and function, but they do not explain how Src activation can be directed down a Rap1 pathway. Clearly, serine 17 phosphorylation of Src is important for directing its signaling toward Rap1. Understanding the mechanism of Src signaling following PKA phosphorylation, and its specificity, is a goal of future studies in the laboratory.

FUTURE DIRECTIONS

How does serine 17 phosphorylation of Src dictate its downstream signaling?

Src has previously been shown to be activated by growth factor stimulation furthermore, this activation has been shown to contribute to ERK activation and cell growth in fibroblasts (Broome and Hunter, 1996; Roche et al., 1995a; Roche et al., 1995b). One notable and interesting finding in this thesis was that Src activation by cAMP/PKA inhibited cell growth. This data would suggest that Src activation by PKA provides a unique regulation of Src signaling pathways. This raises the interesting question of how Src activation by different stimuli leads to distinct cellular outcomes? One way to try and address this question is to examine Src-dependent signaling pathways. Interestingly, work provided in this thesis and elsewhere suggest that stimulation of both Rap1 and Ras by the β-adrenergic receptor agonist, isoproterenol, requires Src (Daaka et al., 1997; Schmitt and Stork, 2002). To this end, we have examined whether Src is necessary and sufficient to activate Rap1 and Ras following isoproterenol stimulation (manuscript in preparation). In Hek293 cells both endogenous Rap1 and Ras activation were blocked in the presence of pharmacological inhibitors of Src. In both Hek293 cells and SYF cells, isoproterenol stimulation of Rap1 but not Ras was blocked in cells expressing SrcS17A. In addition, SrcS17D does not activate Ras but is sufficient to activate Rap1. This data, coupled with our previous results, would suggest that Src is required for isoproterenol's activation of both the Rap1/B-Raf/ERK and Ras/AKT pathways in Hek293 cells. Indeed, unpublished work suggests that PKA activation of Src specifically activates the Rap1/B-Raf/ERK pathway but does not effect Ras/AKT signaling (See Figure 5.4). It is worth noting that while Ras/AKT could be

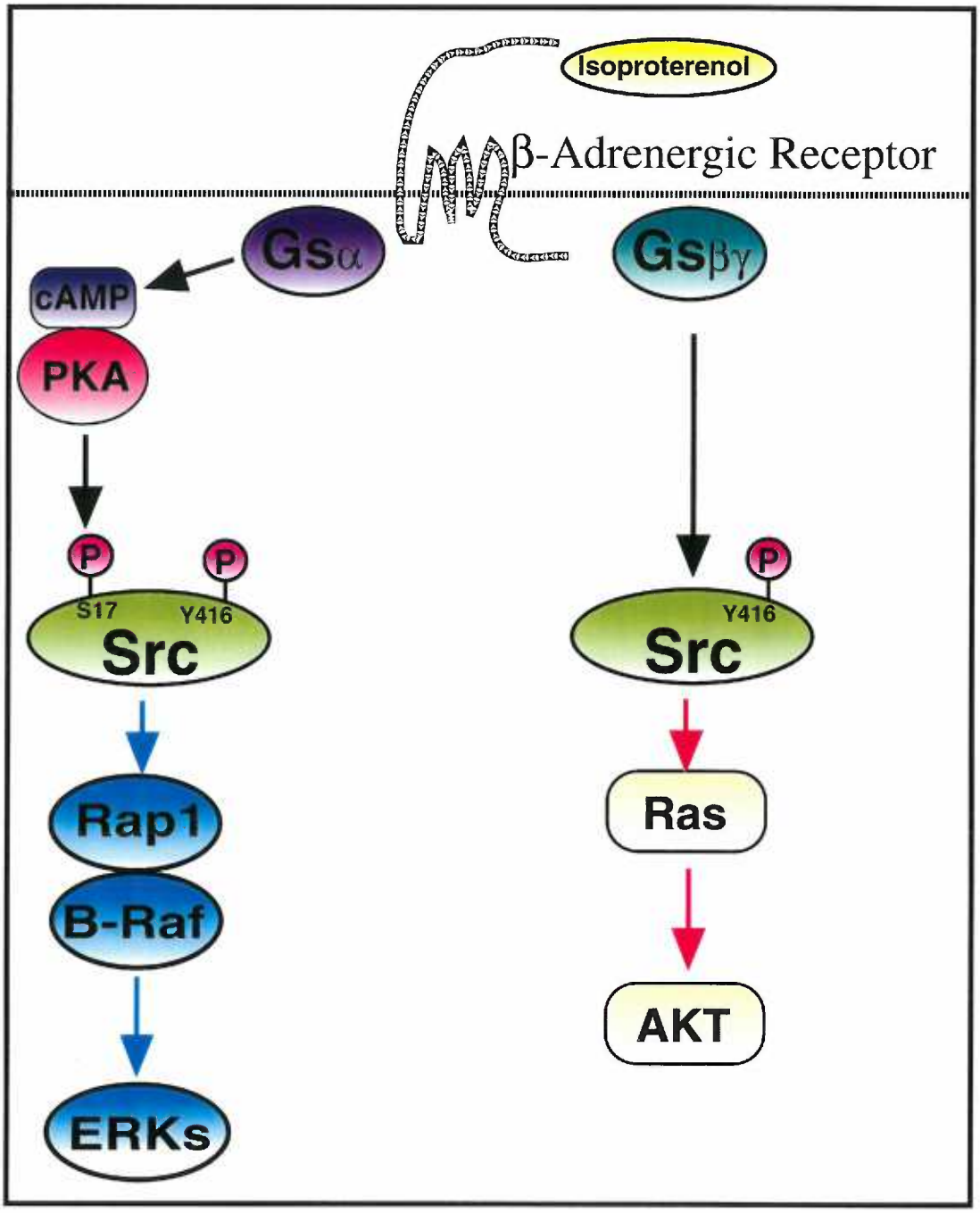

Figure 5.4. **Src phosphorylation dictates its downstream signaling.** Stimulation of the β-adrenergic receptor with isoproterenol activates two distinct Src-dependent pathways, in Hek293 cells. One pathway utilizes Gsα, PKA, Src, Rap1, to activate ERKs which is dependent upon Src phosphorylation at serine 17 (S17). An additional pathway utilizes the Gsβγ subunits to activate Src, Ras, and AKT however, serine 17 phosphorylation of Src does not contribute to Ras/AKT activation.

activated by isoproterenol, Ras/AKT activation could be augmented by overexpressing any of the Src plasmids. This data would suggest that Src is utilized by the Ras pathway and that the Src mutants function as wild type Src in pathways other than Rap1 activation. The physiologic consequences of the activation ERKs and AKT in Hek293 cells or other cell types are an area for future examination in the laboratory.

The distinct actions of Src may be achieved by its interaction with a specific cofactor(s). In other words, does Src phosphorylation by PKA induce a novel protein-protein interaction? Current studies in the lab have been directed at examining Src's interaction with proteins following its phosphorylation by PKA (See Figure 5.5). One approach we have taken is to transfect Hek293 cells with Flag-Src, Flag-SrcS17A, Flag-SrcS17D, or pcDNA3 and stimulate the cells with Forskolin or leave them untreated. Following a Flag immunoprecipitation from cell lysates, the proteins have been subjected to SDS polyacrylamide gel, and silver-stained for unique protein band associations. Preliminary results suggest that Src may be interacting with a protein in the 80-90 kilodalton size range. Examination of the literature did not suggest any known Src interacting proteins in that range therefore, the protein was purified and analyzed by mass spectroscopy. Future work will be directed at analyzing the protein masses to determine the interacting protein. If a candidate protein is identified, a number of interesting hypotheses could be imagined. For example, is this protein required for Rap1 activation by PKA and Src? Does this protein confer Src signaling specificity to Rap1 and inhibition of ERKs and cell growth? How does this protein interact with Src and what are the structural requirements for this interaction? Examining these and other questions

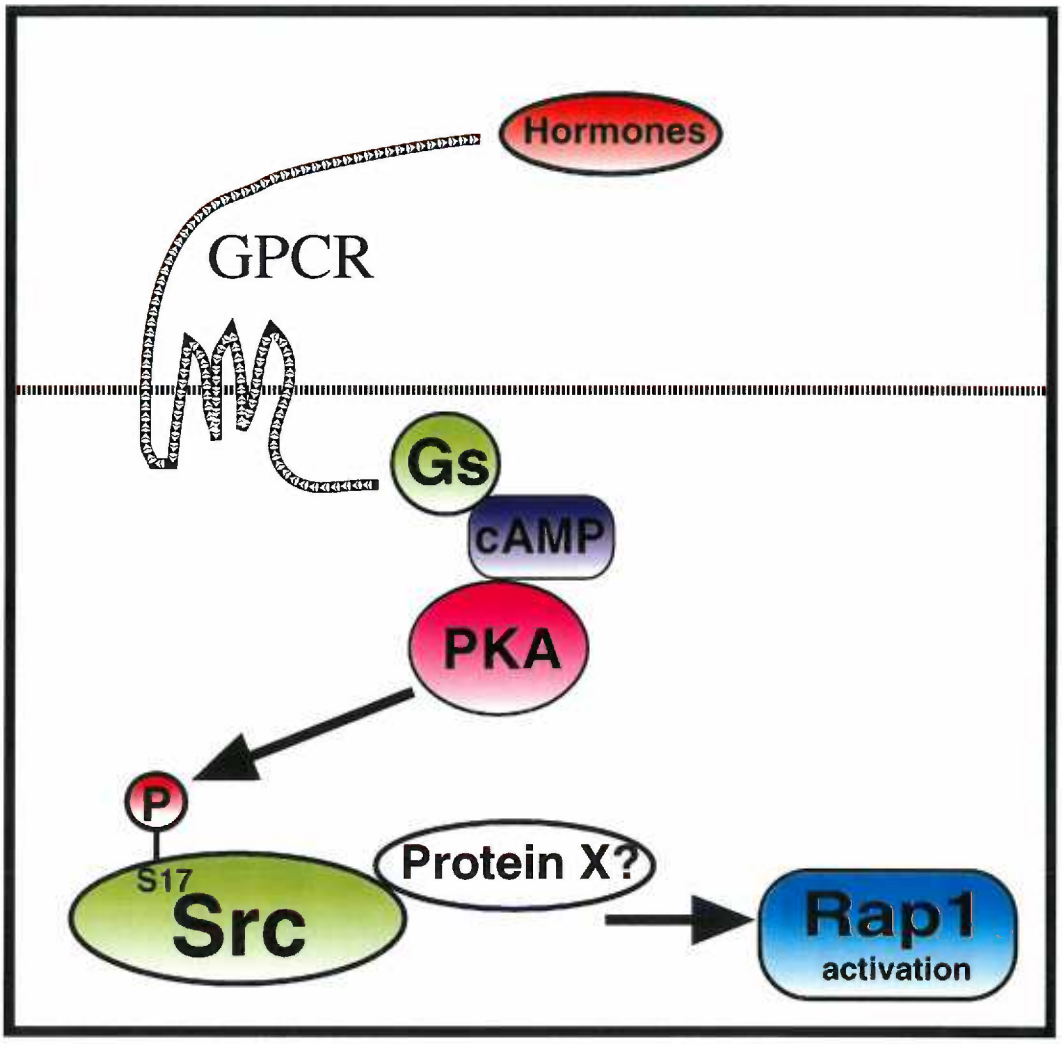

Figure 5.5. **PKA phosphorylation of Src may mediate Src's association with an unknown protein.** It is possible that serine 17 phosphorylation of Src by PKA may serve to recruit a cofactor(s) to Src. These proteins may regulate Src's ability to selectively activate Rap1.

may provide simple yet important clues for further understanding how hormones and cAMP regulate Rap1, ERKs, and cell growth.

In conclusion, the primary focus of this thesis was to further understand the role of Rap1 in cAMP signaling. In this regard, work presented here demonstrates that cAMP's activation of PKA, Src, and Rap1 mediate its signaling to ERKs. In addition, activation of Src and Rap1 by PKA in fibroblasts inhibited ERKs and cell growth. These studies provide a strong groundwork for future studies examining Src and Rap1 signaling in fibroblasts as well as other cell types.

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