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Regulation of *ras* signaling by Gs-alpha

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REGULATION OF *RAS* SIGNALING BY Gs-ALPHA

by

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ABSTRACT

REGULATION OF *RAS* SIGNALING BY Gs-ALPHA

By

JIANGHAO CHEN

Advisor: Professor Ravi Iyengar

Mutant activated α-subunits of G proteins (α*) have been postulated to be oncoproteins. α_s* and α_i* have been found in human pituitary and ovarian tumors respectively. α_i*, α_o* and α_q* are oncogenic as assessed by *in vivo* and *in vitro* experiments. Although α_s* was the first mutant α-subunit identified in tumors, it has not been shown to have oncogenic potential. I have examined whether α_s* had this transforming ability. Two cell types, NIH-3T3 and RAT-1, that show different proliferative responses to cAMP were used in my study. In NIH-3T3 cells, increases or decreases in cellular cAMP levels alone do not have any effects on cell proliferation. On the other hand, lowering of cellular cAMP levels strongly stimulates cell proliferation in RAT-1 cells. Clonal NIH-3T3 and RAT-1 cell lines that expressed α_s* were established and examined. Expression of α_s* in these cell lines modestly raises cellular cAMP levels; however, this expression did not result in transformation of

these cells and did not affect mitogenesis. α_s* may be nontransforming by itself because it is a weak oncogene that functions best in the presence of another oncogene. Hence, I tested whether α_s* promotes H-*ras*-induced transformation. Surprisingly, α_s* substantially blocked *ras*-induced transformation of NIH-3T3 cells and inhibited *ras*-stimulated DNA synthesis in these cells. Expression of α_s* did not affect transfection efficiencies or expression of H-*ras* protein. Similarly, H-*ras*-induced transformation of RAT-1 cells is also blocked by α_s*.

I studied the possible mechanisms which underlie the inhibitory effects of α_s* on *ras*-induced transformation. It has been demonstrated that proliferative signals from *ras* are routed through the MAP kinase pathway. I found that *ras*-stimulated MAP kinase activity was significantly reduced by α_s*. Furthermore, I have also shown that a cAMP analog, 8 Br-cAMP, suppressed *ras*-induced transformation of NIH-3T3 cells and decreased MAP kinase activity. The effect of α_s* on the *ras*-induced transformation can be relieved by dominant negative regulatory subunits of protein kinase A. These data suggest that α_s inhibits proliferative signals from *ras* by stimulating cAMP production and activating protein kinase A. The antiproliferative effect of α_s may be achieved by attenuation of *ras* signaling through the MAP kinase pathway.

DEDICATION

This dissertation is dedicated to my husband, Xingqiang Li, and
my adorable daughter, Jennifer, and
my father and my mother.

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FORMAT OF THESIS

This thesis is prepared according to the guidelines of Graduate School of Biological Sciences, Mount Sinai School of Medicine, City University of New York. The thesis has a general introduction with literature review, several chapters summaring the experimental results and a discussion section.

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CHAPTER 1

INTRODUCTION

All living cells are capable of receiving and processing information from their surroundings. Light, odorants and dietary chemicals are external signals which stimulate target cells in specialized sensory organs. Hormones, neurotransmitters and growth factors released from target cells, on the other hand, carry messengers from one cell to another cell. The biochemical basis of signal transduction across cell membranes has long been a subject of intense research. The major components of the signaling systems that eventually lead to the regulation of physiological processes such as cell to cell communication, cell proliferation and cell differentiation are now known. One commonly used method of cellular communication involves transduction at the cell surface. In such systems, the extracellular messengers first interact with their specific receptors at the cell surface. Stimulation of these receptors often results in activation of effector proteins, such as enzymes or ion channels. These effectors then mobilize chemical "second messengers" that initiate specific actions within the cell. A family of heterotrimeric GTP-binding and hydrolyzing proteins (G proteins) is one set of proteins which act as switches that regulate information circuits connecting cell surface receptors to a variety of effectors in all eukaryotic organisms. This family of G proteins belongs to a superfamily of GTPases that oscillates between active and inactive states depending on whether GTP or GDP is bound. The superfamily of GTPase also includes small GTPases of low molecular weight (about 20-30 KDa) which have variety of different cellular functions.

A. GTPase superfamily

1) Function of GTPase superfamily.

The GTPase superfamily can be divided into several families according to their cellular functions (Bourne et al., 1990).

The first group includes elongation and initiation factors which are involved in many steps of protein synthesis. These GTPases play a role in formation of initiation complex, kinetic proofreading of the match between codon and anticodon and translocation of peptidyl-tRNA from the A to the P site on the ribosome during protein synthesis.

G proteins are heterotrimeric proteins composed of α–, β–, and γ–subunits (Iyengar, 1991). They are associated with transduction of specific signals across cell membrane. The α-subunits bind and hydrolyze GTP and share regions of homology, particularly GDP/GTP binding and GTP hydrolysis, with other GTPases.

The *ras* family of GTP-binding proteins includes more than fifty proteins of low molecular weight (about 20-29 KDa) (Bourne et al., 1990; Bokoch and Der, 1993). For example, the three *ras* genes of mammals, H-*ras*, K-*ras* and N-*ras*, encode 21K proteins that control regulatory pathways critical for proliferation and differentiation of cells. *Ras* 1 and *Ras* 2 gene products stimulate adenylyl cyclase in yeast. Rap 1 is highly homologous to *ras* protein and can revert transformation by K-*ras* when overexpressed. Rab and Sec 4 are involved in intracellular vesicular trafficking. Rho plays a role in cytoskeletal control. ARF is an ADP-

ribosylation factor and is required for ADP-ribosylation of α_s (one of the G protein α-subunits) catalyzed by *cholera toxin* (Bourne et al., 1990).

2) The universal GTPase cycle.

All GTPases share some conserved features. They all have GTP-binding sites and GTPase activity. The GTPases go through a similar cycle of reactions to exert their functions (Fig. 1) (Bourne et al., 1990). The protein in GDP-bound form is inactive and active in the GTP-bound form. The 'empty state' usually serves as a transient intermediate between the two guanine nucleotide binding states. The irreversibility of GTP hydrolysis makes the cycle unidirectional.

Generally, the concentration of GTP in the cell is about 100 μM and is ten times higher than GDP concentration in cells (Gilman, 1987). It is likely that GTP rapidly binds to the empty guanine nucleotide binding site of the α-subunit. The fraction of GTPases in the active state, GTP-bound form, is determined by the outcome of two reactions. First, it depends on rate of dissociation of GDP from the GTPase binding site. The rate of this reaction is characterized by rate constant $K_{diss \cdot GDP}$. The second one is the hydrolysis of bound GTP. This rate is characterized by $K_{cat \cdot GTP}$. Thus the ratio of GTPase·GTP / GTPase·GDP equals the ratio of $K_{diss \cdot GDP}$ / $K_{cat \cdot GTP}$. This equation suggests that increasing the relative portion of the active GTP-bound form of GTPases over the inactive GDP-bound form of GTPases can be achieved either by

accelerating the dissociation of GDP or by reducing the rate of GTP hydrolyzing.

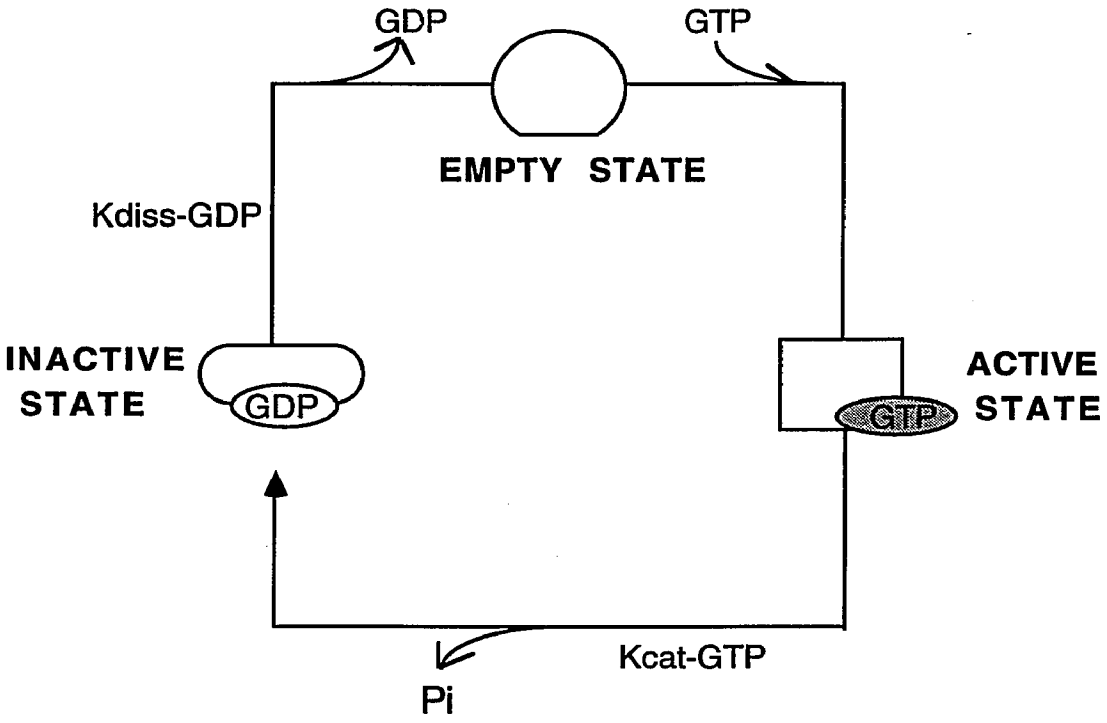


Fig. 1-1. The GTPase cycle.

Three conformational states of the protein are indicated. Release of bound GDP converts the transition of 'inactive' state to 'empty' state. Under normal cellular conditions (see text), GTP is more likely to enter the empty guanine nucleotide binding site of GTPases and the proteins are transformed to the 'active' state. The ratio of GTPase·GTP / GTPase·GDP is determined by the ratio of K_{diss}·GDP/ K_{cat}·GTP.

3) Structure of GTPases.

Knowledge of three-dimensional structure of GTPase is based on the crystal structure of the small GTPase protein, *ras* protein (Pai et al., 1990, Milburn et al., 1990) and of elongation factor Tu (Berchtold et al., 1993). The tertiary structure of the *ras* protein and Tu factor are remarkably similar despite the fact that their primary sequences share only 17% identity (Halliday et al., 1984; Conklin and Bourne, 1993). It is therefore assumed that all the GTPase proteins may have a similar guanine nucleotide binding domain.

The core structure of $p21^{ras}$ contains the consensus sequences necessary for GTP/GDP binding including those for binding phosphate and for interacting with the ribose sugar and the guanine base, and for participating in GTP hydrolysis (Bourne et al., 1991). The core structure can be divided into G regions. G1 to G5 represent conserved sequences that are dispersed in the primary sequence and are brought together in the tertiary structure of the $p21^{ras}$.

The G1 region, including residues 10-17 in $p21^{ras}$ (GAGGVGKS), forms bonds with the α- and β- phosphates of GDP or GTP. Crystal structures of Gly12Arg and Gly12Val mutants show that mutations in mutant $p21^{ras}$ prevent attacking the catalytic water molecule by the Gln61 (Krengal et al., 1990).

The G2 region, containing residues 32-40 in $p21^{ras}$ (YDPTIEDSY), is involved in coordination Mg^{2+} to form Mg^{2+} bridges to

the β- and γ-phosphates of the GTP. This Mg^{2+} is very important in GTP hydrolysis.

The G3 region, including residues 53-62 in $p21^{ras}$ (LDILDTAGQE), is Involved in the conformational switch of GTPase between active GTP-bound form and inactive GDP-bound form. Asp57 binds the catalytic Mg^{2+} through an hydration shell. Gly60 forms a hydrogen bond with the γ-phosphate of GTP. The Gln61 is conserved among all members of GTPase superfamily. The crystal structures of Gln61His and Gln61leu mutants (Pai et al., 1990; Krengal et al., 1990) reveals that this amino acid together with Thr35 could activate a water molecule perfectly placed for nucleophilic attack on the γ- phosphate. However, mutants containing mutations at this position could not activate the catalytic molecule.

The G4 region, including residues 112-119 in $p21^{ras}$ (VLVGNKCD), participates the specific interaction with the guanine ring.

The G5 region, including residues 144-146 in $p21^{ras}$ (TSA), probably contains only one contact site with GTP through Ala146 and is only conserved in the small eukaryotic GTPase subfamily, but not conserved in the GTPase superfamily.

B G proteins

1) G protein regulated signal transduction.

As mentioned above, G proteins are heterotrimers composed of α–, β–, and γ– subunits. G proteins associate with the membrane as γ-subunits, and are prenylated at a C-terminal CAAX site (Inguez-Lluhi et al., 1993). Some of the α-subunits like G_i, G_o and G_t are myristylated (Helper and Gilman, 1993; Simon et al., 1991), and some of the α-subunits like G_s, G_o and G_q are palmitoylated (Parenti et al., 1993). The α-subunit has a single high affinity guanine nucleotide binding site as well as intrinsic GTPase activity (Gilman, 1987; Birnbaumer, 1992). The β– and γ-subunits exist as a tightly associated complex. It appears that the same βγ–subunit complex may be shared among different α-subunits to form the heterotrimer. The unique properties of the individual G proteins appear to be conferred primarily by α-subunits (Helper and Gilman, 1993; Conklin and Bourne, 1993; Birnbaumer, 1992).

G proteins go through a similar GTPase cycle as other members of GTPase superfamily. The guanine nucleotide binding site on the α-subunit in the non-activated state is occupied by GDP, and the α-subunit binds tightly to βγ–subunit complex. During the activation process the hormone receptors promote the release of bound GDP and the binding of GTP (Helper and Gilman, 1993; Bourne et al., 1990). Binding of GTP leads to the dissociation of the βγ–subunit complex from α-GTP. The α-GTP is active and capable of regulating the activity of the effectors, which may be an enzyme that produces intracellular messengers such as

cAMP, IP_3 and DAG, or may be an ion channel. It is believed now that the free $\beta\gamma$-subunit complex in some systems has functions and modulates activity of effectors (Tang and Gilman, 1991; Federman et al., 1992; Iniguez-Lluhi, et al. 1993).

Finally the intrinsic GTPase activity of the α subunit hydrolyzes the bound GTP to GDP resulting in the termination of the activation process. The GDP bound form of α–subunit has a high affinity for $\beta\gamma$–subunit resulting in the formation of the heterotrimeric protein. The G protein then returns to the non-activated state. The entire process is shown below in a schematic fashion in figure 1-2.

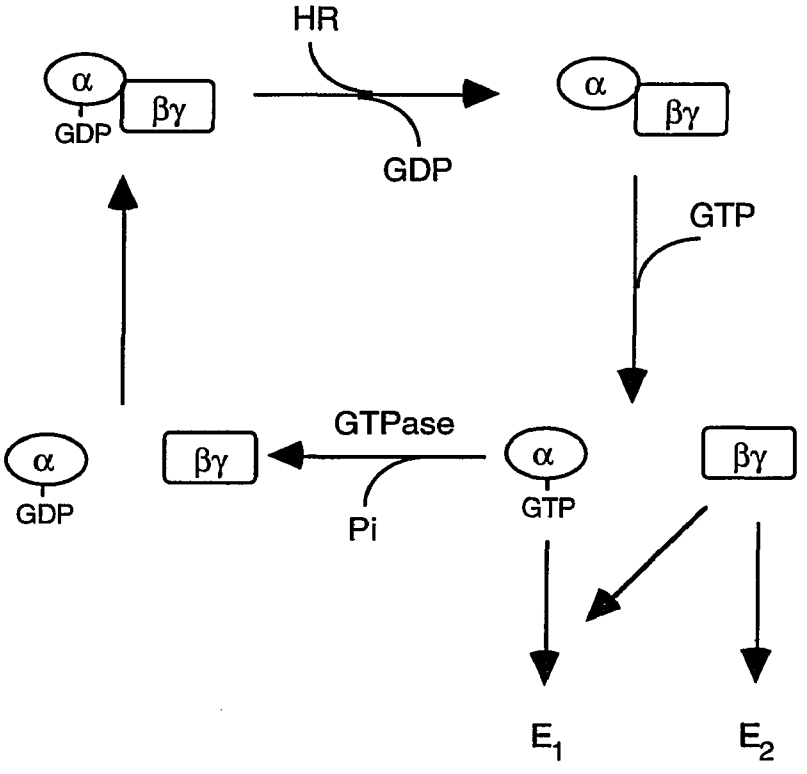

HR
α
βγ
GDP
GDP
α
βγ
GTP
α
GDP
βγ
GTPase
Pi
α
GTP
βγ
E1
E2

Fig. 1-2. G protein-mediated signal transduction.

In the inactive state, the G protein is a trimer composed of the α , β and γ subunit with GDP bound to the α subunit. The interaction of the activated receptor (HR) with the G protein leads to a conformational change and release of GDP from the α subunit. Receptor also promotes the binding of GTP to the vacant nucleotide binding site. This binding results in the disassociation of the G protein from the activated receptor and of the G protein itself to the α–GTP and the $\beta\gamma$ subunit. Primarily the α-GTP, sometimes the $\beta\gamma$, activates the effectors (E1) directly. The $\beta\gamma$ may also modulate independently the activity of the effector (E2). The α subunit has intrinsic GTPase activity which catalyzes the hydrolysis of bound GTP to GDP. The α subunit with GDP bound has a high affinity for $\beta\gamma$, resulting in the reassociation of the α subunit to $\beta\gamma$ to form the heterotrimers again. The system then goes back to the basal state.

HR, hormone receptor complex; E_1, E_2, effectors.

2) Structure of α-subunits.

Currently, the crystal structure of any α-subunit is not published. The model structure of α-subunit, as explained before, is based on an alignment of the primary homologous sequences of the four G regions. These G regions are critical in several reactions such as GDP/GTP exchange, GTP-induced conformational change and GTP hydrolysis (Conklin and Bourne, 1993). Mutational analysis of important regions in α–subunits of G proteins based on the crystal structure of $p21^{ras}$ has confirmed the predicted similarity between the structures of the G proteins and the *ras* protein. Differences between these proteins were also revealed. It should be noted, for example, that the *ras* protein contains 186 amino acids, and mammalian α-subunit on the other hand contains 350-394 amino acids including extra N and C terminals and four insertion sequences.

The C-terminus and N-terminus of α–subunits are spatially in close proximity. It is predicted that they have important functions. Mutational analysis of these two regions showed that the N-terminus is involved in regulating interaction of α–subunits with βγ-subunits (Navon and Fung, 1987; Hamm et al., 1988; Neer et al., 1988; Linder et al., 1991). The C-terminus contains receptor contact sites (Young et al., 1986; Deretic and Hamm, 1987) and is involved in the regulation of receptor binding and the activation of α-subunits (Weiss et al., 1988). A region close to the C-terminus contains effector-activating residues which are involved in coupling of α-subunits to effectors (Rarick and Hamm,

1992; Berlot and Bourne, 1992). These features of the α subunits are shown in figure 1-3.

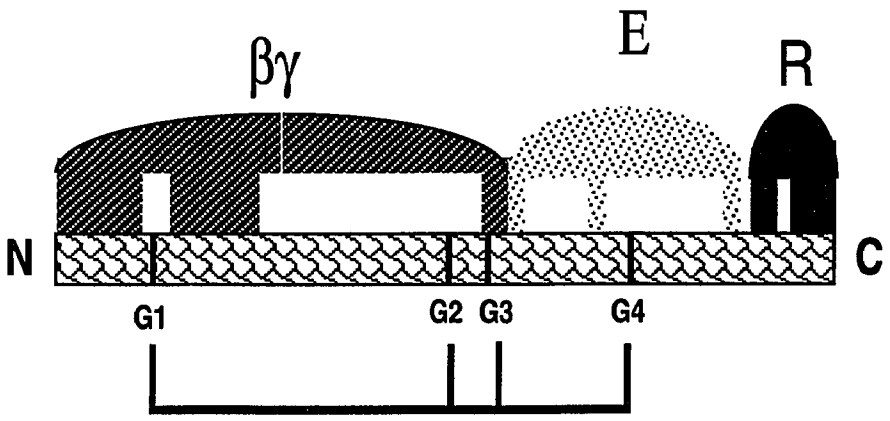


Fig. 1-3. Functional domains of α-subunits of G proteins.

The bar represents the α subunit. G1-4 are four regions which are conserved throughout the G protein superfamily. The positions of these regions are indicated. βγ subunit (hatched region) contacts with the several sites of α subunit. The regions of α subunit which interact with activated receptor (R, the black area) and the effector (E, the dotted area) are also illustrated.

3) Classification of G proteins.

The family of α-subunits of G proteins can be classified according to their structural relationships. Until now, cDNAs that encode 21 different G protein α-subunits have been cloned. They are the products of 17 genes. When the deduced amino acid sequences of all α-subunits are aligned, approximately 20% of the amino acids are found to be conserved (Lochrie and Simon, 1988). According to amino acid sequence similarity, α-subunits of G proteins can be divided into three classes represented by G_s, G_i and G_q (figure 1-4). Each class is composed of several subfamilies of highly similar α-subunits. Generally, different members of a subfamily are all capable of interacting with the same receptors and regulating the same effectors. For example, α_s and α_{olf} are able to stimulate adenylyl cyclases; all three forms of α_i and α_o can stimulate K^+ channels; α_q, α_{11}, α_{14} and α_{16} can stimulate phospholipase C-β_1, β_2 and β_3 (Birnbaumer, 1992; Helper and Gilman 1992).

Over the last few years, it has become evident that individual G proteins can regulate more than one signal transduction pathway. It has been shown that all three forms of α_i mediate inhibition of adenylyl cyclase activity as well as stimulation of potassium channels (Yatani et al., 1987; Kubo, et al., 1993). Similarly, in cardiac myocytes, α_s has been shown to stimulate both adenylyl cyclase and calcium channels (Mattera et al., 1989). A phylogenetic tree of the various α subunits and effectors which are regulated by these α subunits are shown in Fig. 1-4. This figure is adapted from Birnbaumer (1992).

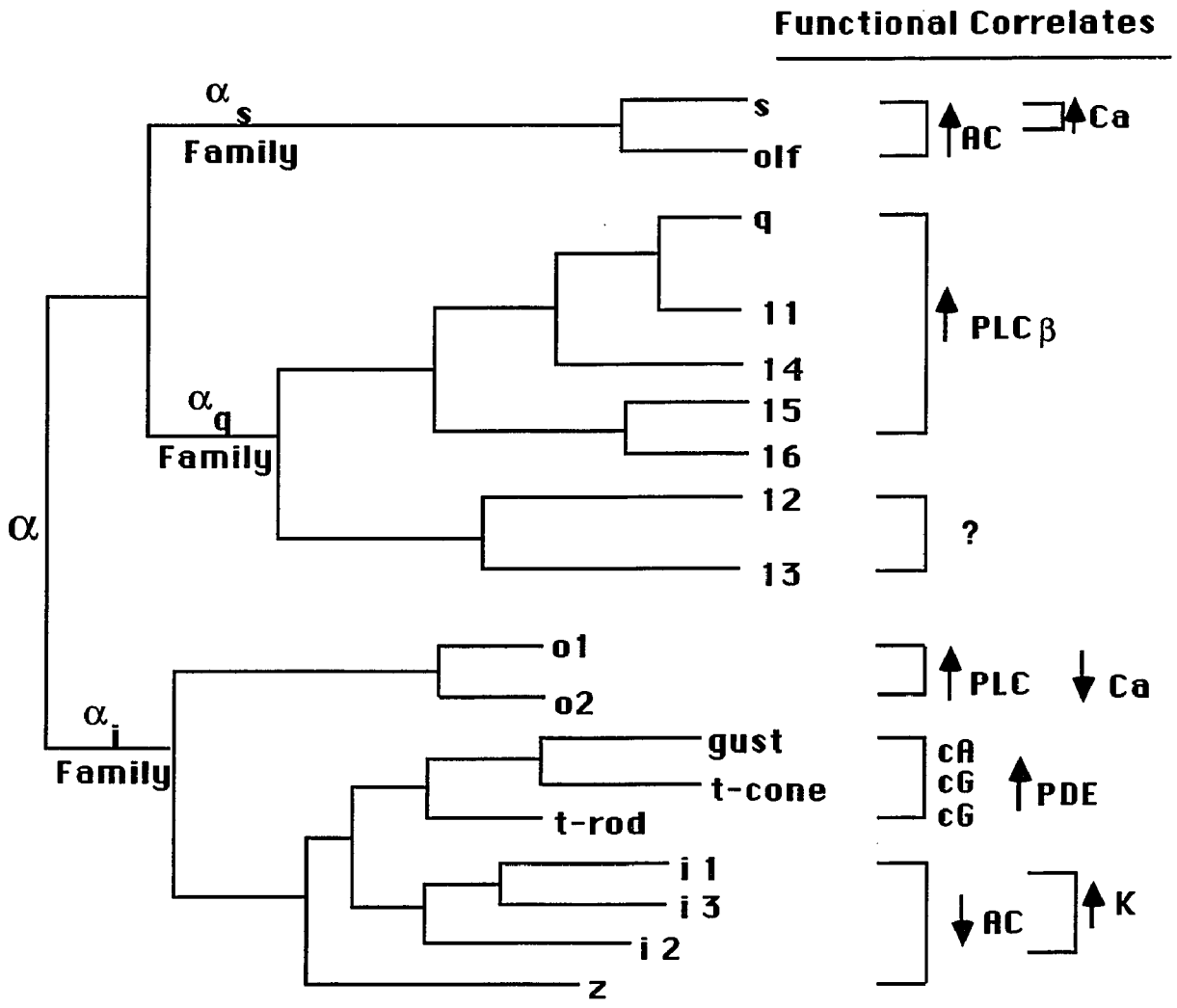

Functional Correlates
α
α s
Family
α q
Family
α i
Family
s
olf
q
11
14
15
16
12
13
o1
o2
gust
t-cone
t-rod
i 1
i 3
i 2
z
AC
Ca
PLC β
?
PLC
Ca
cA
cG
cG
PDE
AC
K

Fig. 1-4. Relationships among mammalian α-subunits of G proteins and their functional correlates.

The α subunits are grouped according to amino acid sequence identity. Abbreviations for α subunits: s, stimulatory; i, inhibitory; o, other; t-cone, transducer of cone photoreceptors; t-rod, transducer of rod photoreceptors; of, olfactory; gust, gustducin. Abbreviations for effectors; AC, adenylyl cyclase; Ca, Ca^{2+} channel; cA, cyclic AMP; cG, cyclic GMP; K, K+ channel; PDE, phosphodiseterase; PLC, phospholipase C.

C. *Ras* genes.

1) *Ras* genes.

Ras genes belong to a ubiquitous gene family. They have been identified in almost all eukaryotic organisms from yeast to human. Their remarkable evolutionary conservation suggests that they have essential cellular functions. The biological roles of *ras* have usually been studied either from the perspective of its involvement in tumor formation or its participation in physiological processes. Three functional *ras* genes: H-*ras* (Harvey, 1964), K-*ras* (Kirste and Mayer, 1967) and N-*ras* (Shimizu, et al., 1983) have been found in the mammalian genome (Barbacid, 1987). The three *ras* genes are on different chromosomes (O'Brien, 1984) and encode highly related proteins known as p21 (Shih et al, 1979). The have a common structure with a 5' noncoding exon and four coding exons. The size and the sequence of introns differ widely while the size of the exons are similar. Their promoters have a high GC content and lack cis elements, TATA box or the CAT box. These features indicate that the *ras* genes may be housekeeping genes and agrees with the notion that at least one of the three *ras* genes is expressed in all cell types (Lowy and Willumsen, 1993).

2) *Ras* proteins.

Ras proteins are synthesized in the cytosol on free ribosomes as pro-p21s which have a half-life of more than 24 hours. They become associated with the inner side of the plasma membrane after post-

translational modifications at the C-terminus. Processing of the pro-p21 begins with the addition of a isoprenoid farnesyl group to the cys186 and is followed by proteolytic removal of the last three amino acids. The cys186 is then carboxy-methylated. $p21^{H\text{-}ras}$, $p21^{K\text{-}rasA}$ and $p21^{N\text{-}ras}$ are further processed with the palmitoylation of two cysteines at positions 181 and 184. These C-terminal modifications increase the hydrophobicity of the proteins and are required for the transforming activity of *ras* (Lowy and Willumsen, 1993; Downward, 1990).

The biochemical properties of *ras* proteins include binding and hydrolysis of guanine nucleotides. Normal and transforming *ras* proteins bind guanine nucleotides with similar affinity, but the intrinsic GTPase activity is impaired in transforming alleles of *ras* proteins. The structure of *ras* has been determined from both mutational studies and from X-ray crystallography. As it has been mentioned before, five G regions of *ras* proteins are conserved within the small GTPase superfamily and are essential for guanine nucleotide binding and for GTP hydrolysis (Bourne et al., 1990). Several other essential regions which have also been identified, as shown in figure 1-5, include the effector domain.

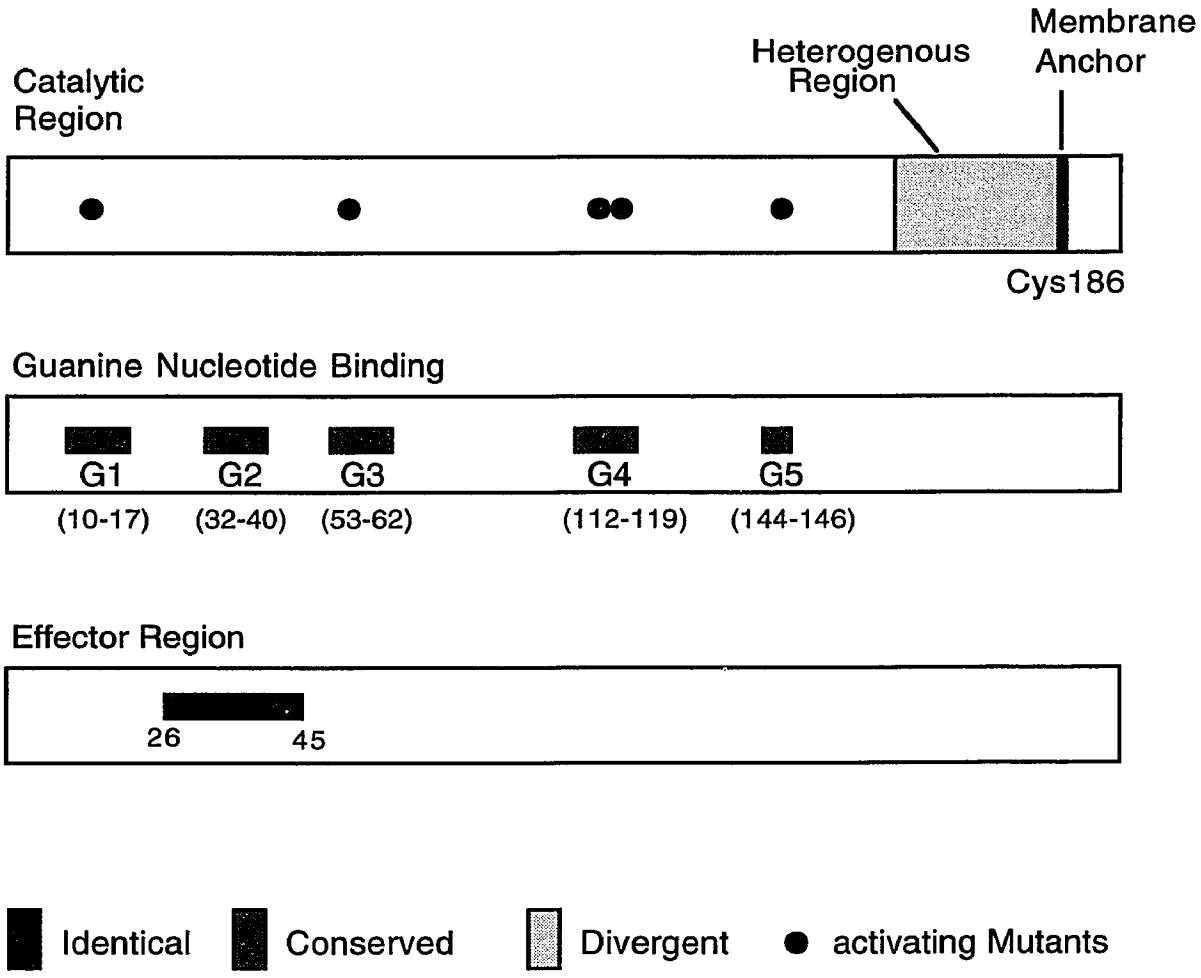


Fig. 1-5. Structure of p21^{ras}.

The bar represents *ras* protein starting from the N-terminus at left and the C-terminus at right. The three amino acids beyond the cys186 is cleaved post-transcriptionally. Thus the mature *ras* protein contains totally 186 amino acids. Through the last cysteine, cys186, *ras* protein is associated to cellular membrane. The residues involved in various functions are shown.

Ras protein functions as a signal transmitter in signaling system. Its well studied functions include: 1) stimulation of growth and transformation of fibroblast cells; 2) regulation of proliferation and differentiation of hematopoietic cells; 3) activation of T lymphocytes and 4) regulation of neuronal differentiation of pheochromocytoma cells (Satohh et al., 1992).

3) *Ras* oncogenes.

Ras genes can acquire transforming properties by several mechanisms. Certain mutations result in converting *ras* genes into potent oncogenes. Mutated forms of *ras* are commonly associated with tumors. Naturally occurring mutations in *ras* oncogenes have been detected in codons 12, 13, 59, and 61. Such mutations generally inhibit GTPase activity or affect the interaction of *ras* proteins with guanine nucleotide and thus presumably trap the proteins in a continuously active state. Mutations of either residue 12 or 61 particularly inhibit the intrinsic rate of the hydrolysis of GTP. Mutations of residues 59 or 61 have been shown to affect guanine nucleotide exchange rates. These point mutations in mammalian *ras* genes are responsible for expression of altered protein products capable of transforming NIH-3T3 cells to a malignant phenotype. Activated *ras* oncogenes have been detected in 20 to 40% of colorectal tumors (Bos, 1988) and in 93% of pancreatic carcinomas (Smit et al., 1988). Detection of activating mutations in a significant percentage of natural tumors indicates that *ras* oncogene

products play a prominent role in neoplastic processes. Overexpression of normal $p21^{ras}$ has also been linked to malignant transformation (Barbacid, 1987).

4) Systems for assaying *ras* transformation.

Many *ras* bioassays depend on the mitogenic activity of *ras* protein. The ability of *ras* protein to induce germinal vesicle breakdown (GVBD) of oocytes is one of the frequently used assays. Because of their large size, it is easy to micro-inject oocytes. Determination of GVBD has become one of the standard assays for assessing biological functions of *ras* protein.

The other methods to quantitate the *ras* gene or protein are based on transformation ability of *ras* proteins, which measure DNA synthesis, formation of foci in confluent cultures, anchorage independent growth as assayed by colony formation in soft agar and tumor formation in nude mice.

Primary cells usually can not be transformed by activated mutant *ras* gene alone. Efficient transformation only occurs when *ras* is cotransfected with a second "immortalizing" oncogene, such as myc or adenovirus E1A which both are nuclear oncogenes (Land et al., 1983). Cell lines such as NIH-3T3 cells and RAT-1 cells are already immortalized. These cells can continuously proliferate and can be transformed by a single oncogene. Hence, *ras* by itself can transform NIH-3T3 or RAT-1 cells.

NIH-3T3 cells, mouse fibroblasts, can be propagated *in vitro* indefinitely. They take up foreign genes quite efficiently and require the expression of only one exogenous oncogene to be transformed (Land et al., 1983). For example, when transfected by an oncogene, these cells can grow in an anchorage independent manner. They can grow, divide and form colonies in soft agar plates. As each colony arises from a single cell, the number of colonies represent the number of transformed cells. Thus scoring the number of colonies formed in certain number of cells plated gives a quantitative estimate of transformation. This assay is widely used as a reliable measure for transformation (Kriegler, 1990). The ability of transfected genes to transform NIH-3T3 cells has been extensively used to detect and isolate oncogenes (Shih et al., 1981, Krintiris and Cooper, 1981; Young et al., 1986; Julius et al., 1989; Lewin, 1991). The T24 H-*ras* gene was isolated from the T24 line of human bladder carcinoma cells by the virtue of its capability to transform NIH-3T3 cells (Goldfarb, 1982).

5) Proteins interacting with $p21^{ras}$.

Comparison of the inactive GDP-bound structure with the activated GTP-bound crystal structure of *ras* protein revealed that the conformational changes only occur in two regions. The first region includes residues 30-38 forming the *Loop* 2 or *Switch* 1. The second region encompasses residues 60-76 known as *Loop* 4 or *Switch* 2 (Lowy and Willumsen, 1993). The N-termini of these two regions have been proposed as possible effector residues (figure 1-5) (Marshall, 1993).

Considerable progress has been made recently in identifying proteins that can interact with $p21^{ras}$. The first one identified was GAP (GTPase activating protein). GAP had been purified from *Xenopus* oocytes and from mammalian cells as a cytoplasmic factor that can significantly accelerate the low intrinsic GTPase activity of normal *ras* protein. Although mutant activated *ras* proteins (mutations of residue 12, 13, 59 or 61) also interact with GAP, they have very low GTPase activity that is not stimulated by GAP (Lowy and Willumsen, 1993).

The product of the NF-1 gene responsible for von Recklinghausen's neurofibromatosis (NF1 disease) has been found to encode a GAP-like protein. This protein is highly expressed in cells of nervous system and is a non-nuclear protein (Lowy and Willumsen, 1993).

Another type of protein that interacts with *ras* protein is the guanine nucleotide exchange proteins or guanine nucleotide release factors (GNRF). They positively regulate the *ras* protein by stimulating the replacement of GTP for GDP of *ras* and, thereby, activating *ras*. These proteins include Sos (son of sevenless) from *D. melanogaster*, Cdc25 from *S. cerevisiae*, and Cdc25Mm which is a murine gene product and shares homology with the catalytic region of Cdc25 (Lowy and Willumsen, 1993, Feig, 1993).

Two GDIs (guanine nucleotide dissociation inhibitors) against members in *ras* superfamily, *rab, rho* and *rac* have been characterized. A cytoplasmic protein that can inhibit the *ras* protein GTPase activity also has been found (Tsai et al, 1990).

Currently, several lines of evidences have suggested that the interaction between *ras* and *raf* may be direct. It is already known that *raf* is an intracellular protooncogene occurring in a wide variety of cells. *Raf* is a protein kinase capable of covalently attaching phosphate groups to serine and threonine residues of the target proteins. In the native form the protein kinase activity of *raf* is tightly regulated, but in the mutant oncogenic form the protein kinase is constitutively activated and cannot be regulated (Li et al., 1991). *Raf* is considered a downstream oncogene because of its location within the cell and because it can be activated by a number of cell surface oncogenes (Morrison et al., 1988) such as *ras* (Kolch et al., 1991). Recently, Van Aelst et al. (1993) have shown that noncatalytic N-terminus of *raf* protein forms a complex with *ras* protein and mutations in the G regions or in the effector domain of *ras* protein abolish this interaction with *raf* protein. Vojtek et al. (1993) screened a mouse cDNA library using a two hybrid system to identify proteins interacting with H-*ras*. They found that about 50% of the clones identified encoded portions of the *raf* proteins. Analysis of these clones revealed that a conserved region at the N-terminus of *raf* protein is the region of *raf* protein interacting with *ras* protein. They also observed that *raf* protein only interacted with wild type or activated but not with the effector domain mutant *ras* protein(Vojtek et al., 1993). Moodie et al. first demonstrated that in mammalian system there was an association between *ras* protein and *raf* protein in a crude brain extract (Moodie et al., 1993).

6) *Ras* protein signaling via MAP kinase pathway

A recent explosion of new information has made it possible to trace the *ras* protein signaling pathway from the cell surface to the nucleus.

Over the last few years, an accumulation of evidence from microinjection experiments using anti-*ras* antibody and from experiments using dominant negative *ras* mutants has demonstrated that *ras* protein is downstream of receptor and non-receptor protein tyrosine kinases but upstream of *raf* protein, mitogen-activated protein kinase kinase (MAPKK or called MEK) and mitogen-activated protein kinase (MAPK). *Ras* protein is thought to be located in the middle of this pathway and regulates the signal transduction by oscillation between active GTP-bound form or inactive GDP-bound form (Lowy and Willumsen, 1993; Schlessinger, 1993). The ratio of these two forms can be regulated by either GNRF, GAP or NF-1.

It has now become clear how receptors, which can bind to a variety of growth, neurotrophic factors and factors involved in immune responses, lead to the activation of *ras* protein. Currently, two proteins, Grb2 (growth factor receptor-binding factor) and mSos (mammalian son of sevenless), have been found to mediate the activation of *ras* by EGF receptor in mammalian cells (Feig, 1993; Schlessinger, 1993; Marx, 1993). mSos is a mammalian homology of Drosophila Sos, which is a GNRF for *ras* protein. Grb2, which is an adaptor protein, contains one SH2 and two SH3 domains. In the unstimulated cells, the SH3 domains bind to a proline-rich region of mSos and form a stable complex in the

cytoplasm. Activated EGF receptor which has intrinsic tyrosine kinase activity can undergo autophosphorylation. One of the phosphorylation sites, Tyr1086, has high affinity for the SH2 domain of Grb2. Autophosphorylation of EGF receptor results in the binding of EGF receptor to Grb2-mSos complex. mSOS is recruited from cytoplasm to the membrane where the *ras* protein is located, thus allowing it to activate *ras* protein by promoting GDP release and GTP binding.

The steps of the pathway downstream of *ras* protein is a series of enzymes each of which phosphorylates and activates the next member of the cascade (figure 1-6). Recent evidence has shown that *raf* protein can directly interact with *ras* protein and becomes the most likely candidate as the direct target protein whose activity is regulated by *ras* protein.

Unlike other kinase substrates which only have transient interaction with enzymes, it is found that *raf* protein can tightly bind to its substrate MAPKK (MEK) both *in vivo* and *in vitro* (Van Aelst et al., 1993; Crews and Erikson, 1993). It appears that *raf* protein functions as a bridge to bring $p21^{ras}$ and MEK together. This is supported by the fact that only in the presence of the full-length *raf* protein, does $p21^{ras}$ form a complex with MEK.

MEK has been purified from human, murine, rabbit and *Xenopus* oocyte (Pelech and Sanghera, 1992[a]) as a 45K protein. Immunoprecipitates of constitutively activated *raf* protein can reactivate the phosphatase 2A inactivated MEK (Kyriakis, 1992). These result suggest that MEK is located downstream of *raf* protein. MEK also can induce both tyrosine and threonine phosphorylation of mutant $p42^{mapk}$

whose autophosphorylation sites have been completely abolished by mutagenesis (Posada and Cooper, 1992; Nakielny et al., 1992).

MAP kinase has been found both in the cytoplasm and nucleus (Davis, 1993). Activation of MAP kinase requires phosphorylation at both tyrosine and threonine residues. Upon mitogen stimulation, there is a translocation of MAP kinase from cytoplasm to the nucleus. MAP kinase links the signaling pathway from the cytoplasm to the nucleus. At least four distinct MAP kinases have been purified. Their molecular weights range from 40K to 54K. Substrates for MAP kinases includes their upstream kinases Sos, *raf* and MEK suggesting that a feed back regulation is possibly involved. Their downstream substrates includes *myc*, *jun, fos,* ATF-2, Tal1, S6 kinase and others. Phospholipase A_2 and EGF receptor are also its substrates, although the significance of phosphorylation of EGF receptor is unclear. MAP kinases are also capable of undergoing auto-phosphorylation. Activation of MAP kinase has been correlated with control of cell cycle progression and its activity is believed to be regulated at the G_0/G_1 transition (Pelech and Sanghera, 1992[b]).

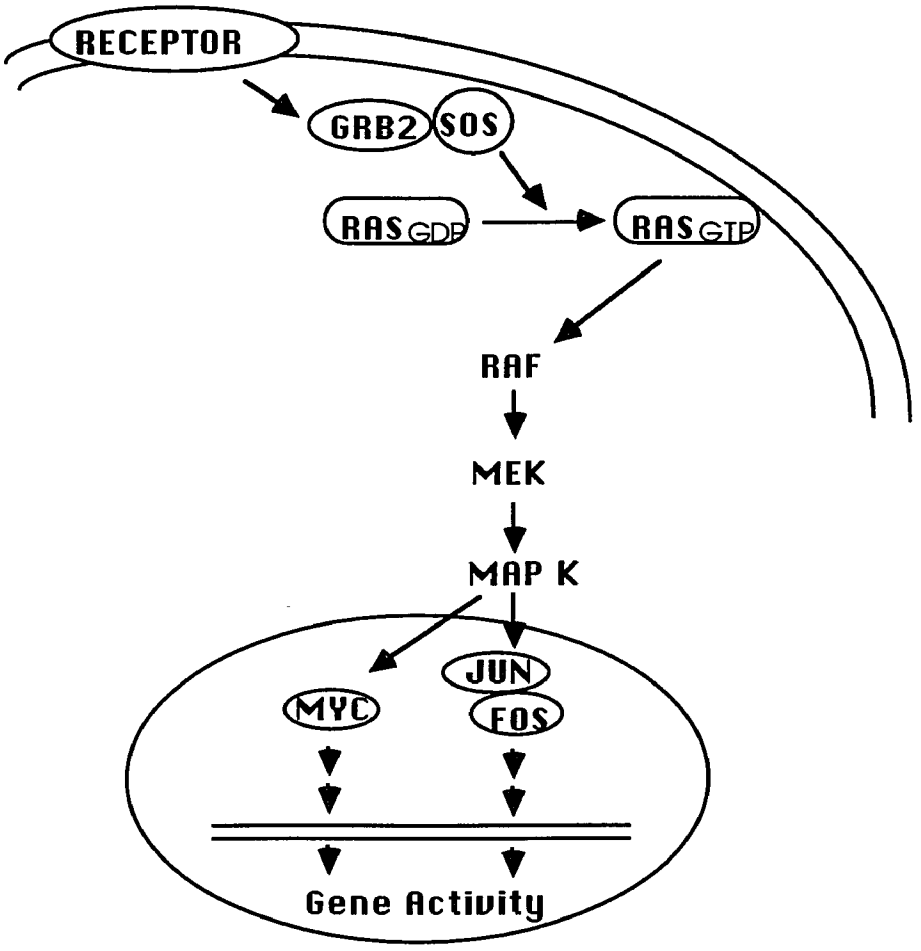


Fig. 1-6. Signal transduction pathways regulating the MAP kinase activity.

The stimulated receptor interacts with GRB2 and SOS complex resulting in the conformational change of *ras* protein from ras_{GDP} to ras_{GTP} form. RasGTP then activates *raf* protein and in turn a series of kinases. Subsequently the nuclear transcription factors myc, and fos and jun etc. are activated. Eventually the specific gene activity is expressed. The dotted lines represent the possible connections of G protein-mediated signal pathways to the *ras* protein signalling pathway.

D. G protein involved in growth signaling pathways

1) Mitogenic pathways mediated by G proteins.

In the past few years, evidence has accumulated to indicate that mutant forms of α_i may be oncogenes. GTPase-inhibiting mutations in the α_{i2} subunit polypeptide have been found in a subset of ovarian and adrenal cortical tumors (Lyons et al., 1990). Gupta et al. (1992) have introduced mutations that inhibit intrinsic GTPase activity in the α_{i2} subunit. These mutations are the same as or similar to those present in the α_i genes found in a subgroup of neuroendocrine tumors (Landis et al., 1989; Lyons et al., 1990). The mutant α_i subunits when expressed in Rat-1 fibroblasts were capable of inducing transformation of these cells. Gupta et al., (1992) concluded that activated α_i mutant polypeptides can induce a loss of normal growth control similar to that induced by other defined oncogenes. Hermouet et al. (1991) also showed that activating mutations of the α_i subunit can induce, and inactivating mutations can inhibit proliferation of NIH-3T3 cells and appearance of transformation-like characteristics. Studies from our laboratory have shown that mutant forms of α_q (De vivo et al., 1992) and α_o (Kroll et al., 1992) are capable of transforming NIH-3T3 cells, though naturally occurring mutations in these α subunits have not been identified. Thus it appears that several G protein signaling pathways are involved in growth regulation though as yet unidentified mechanisms.

2) G_i activation of MAP kinase pathway.

Although it is well known that receptor tyrosine kinases transduce signals via *ras*-coupled MAP kinase, current data also suggest that G protein coupled-receptors can also activate of MAP kinase in some cell types. The receptor for thrombin is an example of receptor whose activation leads to stimulation of MAP kinase in a *ras*-independent manner (Pelech and Sanghera, 1992[a]; Boulton et al., 1991; L'Allemain et al., 1991). Thrombin receptor responses involve G_i and G_q. In RAT-1 cells, the transformation by GTPase-inhibited α_i is independent of *ras* signaling, as the dominant negative mutant *ras* had little influence on the ability of activated mutant α_i to stimulate cell growth (Gupta et al., 1992). Recent reports showed that thrombin receptor and the mutant activated G_{i2} both activate MEK in a *raf*-independent way in fibroblasts (Gardner et al., 1993). MEKK (MEK kinase) has been found as a potential candidate for coupling the activation of MAP kinase by the G protein-involved pathway (Lange-Carter et al., 1993). The gene for MEK kinase was cloned based on the sequence similarity to the yeast protein kinase Byr 1 and Ste 7, which are yeast homologies of MAP kinase activators. This MEK kinase can phosphorylate and activate MEK independent of *raf*. Lang-Carter et al. (1993) speculated that this MEK kinase may mediate primarily signals originating from receptors that activate some G proteins and protein kinase C, as evidence showed that protein kinase C also can activate MAP kinase through tyrosine kinase-independent pathway (Pelech and Sanghera, 1992[a]; deVries-Smits, et al., 1992).

Besides activation of MAP kinase by G protein coupled-receptors independent of the *ras* or *raf*, new evidence has shown that *ras* and *raf* are involved in the acetylcholine muscarinic m2 receptor (m2R) and LPA (lysophosphaticidic acid) receptor activation of MEK and MAP kinase (Winitz et al., 1993; Howe and Marshall, 1993; Cook et al., 1993). LPA receptor couples to G_i and G_q and the m2R couples selectively to the pertussis toxin sensitive G proteins G_i and G_o. The mechanism of how activation of G protein is coupled to the activation of *ras* protein is still not known.

The information about the role of G proteins in the MAP kinase signaling pathways seems controversial thus far. This is probably due to the fact that one receptor can couple to several G proteins and the various G proteins can use different effectors to couple to growth signaling pathways. Thus the overall outcome are dependent on the interactions among different pathways and become even more complex. As more information accumulates, a better picture may be obtained.

E. α subunit of G_S and cAMP-mediated regulation of cell growth.

1) cAMP-mediated regulation of cell proliferation.

Many hormones and neurotransmitters including adrenaline, glucagon and vassopressin exert their cellular effects through stimulating the production of intracellular cyclic adenosine monophosphate (cAMP). cAMP, discovered by Sutherland in 1957, was the first identified second messenger for hormone action (Robison, 1973). Generally hormonal stimulation of the cAMP pathway leads to a variety of acute physiological effects. A good example is the aerosol spray used for temporary relief of asthma attacks. It contains an agonist that binds to β-adrenergic receptors and stimulates the production of cAMP. This results in the relaxation of the airway passages. However, long term changes in cellular cAMP levels have also been known to affect many cellular processes. The best characterized examples is the lowering of cellular cAMP levels with development of opiate addiction. In addition, depending on the cell types, cAMP can have varied effects on cell proliferation. Increased cAMP can contribute to the proliferation of certain cells such as thyroid and pituitary cells. The thyroid-stimulating hormone (TSH) receptor stimulates cell proliferation in a cAMP-dependent manner in thyrotrophs and FRTL5 rat thyroid cells (Billestrup, 1986; Dumont et al., 1989). Lowering of cellular cAMP often constitutes a powerful proliferate signal in some other cell types, such as the Rat-1 cells, where pertussis toxin inhibits mitogenic responses of LPA (van

Corven et al., 1989). However, many cells cAMP has no effect on cell proliferation.

cAMP also has been shown to inhibit cancer cell growth (Cho-Chung, 1992). These cancer cell lines include breast, colon, lung and gastric carcinomas, fibrosarcomas, gliomas and leukemias. This growth inhibition effect of cAMP is thought to be selective toward the transformed state of cancer cells.

2) Adenylyl cyclase and cAMP-dependent protein kinase A.

The link between the activation of receptors and the regulation of intracellular cAMP levels is membrane associated adenylyl cyclases. Mammalian adenylyl cyclases are glycoproteins that have two sets of six transmembrane domains. The central cytoplasmic domain and the carboxy-terminal tail are thought to contain the nucleotide-binding site(s) and the catalytic site(s) (Pieroni et al., 1993; Tang and Gilman, 1992). All six of the known mammalian adenylyl cyclases can be stimulated by G_S. G_i may inhibit all the adenylyl cyclases.

cAMP-dependent protein kinases (protein kinase A) are the major transmitting signals from cAMP in eukaryotic cells. The increased cAMP level results in the stimulation of protein kinase A. In the absence of cAMP, protein kinase A is a tetrameric holoenzyme consists two regulatory (R) subunits and two catalytic (C) subunits. Each R subunit has two cAMP binding domains which are highly conserved. Each binding domain contains one cAMP binding site. These two binding sites

are called A and B. Site A is located almost in the middle but near the carboxy terminus of the linear sequence of polypeptide chain, while site B is in the carboxyl-terminus one fourth of polypeptide chain. The positively cooperative binding of cAMP changes the conformation of R subunits and results in the dissociation of two monomeric C subunits from the regulatory-subunit dimer. The free C subunits are active and catalyze the transfer of the terminal phosphate group from ATP to specific serine or threonine residues of selected proteins. Such a modification often leads to changes within a consensus sequences in protein function that results in altered physiology (Krebs and Beavo, 1979; Weber et al., 1982).

Two types of the protein kinase A had been found based on differences in the structure of their R subunits, termed RI and RII subunits (Krebs and Beavo, 1979). RI subunits have MgATP binding sites. Binding of MgATP stabilizes the holoenzyme by raising the threshold of cAMP concentration required to cause activation. RII subunits do not bind ATP but contain autophosphorylation sites and can be phosphorylated by C subunits. Multiple isoforms of RI, RII and C subunits have been cloned including RIα, RIβ, RIIα, RIIβ, Cα, Cβ and Cγ. The functional significance of these isoforms is still not clear. It is also not known if there is preferential coexpression of any of these C subunits with either RI or RII subunits (Borrelli et al., 1992). Infection of inducible retroviral vector containing human RIIβ cDNA brought more than 50% growth inhibition in several cancer cell lines: SK-N-SH neuroblastoma, MCF-7 breast and LS-174T colon carcinoma, and K-*ras*-transformed NIH-3T3-clone DT (Cho-Chung, 1993).

Protein kinase A phosphorylates and regulates a large number of target proteins which include cytoplasmic proteins and nuclear transcription factors. Free active C subunits translocate to the nucleus where they phosphorylate cAMP-regulated transcription factor (CREB) on Ser133. Phosphorylation of this site will not change the DNA binding affinity but will increase activity of the trans-activation domain of CREB presumably through the protein conformation changes. CREB can bind to cAMP response elements (CREs), which are located in the promoters of cAMP-inducible genes, and mediate their induction in response to activation of protein kinase A pathway (Karin and Smeal, 1992). Transgenic mice expressing a Ser133Ala mutant CREB protein shows dwarf phenotype as a result of somatotrope hypoplasia (Struthers et al., 1991). This experiment has confirmed the requirement of cAMP for normal pituitary cell proliferation.

The protein kinase A target proteins also include Rap1 and Rap-GAP. Rap 1 when overexpressed is able to revert transformation by K-*ras*, as mentioned previously. A Rap1 mutant RapV12, which is insensitive to Rap-gap, antagonizes *ras*-dependent activation of MAP Kinase 1 and MAP Kinase 2 by LPA and EGF in RAT-1 cells (Cook et al., 1993). McCormick hypothesized that the activity of either Rap1, Rap-Gap or both may be regulated by pKA.

3) Function of α subunit of G_S.

G proteins which are involved in hormonal stimulation of adenylyl cyclase belong to the G_S family. This family includes both α_S and α_{olf}. α_{olf} shows 88% amino acid sequence identity with α_S and α_{olf} is only expressed in special neural tissues (Simon et al., 1991). Currently four members of α_S have been identified by molecular cloning techniques. Calculated molecular weights of human α_S subunit are 44,093D, 44,180D, 45,578D and 45, 665D. Their mRNAs are products of alternative splicing from a single precursor mRNA. All four proteins have been shown to stimulate adenylyl cyclase (Mattera et al., 1989). In reconstituted systems, purified α_S can stimulate dihydropyridine-sensitive Ca^{2+} channels in excised patches from skeletal muscle (Mattera et al, 1989) and inhibit cardiac Na^+ channels (Schubert et al., 1989). In addition to that, antisense oligodeoxynucleotides to α_S-subunit sequence was found to accelerate differentiation of 3T3-L1 fibroblasts to adipocytes and this effect is independent of increased intracellular cAMP (Wang et al., 1992). Figure 1-7 summarizes the functions of α_S-subunits of G proteins.

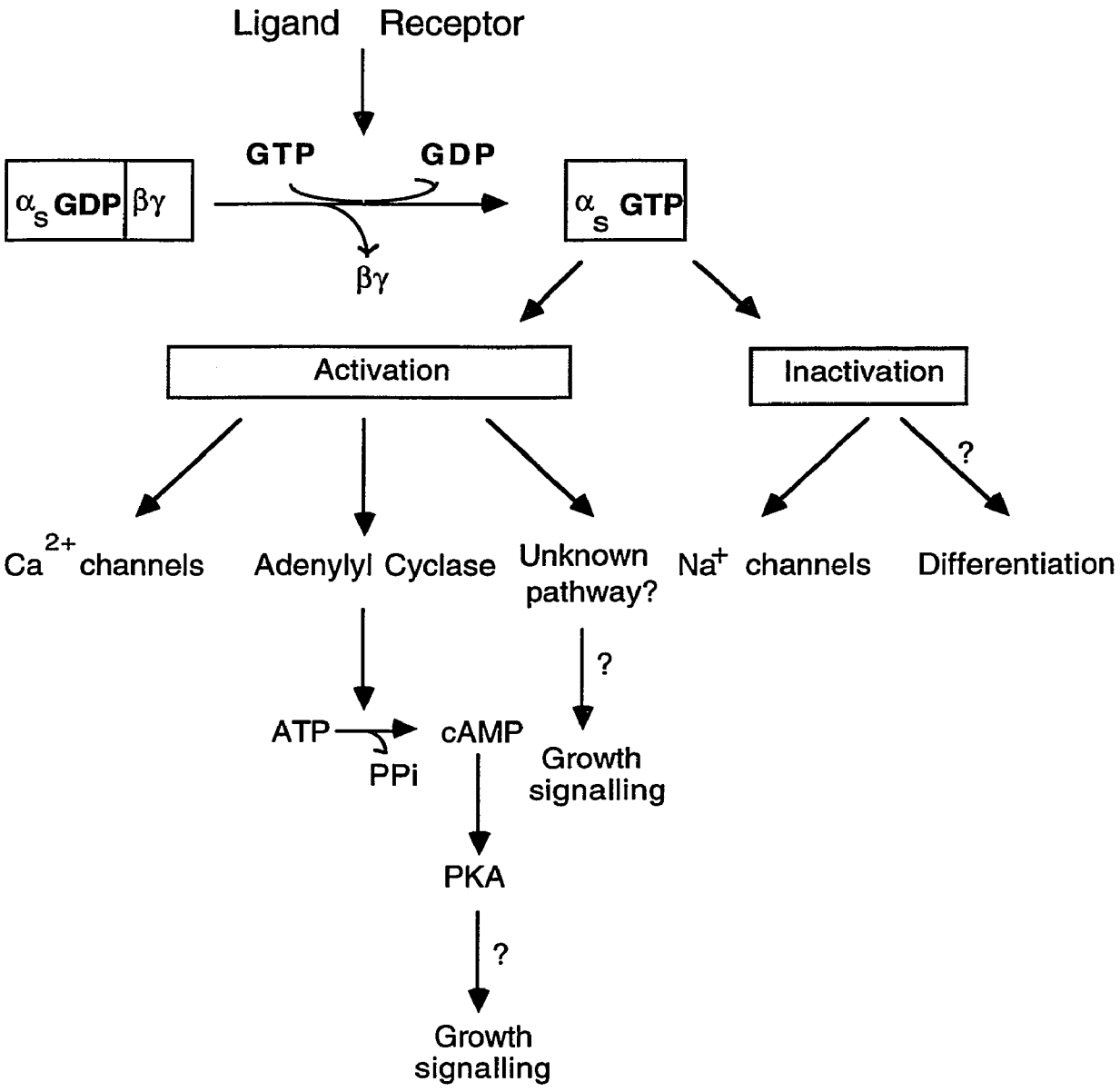

Ligand
Receptor
GTP
GDP
α_s GDP βγ
α_s GTP
βγ
Activation
Inactivation
?
Ca^{2+} channels
Adenylyl Cyclase
Unknown pathway?
Na^+ channels
Differentiation
?
ATP
cAMP
PPi
Growth signalling
PKA
?
Growth signalling

Fig. 1-7. The functions of α_S-subunits of G proteins.

The binding of ligand to the receptor activates the receptor which promotes the release of GDP, and subsequently disassociation of α and $\beta\gamma$ complex. α_S binds to GTP immediately. α_S GTP can activate several effectors including Ca^{2+} channels and adenylyl cyclase. α_S GTP may also be involved in growth control. On the other hand, α_S GTP causes inactivation of Na^+ channels and other effectors.

Interest in the effects of cAMP pathways on cell proliferation was revived when Bourne and co-workers showed the presence of a mutated α_s-subunit which is continuously active in a subset of pituitary tumors (Landis et al. 1989; Climenti et al., 1990). This mutation results in elevated levels of cAMP and consequently enhanced secretion of growth hormones from these cells. This finding was later extended to show that such mutant forms of α_s could also be found in thyroid tumors (Lyons et al. 1990; Suarez et al., 1991). The activated mutants of α_s found in tumors contained mutations in two positions. The Gln residue in position 227, which is equivalent to Gln^{61} of $p21^{ras}$, is within the GTP binding domain of the α_s-subunit and is conserved through out the GTPase superfamily. The Arg residue in position 201 is thought to be ADP-ribosylated by the exotoxin of *V. cholerae*. The substitutions of these residues result in α-subunits of impaired GTPase activity and trapping of the protein in its active, GTP-bound form. Based on these observations, Bourne and co-workers have proposed that the gene encoding the mutant α_s-subunit could be an oncogene especially in cells in which cAMP acts as a mitogenic agent (Landis et al. 1989; Spade et al., 1992). However, for α_s-subunits there is no evidence to show that these mutations can convert them into a transforming agent.

CHAPTER 2

MATERIALS AND METHODS

1. Site directed mutagenesis.

Primers 5'-GCAGATGAGGATCCTGCATGTTAATG-3' and 5'-CGTTGAAGCACTGGATCCACTTGCGGCGTTCATCGCGAAGGCCACC CACG-3' were synthesized on an Applied Biosystems DNA synthesizer. oligonucleotides were purified by separation on 15% polyacrylamide gels (acryl:bis 40:2) containing 7 M urea in TBE buffer (0.09 M Tris-phosphate, 0.002 M EDTA) at 500 V. DNA bands were visualized with a short-wave length UV light, excised, incubated in 2 ml NTE buffer [0.1 M NaCl, 10 mM Tris·Cl (pH 7.2), 1 mM EDTA (pH 8.0)] and shaked at room temperature overnight. After extraction with equal volume of phenol/chlorform, oligonucleotides were precipitated with ethanol and resuspended with H_2O at a concentration of 1 μg/μl.

Mutant α_s cDNA was made by site directed mutagenesis using polymerase chain reaction (PCR). The template used to generate the mutant cDNA fragment are wild type $G\alpha_s$ in the bacterial expression vector pT7-7. Heat-stable DNA polymerase *Taq* DNA polymerase (from Promega) was applied for PCR. Reaction mixture (99 μl) contained PCR buffer [50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.0 mM $MgCl_2$. 0.1% gelatin], 200 μM of each dNTP (neutralized), 600 ng of each primer, 30 ng of plasmids. After denaturing the DNAs by heating the mixture 7 minutes at 94°C and cooling down on ice, 2.5 units of Taq polymerases were added. Reaction mixtures were covered with 100 μl of mineral oil to prevent evaporation and subjected to 30 cycles of denaturation (94°C 30 sec), annealing (55°C 1 min) and extension (72°C 2 min) in a Perkin-Elmer Cetus thermal cycler. Reaction mixtures were then extracted twice

with equal volume of phenol/chlorform. The aqueous phases were dried in a Speed-vac, resuspended in sample buffer and subjected to electrophoresis in NuSieve agarose gels containing TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). PCR fragments were recovered from agrose by using Gene-Clean kit (from Bio 101). Two changes (C to A and T to A) were made at the underlined positions of primer, resulting in changing of Gln-227 to Leu and create a new Nru I site in the mutant fragment.

2. Construction of the mutant α_s.

pT7-7-α_s and PCR products were digested with BamHI. Vectors and inserts were subjected to electrophoresis in NuSieve agarose gels containing TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and were recovered from agrose by using Gene-Clean kit (from Bio 101). 20 ng of vectors and 100 ng of inserts were ligated using 4.5 units phage T4 DNA ligase (from Promega) in 15 µl T4 DNA ligase buffer (from Promega) at 4°C overnight.. The ligated DNAs were transformed into competent XL1 Blue E. *coli* (from Stratagene) (100 µl) by mixing at 4°C for 30 min and heated at 42°C for 45 sec. Transfected bacteria were amplified for one hour at 37°C and were spread onto YT plates (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl, 1.5% bacto-agar) with ampicillin (100 µg / ml). Minipreps of colonies were prepared by the alkaline lysis method.(Ausubel, et al., 1987). Clonies were grown overnight in 1.5 ml YT medium. Cells were pelleted and resuspended in 100 µl TE buffer [10 mM Tris·Cl (pH 7.6), 1 mM EDTA (pH 8.0)]. Bacteria were lysed by addition of

200 µl of NaOH/SDS solution (0.2N NaOH, 1% SDS), mixed gently and placed on ice for 5 min. 150 µl 3 M potassium acetate solution (pH4.8) was added. Cell debris and chromosomal DNAs were removed by spin 1 min in microcentrifuge. Supernatants were transferred to fresh tubes. Plasmids were precipitated by ethanol and resuspended in 20 µl H_2O.

The colonies containing wild type region of pT7-7-α_s replaced by the BamH1 digested PCR products with right orientation were isolated by restriction enzyme analysis. The NruI and NcoI digestions were done to check the existence of mutations and the orientation of the inserts. Full length Q227L-α_s cDNAs with the presence of the mutation were then confirmed by DNA sequencing of the coding region for α_s with Q227L-α_s cDNA specific primers.

3. DNA sequencing.

Doubled stranded plasmid DNAs (2 µg) were denatured by treatment with 0.2 N NaOH for 5 min and followed with addition of one tenth volume of 0.3 M NH_4OAC (pH4.5). Denatured DNAs were sequenced using modified T7 DNA polymerase (US Biochemical) and [α-^{32}p]dATP (650 Ci/mmol) and DNAs were resuspended in sequence reaction buffer with 1 pmol primers and annealed by incubation at 37^{o}C for 15 min and extended for 5 min. Reactions were terminated according to the manufacture's protocol and were denatured by heating to 100^{o}C for 2 min. Samples were loaded on 8% polyacrylamide sequencing gels

containing 7 M urea and TBE buffer. Gels were run at 1500-2000 V and dried under vacuum for autoradiography.

4. Synthesis of 5'-capped RNA transcripts *in vitro*.

HindIII-linearized pT7-7-α_S or pT7-7-Q227L-α_S DNAs (5 μg) as templates were incubated in 50 μl of transcription buffer (40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl), 0.5 mM of each ATP, CTP, and UTP, 0.05 mM of GTP, 0.5 mM of GpppG, 0.01 mM of DTT, 80 units of RNasin ribonuclease inhibitor and 40 units of T7 polymerase. The mixtures were incubated for 2 h at 37°C. At the end of the incubation, 5 units of RNase-free DNases were added and reactions were incubated for a further 15 min at 37°C to digest the template DNAs. The reaction mixtures were diluted with 100 μl of autoclaved H_2O, extracted twice with equal volume of phenol:chloroform (1:1) and precipitated with ethanol.

5. Translation of the *in vitro* synthesized mRNA.

2 μl (10 μg) of template mRNAs transcribed from wild type (pT7-7-α_S) or mutant (pT7-7-Q227L-α_S) cDNAs were heated at 67°C for 10 min and immediately cooled on ice. The denatured mRNAs were incubated with the following mixtures containing 35 μl of nuclease treated rabbit reticulocyte lysate, 7 μl of H_2O, 1 μl of 40 u/μl RNasin ribonuclease inhibitor, 1 μl of 1 mM amino acid mixture (minus methionine), and 4 μl of

^{35}S-methionine (1,200 Ci/mmole) at 10 mCi/ml at 30°C for 60 minutes. The translated products were directly loaded on to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel using the similar method as described in immunoprecipitation. After electrophoresis, the gel was dried and exposed to X-ray film.

6. Construction of α_s mammalian expression vectors.

The wild-type and mutant α_s cDNAs were released from pT7-7-α_s (4 μg) or pT7-7 Q227L-α_s (4 μg) by NcoI and Hind III digestion in a 20 μl reaction. DNAs were rendered blunt by adding 1 unit of Klenow fragments of E. *coli*, 1 μl of 5 mM each dNTP at 30°C for 15 min. Mammalian expression vector MAMneo (pMN) contains an inducible mouse mammary tumor virus promoter, which is a glucocorticoid responsive element, and a neomycin resistance gene which is expressed under the control of SV40 promoter. pMN were digested with XhoI and rendered blunt by using Klenow fragments. The α_s cDNAs were ligated with the vectors and were transformed into competent XL-1 blue E. *coli* cells. The plasmids were selected with restriction enzyme digestion first and then confirmed by DNA sequencing.

7. Cell cultures.

NIH-3T3 cells and RAT-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (from Gibco) containing 10% calf serum

(from Hyclone). All the reagents and solutions used in mammalian cell culture were either filter sterilized (filter < 0.2 μm) or purchased sterile.

8. Calcium phosphate transfection of DNA into mammalian cells.

Cells were split into 10-cm dishes at a density of 2 x 10^5 cells / dish 24 hours before transfection and changed with 6 ml fresh DMEM containing 5% calf serum 4 hours before transfection.

For establishment of clonal cell lines, 20 μg of plasmid DNAs diluted in 220 μl of 0.1 x TE [1 mM Tris·Cl (pH 7.6), 0.1 mM EDTA (pH8.0)] and mixed with 250 μl 2 x HEPES-buffered saline (2 x HBS) (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, 50 mM HEPES, adjusting pH to 7.05 with 0.5 N NaOH) in a sterile 15 ml plastic tube. 31 μl of 2 M $CaCl_2$ was slowly mixed with DNA-HEPES solution for about 20 sec. Transfection mixtures were washed for 2 hours at room temperature and evenly distributed over the 10-cm dishe. Cells were incubated for 24 hours and then fed with fresh 10 ml DMEM containing 10% calf serum.

For all the other assays, 20 μg of ethanol precipitated DNA containing carrier genomic DNA and indicated amounts of plasmids were diluted in 220 μl of 0.1 x TE and mixed with 31 μl of 2 M $CaCl_2$. 250 μl of 2 x HBS was placed in a sterile 15 ml plastic tube and bubbled with a plugged 1 ml pipette along with the DNA/$Cacl_2$ solution. Transfection mixtures were allowed to sit for 30 min at room temperature and evenly

distributed over the 10-cm dishe. Cells were incubated for 24 hours and then fed with fresh 10 ml DMEM containing 5% calf serum.

9. Establishment of clonal cell lines.

Individual clonal cell lines were derived from cells transfected with pMN, pMN-α_S, or pMN-Q227L-α_S (pMN-α_S^*). Transfected cells were subjected to G-418 selection (800 μg / ml). Mediums containing 10% calf serum and 800 μg / ml G-418 were changed every third day. Colonies were picked two or three weeks after selection by placing cloning cylinders around clearly separated colonies. Cells were trypsinized by adding 100 μl of Trypsin/EDTA (from Gibco) and incubated for 3 min at room temperature. Cells were then transferred into 6-well plates with 3 ml medium containing 10% calf serum and 800 μg / ml G-418. After cells were subconfluent, all clonal cells were kept in medium containing 10% calf serum and 400 μg/ml G-418 for further experiments.

Varies cell lines were established including: n-1, n-3, pMN transfected NIH-3T3 cell line; α_S-2, α_S-3, pMN-α_S transfected NIH-3T3 cell line; α_S^*-1, α_S^*-3, α_S^*-12, α_S^*-14, pMN-α_S^* transfected NIH-3T3 cell lines; R-n-1, pMN transfected RAT-1 cell line; R-α_S-5, pMN-α_S transfected RAT-1 cell line; R-α_S^*-1, R-α_S^*-2, R-α_S^*-7, pMN-α_S^* transfected RAT-1 cell lines;

NIH-3T3 clonal cells and Rat-1 clonal cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum.

10. [^{3}H]cAMP accumulation assays.

Cells were treated with 1 μM of dexamethasone (DEX) in Dulbecco modified Eagle medium (DMEM) plus 10% bovine calf serum for one week and then seeded subconfluently (about 2 x 10^5 cells / well) in 24-well plates. 5 μCi/ml of [^{3}H]adenine were used to label cells for 24 h. cAMP accumulation was measured as described (Chen and Iyengar, 1993). Wells were washed once with 1 ml of HEPES-buffered medium [DMEM with 20 mM HEPES (pH7.4)] and incubated at same medium containing 1 mM IBMX for 30 min at 37°C. Reactions were stopped by aspiration of medium and addition of 1 ml ice cold 5% TCA. Plates were placed at 4°C for 30 min and samples were added to columns to separate cAMP from ATP using the method modified from Salomon et al. (1974).

Samples were first loaded onto regenerated Dowex AG-50 columns (prewashed with 10 ml of dH_2O, 2N NaOH, dH_2O, 2N HCl, dH_2O, dH_2O, dH_2O). Dowex columns were washed with 3 ml of dH_2O. These 4 ml elution was ATP counts and measured by scintillation counting. Dowex columns were then placed over Alumina columns [neutralized with 10 ml of imidazole solution (0.1 M imidazole, pH7.5)] and washed with 10 ml of dH_2O. Alumina columns were further eluted with 6 ml of imidazole solution. This 6 ml of elution was cAMP counts and measured by scintillation counting. cAMP accumulation assays were carried out in triplicates and cAMP accumulations were calculated by the ratio of [^{3}H]cAMP/([^{3}H]cAMP+[^{3}H]ATP)(x 10^{-3}).

11. Adenylyl cyclase assays.

Cells were treated with 1 μM of dexamethasone (DEX) in Dulbecco modified Eagle medium (DMEM) plus 10% bovine calf serum for one week and then seeded subconfluently (about 2 x 10^5 cells / well) in 24-well plates. Wells washed with 500 μl of lysis buffer (25 mM Hepes pH7.5, 1.5 mM EDTA, 0.1 mM PMSF). Cells were lysed by addition of 100 μl lysis buffer and incubation at 32°C for 22 min with shaking. Adenylyl cyclase activity was assayed by addition of 50 μl of 3 x IM/RS/ATP incubated for 10 min at 32°C. IM/RS/ATP contained 25 mM of NaHEPES (pH 8.0), 1 mM of EDTA, 1 mM of [2,8-^{3}H]cAMP (10,000 cpm / assay), 20 mM of creatine phosphate, 50 μg / ml (10 units / mg) of creatine phosphokinase, 1 μg/ml (100 units / ml) of myokinase, 100 μM of [α-^{32}P] ATP (10^5 cpm / μl). Reactions were stopped by addition of 100 μl adenylyl cyclase stop solution (1 mM of cAMP, 1 mM of ATP, 1% of SDS). cAMP was separated on Dowex AG-50 and neutralized Alumina columns as described by Salomon et al. (1974), and measured by scintillation counting. Experiments were carried out in triplicates Counts were converted to enzyme activities based on the recovered [^{3}H] cAMP standard and correction of the measured specific activity of [α-^{32}P] ATP in each assay.

12. Carrier DNA extraction.

1 x 10^8 cells (NIH-3T3 cells or RAT-1 cells) were trypsinized and spun at 4°C for 15 min. Carrier DNA extraction method was modified from

Stratagene's protocol. Pellets were washed once with ice-cold PBS and added to 11 ml of solution 2 [50 mM Tris·Cl (pH7.6), 20 mM EDTA (pH8.0), 2 % SDS]. Cells were homogenized with a Dounce homogenizer. Cellular proteins were digested by Pronase (100 μg / ml) at 37°C with shaking overnight. Mixtures were placed on ice for 10 min, treated with 4 ml of solution 3 (saturated NaCl solution) at 4°C for 5 min, and spun for 5 min at 4, 000 rpm at 4°C. Supernants were treated with RNase (20 μg / ml) at 37°C for 15 min. Genomic DNAs were precipitated by ethanol, resuspended in 1 ml of TE , sheared through a 20G1/2 needle twice and reprecipitated by ethanol.

13. Transfection efficiency assays.

Transfection efficiency was measured by counting hygromycin B-resistant colonies formed in NIH-3T3 cell lines: n-1, n-3, α_S-2, and α_S^*-3 and in RAT-1 cell lines: n-1, α_S-5, and α_S^*-7. Procedures for transfection were described in the transfection methods. 1 μg of pRSV1.1 along with 20 μg of genomic DNAs from either NIH-3T3 or RAT-1 cells were used in transfection. After transfection, the cells were split and grown in media containing 400 μg/ml hygromycin. After two weeks, the media was aspirated and plates were stained with Hank's buffed saline containing 1.5% glutaraldehyde and 0.06% methylene blue at room temperature for one hour. The plates were then washed with large amounts of water and dried in a hood. The number of colonies were counted as the transfection efficiency. Experiments were carried out in triplicates.

14. [^{3}H] thymidine incorporation.

All the clonal cell lines were grown for four days in the presence of 1 μM DEX on alternate days to induce the expression of wild type α_s or α_s^*. Cells were seeded at a density of $2x10^5$ cells per 100-mm plate and were transfected with 20 μg of NIH-3T3 genomic DNA as carrier with or without 1 μg of plasmid T24 DNA which contains an activated c-H-*ras* oncogene isolated from a human bladder cancer (Goldfarb et al., 1982). The medium was changed next day. Cells were then induced by 1 μM of DEX for two more weeks on alternate days, plated at 10, 000 cells / well in 24-well plates with 1 ml of DMEM containing 10% calf serum and induced with DEX on the second day. On the day 3, the medium was replaced with DMEM containing 1% calf serum. On the day 4, 1 μCi of [^{3}H]-thymidine and 4 μl of 250 μM Dex were added to each well. Cells were incubated for 24 hours and harvested according to the following procedure.

Cells were washed once with 2 ml of cold PBS, twice with 1 ml of cold 5% TCA, then solubilized with 250 μl of 0.25 M NaOH for 30 minutes at 55^{o}C. The [^{3}H]cpm in cell lysate was measured by scintillation counting. Experiments were carried out in triplicates.

15. Protein assays.

Protein concentrations in MAP kinase assay were determined by using the BCA protein assay reagent (from Pierce). Reactions contained 100 μl of either diluted soluble cellular extracts or standard BCA in 0.2 x

lysis buffer [0.1% of Triton X-100, 10 mM of β-glycerophosphate (pH 7.2), 20 μM of sodium vanadate, 0.4 mM of $MgCl_2$, 0.2 mM of EGTA, 0.2 mM of DTT, 0.4 μg / ml of leupeptine, 0.4 μg / ml of aprotinin]. Reactions also contained 2 ml of working solution (from Pierce) and were incubated at 37°C for 30 min. Protein concentrations were calculated by measuring the absorbance at 562 nm using BSA as a standard.

16. MAP kinase assays.

For the effect of expression of α_s^* on *ras*-stimulated MAP kinase assays, all the clonal cell lines were grown for four days in the presence of 1 μM of DEX on alternate days to induce the expression of wild type α_s or α_s^*. Cells were seeded at a density of $2x10^5$ cells per 100-mm plate and were transfected with 20 μg of NIH-3T3 genomic DNAs as carriers with or without 1 μg of plasmid T24 DNAs. The media were changed next day. Cells were then induced by 1 μM DEX for two more weeks on alternate days. $4x10^6$ cells were plated per 100-mm plate with 10 ml of DMEM containing 10% calf serum and 1 μM of DEX. Media were changed on the second day without DEX. On the third day, the cells were incubated in DMEM containing 0.1% BSA and 1 μM of DEX for 20 hours to achieve quiescence and subjected to do MAP kinase assays.

For the effect of 8 Br-cAMP on *ras*-stimulated MAP kinase assays, vector transfected NIH-3T3 cells, n-1, were grown for four days in the presence of 1 μM of 8 Br-cAMP. Cells were seeded at a density of $2x10^5$ cells per 100-mm plate and were transfected with 20 μg of NIH-3T3

genomic DNAs as carriers with 1 μg of plasmid T24 DNAs. The media was changed next day. Cells were then induced by 1 μM of 8 Br-cAMP for two more weeks. $4x10^6$ cells were then plated per 100-mm plate with 10 ml of DMEM containing 10% calf serum and 1 μM of 8 Br-cAMP for two days. The cells were incubated in DMEM containing 0.1% BSA and 1 μM of 8 Br-cAMP for 20 hours to achieve quiescence and used for MAP kinase assays.

For the phorbol 12-myristate 13-acetate (PMA) stimulation assays, cells were challenged with PMA (200 nM) for 10 min. Cells were grown for one week in the presence of either 1 μM of DEX on alternate days to induce the expression of wild type α_s or α_s^* or 1 μM of 8 Br-cAMP. Cells were plated at a density of $4x10^6$ cells per 100-mm plate with 10 ml of DMEM containing 10% calf serum and 1 μM of DEX or 1 μM of 8 Br-cAMP. Media were change on the second day without DEX. On the third day, the cells were incubated in DMEM containing 0.1% BSA and 1 μM DEX or 1 μM of 8 Br-cAMP for 20 hours to achieve quiescence. Cells were challenged with 200 nM of PMA and then used for MAP kinase assays.

The MAP kinase assay method is modified from Johnson's group (Heasley and Johnson, 1992). Plates were rinsed once with ice-cold phosphate buffered saline (PBS) (1 mM of KH_2PO_4, 10 mM of Na_2HPO4, 0.137 M of NaCl, 2.7 mM of KCl pH7.4) and followed by the addition of 1 ml of lysis buffer [0.5% of Triton X-100, 50 mM of β-glycerophosphate (pH 7.2), 100 μM of sodium vanadate, 2 mM of $MgCl_2$, 1 mM of EGTA, 1 mM of DTT, 2 μg / ml of leupeptine, 2 μg / ml of aprotinin]. The attached cells were scraped from the dishes, collected into a 1.5 ml-microcentrifuge

tubes and spun for 15 min at 15,000 rpm at 4°. Soluble extracts were filtered using low protein binding MILLEX®-GV 0.22 μM filter unit (from MILLIPORE), normalized for protein content as described in protein assays, diluted into 6 ml and injected into a preequilibrated Mono Q® HR 5/5 FPLC column. Equilibration buffer or called low ionic strength buffer contained 50 mM of β-glycerophosphate (pH 7.2), 100 μM of sodium vanadate, 1 mM of EGTA and 1 mM of DTT. The column was washed with 10 ml of low ionic strength buffer and the bound proteins were eluted from FPLC with 20 ml linear gradient of NaCl (0-0.4 M) in the same equilibration buffer. 20 μl of aliquots were taken from 2 ml of fractions and incubated with 20 μl of reaction solutions for 15 min at 30°C. Reaction solutions contained 50 mM of β-glycerophosphate (pH 7.2), 100 μM of sodium vanadate, 1 mM of EGTA, 1 mM of DTT, 20 mM of $MgCl_2$, 0.5 mg of $EGFR_{662-681}$ peptide (RRELVEPLTPSGEAPNQALLR), and 200 μM [r-^{32}P]ATP (1 Ci / mmol). Reaction were terminated by addition of 10 μl of 25% trichloroacetic acid (TCA) and 45 μl of reaction mixtures were spotted onto 2 cm squares of P-81 phosphocellulose paper (from whatman). The papers were washed three times with 400 ml of 75 mM phosphoric acid with gently shaking for 5 min each. Papers were dehydrated by washing with 50 ml of acetone and were dried in the hood and counted by liquid scintillation counting.

17. soft agar assays.

After treated with 1 μM DEX on alternate days for four days, cells were transfected with 20 μg of NIH-3T3 genomic DNA or RAT-1 genomic

DNA as carrier with or without the indicated amount of plasmid T24 DNAs as described in transfection method. After transfection, the media were changed next day. Cells were then induced by 1 μM DEX one more day for NIH-3T3 cell lines or three more days as in RAT-1 cell lines. 5 x 10^4 treated cells were suspended in 3 ml of medium (DMEM with 20% fetal bovine serum) containing 0.3% agar. The mixtures were added over a layer of 5 ml of 0.5% agar in the same medium on a 60-mm plate. Triplicate plates were set up for each group. Plates were fed with 1 ml medium (DMEM with 20% fetal bovine serum) plus 1 μM DEX once a week. Three weeks later, plates were stained with the vital stain 2-(*p*-isodophenyl)-3-(*p*-nitrophenyl)-5-phenyltertrazolium chloride hydrate (INT) for two days. Colonies larger than 0.15 mm in diameter were scored.

For the 8 Br-cAMP assays, cells treated with or without 1 μM cAMP for four days before transfection. After transfection with indicated amount of pT24 and carrier DNA, cells were induced with 8 Br-cAMP two more days for NIH-3T3 cell lines or four more days for in RAT-1 cell lines. Then 5 x 10^4 treated cells were plated for soft agar assay as described above.

The pKA soft agar assay is similar, except that the transfection contains not only the pT24 and carrier DNA but also contains pHLREV$_{Wt}$neo or pHLREV$_{AB}$neo, which are expression vectors coding for a wild type or mutant type 1 regulatory subunit of cAMP-depenedent protein kinase (pKA) respectively (Schecterson and McKnight, 1991).

18. Measurement of Cell growth.

For measuring the effect of the expression of α_s^* on the proliferation of NIH-3T3 and RAT-1 cells, cells were seeded into 24-well plates at a density of $6x10^3$ cells/well in 1 ml of DMEM plus 10% bovine calf serum. Cells were induced by 1 μM DEX on alternate days and the cell numbers were determined daily for 5 consecutive days.

For measuring the effect of addition of 1 μM 8-Br-cAMP on the proliferation of NIH-3T3 and RAT-1 cells, cells were seeded into 24-well plates at a density of $1x10^3$ cells/well in 1 ml of DMEM plus 10% bovine calf serum. Cells were grown in the presence of 1 μM 8-Br-cAMP and the cell numbers were determined on day 1, 3, 4 and 5.

Each group had four replicate wells. Doubling time was calculated using the equation $Y=Y_o(e^{kt})$. The doubling time equals (0.693/k)x24 h.

19. Immunoprecipitation.

[^{35}S]methionine labeled H-*ras* p21 proteins were immunoprecipitated from extracts of NIH-3T3 cell lines, n-1 and α_s^*-3, which were transfected with 20 μg of genomic NIH-3T3 DNA, 1 μg of pRSV1.1 DNA and with or without 5 μg of pT24 DNA. After transfection as described in the transfection method, cells were induced by 1 μM of DEX for one day and grown in media containing 400 μg/ml of hygromycin B. Media were changed every three days. After one week, cells were induced by 1 μM of DEX once again. One week later, $1x10^7$ cells per 25

cm-dish were placed and grown overnight. On the next day, cells were washed once with 10 ml of met-free DMEM (from Gibco), incubated with 10 ml of met-free DMEM containing 5% dialyzed fetal calf serum at 37°C for 1 hour, labeled with 1 mCi of [^{35}S]methionine at 37°C for 3 hours with gently shaking every 30 min. Plates were washed twice with 5 ml of PBS, lysed by addition of 3 ml of cold PBSTDS (1% of Triton X-100, 0.5 % of sodium deoxycholate, 0.1 % of SDS and 5 mM of PMSF in PBS buffer) and incubated on ice for 10 min. Cells were harvested, spun and supernatants were transferred into three 1.5 ml- Eppendorf tubes for immunoprecipitation assays.

Cellular extracts in Eppendorf tubes were normalized by ^{35}S labeled protein counts and mixed with 10 µl of monoclonal antibody [v-H-*ras* (Ab-1)] Y13-259 (from Oncogene Science) incubated with agitation in cold room. After one hour, 15 µl of Protein G plus-Agarose (from Oncogene Science) were added into each Eppendorf tube by using an end shorted tip and reactions were incubated with agitation in cold room for another two hours. Immunoprecipitates were collected by centrifugation at 15, 000 rpm for 15 sec in cold room. Pellets were washed four times with PBSTDS and resuspended in 40 µl of sample buffer (0.0625 M Tris·Cl (pH 7.6) 1% SDS, 10% glycerol, 0.001% pyronin Y, 10% β-mercaptoethanol), boiled for 5 min and subjected into sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

For analysis of *ras* proteins, 15% separating gels (acryl:bis=30: 0.4), 5% stacking gels with 0.1% SDS in either separating buffer (0.375 M Tris·Cl pH 8.8) or stacking buffer (o.125 M Tris·Cl, pH 6.8) were used.

Rainbow colored low molecular weight standard proteins were from Amersham. Gels were run at about 100 V for four hours, fixed with fixing solution (10% glycerol, 10% acetic acid) for 30 min, soaked in Amplify (from Amersham) with agitation for 15 min and dried under vacuum. Dried gels were hold in close contact with Hyperfilm-MP (from Amersham) at -80^{o}C overnight and developed.

CHAPTER 3

STATEMENT OF PROBLEMS AND SPECIFIC AIMS

Cancer is one of the leading causes of death in the United States. A detailed understanding of the molecular events that underlie the development of cancers may lead to the design of rational strategies for both prevention and cure. Over the past twenty five years, many cellular genes involved in the conversion of the normal cell to a cancer cell have been identified. The proteins that these genes encode and the biochemical basis of their actions have also been described, albeit partially. The majority of these proteins are involved in regulation of cell growth. Some of these gene products, like $p21^{ras}$ when activated, promote the uncontrolled proliferation of cells whereas others serve as "brakes" to regulate cell proliferation (Marshall, 1991).

The interactions of many hormones such as adrenaline, vasopressin and glucagon with their respective receptors on the cell surface lead to activation of the G_S-α signaling pathway. In turn, that activation produces the intracellular messenger cyclic adenosine monophosphate (cAMP). Eventually many cellular activities are regulated. The signal transducing protein, α_s, is responsible for converting the extracellular hormone signals into the intracellular cAMP messenger by stimulating the enzyme adenylyl cyclase. My work, however, has focused on studying the function of this protein in regulation of cell proliferation. Prior studies have shown that some tumors harbor mutant, activated forms of α_s (Landis et al., 1989). It had been suggested that this mutant protein may help to promote the conversion of these cells to a cancerous state.

Based on the prior observation, I have attempted to address the following questions:

1. Can the mutant activated α_s transform cells ?

This question is addressed by first isolation of several inducible, mutant, activated α_s-expressing cell lines, in both NIH-3T3 and RAT-1 cells, and determining the effects of activated α_s on cell growth and cell transformation in both cells.

2. Can the mutant activated α_s promote or block the transformation of NIH-3T3 cells by the oncogene *ras*?

To approach this question, cell lines which express the mutant activated α_s will be transfected with *ras* and the growth of cells will be characterized. Two commonly used criteria will be employed to assess effects of expression the mutant activated α_s on the transformation of cells by H-*ras*. These assays include determination of DNA synthesis rates by measuring [^{3}H] thymidine incorporation, and of anchorage independent growth ability by colony formation in soft-agar.

3. Are the effects of the mutant α_s general or cell type specific?

To answer this question, the effects of mutant α_S on *ras*-induced transformation were studied in two different types of cells: NIH-3T3 and RAT-1 cells. These two cell types show a different proliferate response to cAMP. Changes in cellular cAMP level have two types of effects on these cells. In NIH-3T3 cells, increases or decreases in cellular cAMP by itself do not have any effects on cell proliferation. On the other hand, lowering of cellular cAMP levels strongly stimulates cell proliferation in RAT-1 cells. In this setting, using these two cell types would shed light on question if the effects of the mutant α_S on *ras*-induced transformation are restricted to specific types of cells or are widely distributed.

4. Are the effects of mutant α_S dependent on the cAMP pathway or is the mutant α_S using an as yet unidentified growth signaling pathway?

This question will be explicitly tested in two ways: 1) If the effects of the mutant α_S are solely due to the production of cAMP then culturing cells in the presence of 8 bromo-cAMP (8 Br-cAMP), a biologically active, cell permeable analog of cAMP, should have the same effects as expressing the mutant α_S. 2) I will also tested whether over-expression of the dominant negative regulatory subunits of protein kinase A can relieve the effects of the mutant α_S. The reasoning behind this set of experiments is the following. Protein kinase A is the only intracellular enzyme that is regulated directly by cAMP. In most cell types, all of the effects of cAMP are mediated through this enzyme. Hence if the cAMP pathway is responsible for the effect of α_S on cell proliferation, then

expression of the dominant negative regulatory component of protein kinase A along with activated α_s should yield opposite effects as compared to the expression of the activated mutant α_s alone.

5. Can the mutant α_s also decrease the *ras*-stimulated MAP kinase activity?

It has been shown that MAP kinase is a crucial intermediate enzyme which carries signal from *ras* (Davis, 1993; Marx, 1993). As α_s and *ras* are both cytoplasmic proteins, I have hypothesized that α_s may interact directly or indirectly with intermediate(s) resulting in alteration of *ras*-stimulating MAP kinase activity. To address this question, the effects of expression of mutant activated α_s and 8 Br-cAMP on *ras*-stimulating MAP kinase activity will be studied.

CHAPTER 4

EXPRESSION OF MUTANT ACTIVATED α_s CAN NOT TRANSFORM EITHER NIH-3T3 OR RAT-1 CELLS

A. Construction of the mutant activated α_s expression vectors

1) Construction of the mutant activated α_s protein.

The wild-type α_s cDNA in pT7-7 vector was used as the template. In order to introduce mutations into the α_s coding region, a primer containing the specific mutations was synthesized and used in PCR reaction (see Chapter 2 for details). These two nucleotide changes also generated an unique NruI site. A schematic diagram outlining the procedure used for the construction of the mutant α_s is shown in Figure 4-1.

The PCR product was digested with BamHI and was inserted to pT7-7-α_s digested with BamHI to replace the corresponding wild-type fragment. Digestion with NruI and NcoI digestion were used to check the existence of mutations and the orientation of the insert respectively. In order to ascertain that the mutant α_s has the correct sequence, the region replaced was sequenced and it was confirmed that the mutations were present.

2) Translation of the wild type and mutant proteins *in vitro*.

To further verify that the constructs containing the wild type and the mutant α_s were correct, mRNA molecules had been transcribed *in vitro*

from these plasmids containing the wild type and mutant α_S coding sequences using T7 RNA polymerase, as these vectors contain the T7 promoter (Fig. 4-1). mRNAs were then translated *in vitro* using nuclease-treated rabbit reticulocyte lysate. ^{35}S-methionine was included in the reaction to label translational products. The proteins synthesized were analyzed directly by SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 4-2. In *vitro* translation results in the appearance of a major protein band in lane 3 and 4 which comigrates with the control protein (lane 2). The size of these proteins are the same as the α_S protein determined by protein markers. The general background bands are seen in all lanes due to nonspecific translation products (Fig. 4-2). These results showed that the constructs containing the α_S cDNAs produce correct proteins *in vitro*.

3) Construction of mammalian transfection vectors which contain the cDNAs encoding the native and mutant α_S.

To express the mutant α_S in mammalian cells, a mammalian expression vector, pMAM-neo (pMN) was utilized. The pMN is an inducible vector where the cDNA of interest can be placed downstream of the mouse mammary tumor virus promoter which is a glucocorticoid responsive element. Synthesis of the α_S can be induced by the glucocorticoid receptor when its ligand dexamethasone (Dex) is present. The pMN vector also contains a neomycin (neo) resistance gene which is expressed under the control of SV40 promoter. The neo gene is constitutively expressed in cells transfected with pMN and this

characteristic can be used to select stable transformants. G-418, which is an aminoglycoside, blocks protein synthesis in mammalian cells by interfering with ribosomal function. Expression of the neo gene, which is an aminoglycoside phosphotransferase, results in breakdown of G-418 and therefore protects the cells against G-418 (Southern and Berg, 1982).

cDNAs of the wild type and the mutant type α_S were excised from the pT7-7 plasmids by NcoI and HindIII digestion and rendered blunt by Klenow fragment (Sambrook et al., 1989) (Fig. 4-3). pMN was digested with XhoI and also was blunt-ended using the same method. The cDNAs and vectors were ligated and transformed into competent cells (Fig. 4-3). The plasmids containing the α_S cDNAs were selected by restriction enzyme digestion. The resulting two plasmids are pMN-α_S containing the coding sequence for the wild type α_S and pMN-Q227L-α_S, the mutant α_S.

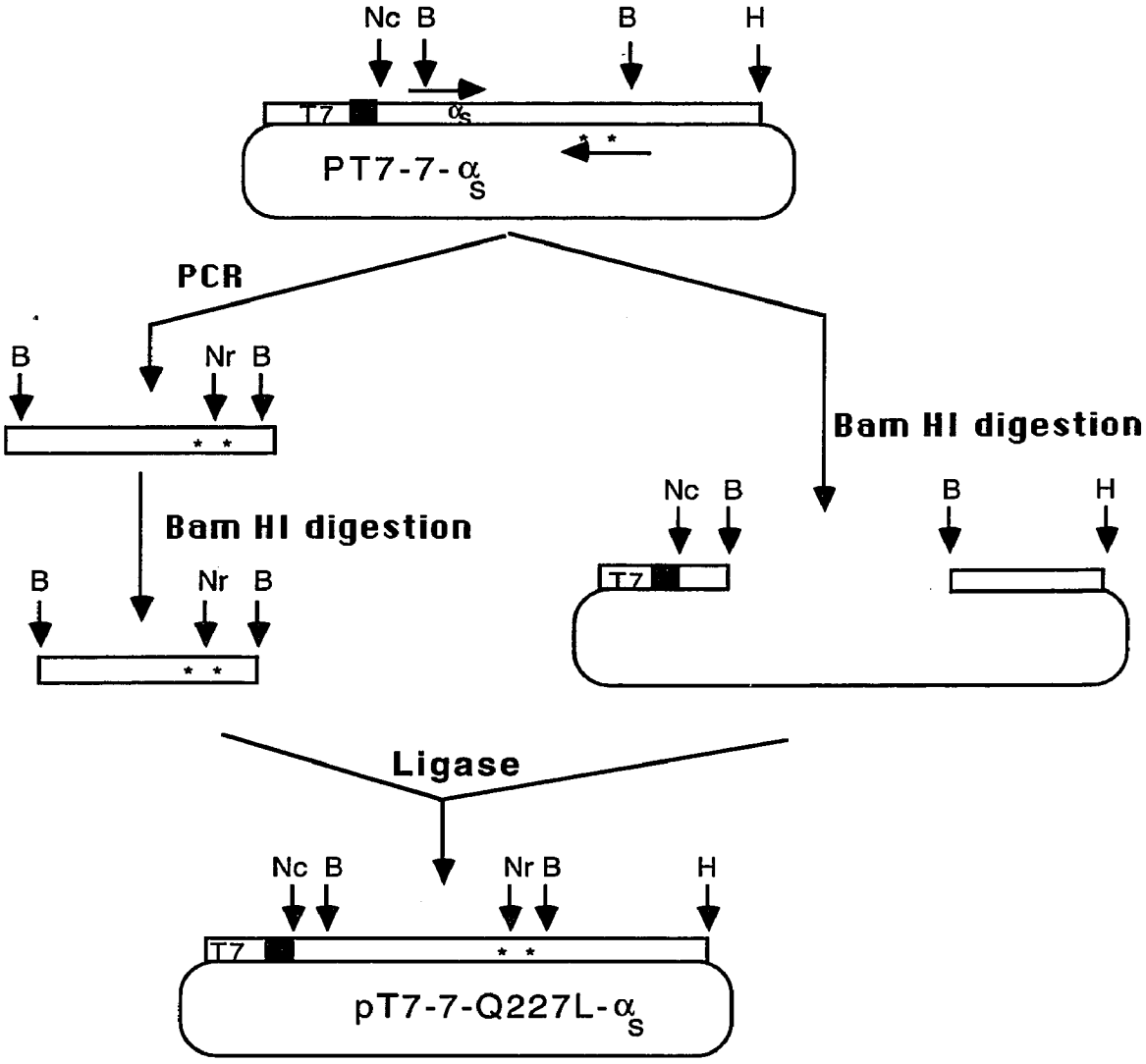

Nc B
B
H
T7
α_s
PT7-7-α_s
PCR
B
Nr B
Bam HI digestion
B
Nr B
Bam HI digestion
Nc B
B
H
T7
Ligase
Nc B
Nr B
H
T7
pT7-7-Q227L-α_s

Fig. 4-1 Strategy of introducing mutations into α subunit of G_S by utilization of polymerase chain reaction (PCR).

Two synthetic oligonucleotides (indicated by horizontal lines and arrows) were used as primers in PCR to amplify the BamH1 fragment from pT7-T7-α_S. The two asterisks represent two nucleotide changes. The PCR product was digested with BamHI and was purified. The pT7-7-α_S was also digested with Bam HI. The vector fragment was isolated and was ligated with the purified PCR product. The plasmid was selected by NruI and NcoI digestion. T7, T7 promoter; shadowed box, the untranslated region; B, BamHI; Nc, NcoI; Nr, NruI.

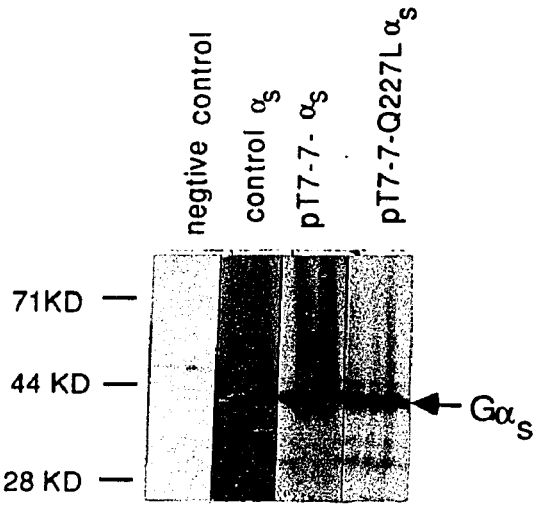


Fig. 4-2. Electrophoretic resolution of the *in vitro* translated proteins.

The mRNAs transcribed *in vitro* from native (pT7-7-α_S) and mutant (pT7-7-Q227L-α_S) were translated in rabbit reticulocyte lysate using ^{35}S-methionine to monitor the synthesis. The synthesized samples and additional controls (no mRNA-negative control, and native protein control-α_S) were analyzed by SDS-PAGE and autoradiography. Lane 1, no RNA was added; lane 2, positive mRNA was added; lane 3, the mRNA transcribed from pT7-7-α_S; lane 4, mRNA prepared from pT7-7-Q227L-α_S was included. The arrow indicates the size of α_S protein. The positions of protein markers are shown at the left.

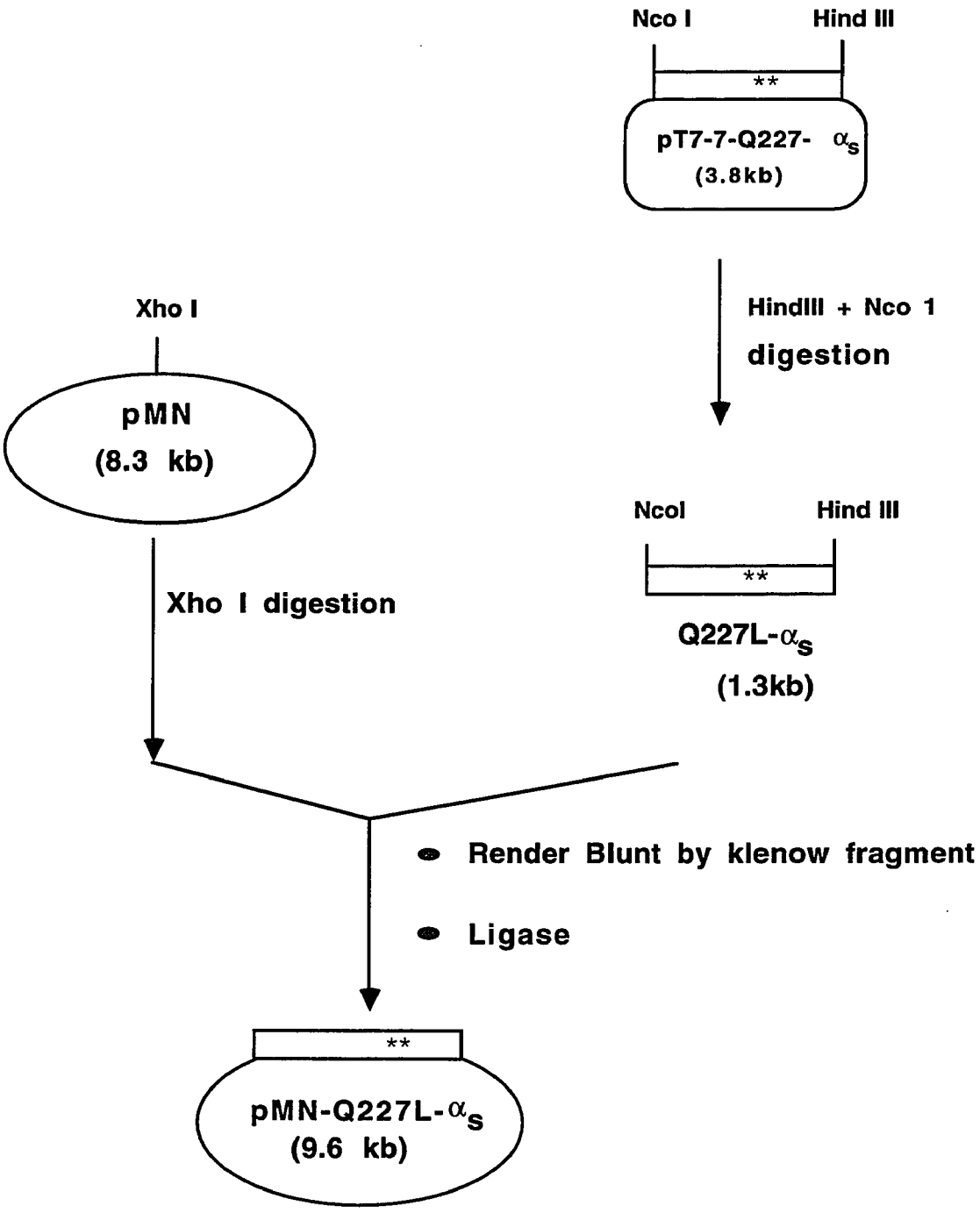

Nco I
Hind III
**
pT7-7-Q227- α_s
(3.8kb)
HindIII + Nco 1
digestion
Xho I
pMN
(8.3 kb)
NcoI
Hind III
**
Q227L-α_s
(1.3kb)
Xho I digestion
Render Blunt by klenow fragment
Ligase
**
pMN-Q227L-α_s
(9.6 kb)

Fig. 4-3. Construction of the pMN-Q227L-α_S.

cDNAs encoding the wild type and mutant α_S were excised from the pT7-7 vector by digestion with Nco 1 and Hind III. The enzyme Nco 1 cuts at the first base of the open reading frame while Hind III cuts in the 3' untranslated region. Thus a cDNA fragment encoding the entire the wild type or the mutant α_S protein in the correct reading frame was obtained. The jagged ends were filled in by Klenow fragment enzyme. pMAM-neo (PMN) was opened with Xho I in the multiple cloning site and the jagged ends were also filled in. The two fragments were combined by blunt end ligation and were propagated in bacteria. The plasmids were purified and used for transfection in mammalian cells.

B. Establishment of the mutant activated α_s expression cell lines

1. Selection of clonal cell lines.

It has been shown that some pituitary tumors harbor mutations in $\alpha_{s,}$. resulting in a continuously activated α_s protein. It has been hypothesized that these mutant activated proteins might participate in transformation of these cells into cancerous state. To study whether the α_s can transform cells in *vitro* directly, cell lines expressing the mutant activated α_s were established. The procedure was illustrated in Fig. 4-4. NIH-3T3 cells which took up these plasmids expressed the neo resistance gene and thus were resistant to G-418, a neomycin analog. The presence of G-418 in medium killed cells that had not taken up the exogenous DNAs. The surviving cells were cloned by isolation of single colonies. Clonal cell lines from RAT-1 cells were also obtained using the same procedure. As clonal cell lines containing the transfected plasmids might express α_s at different levels, these cell lines were subjected to further analysis.

Some of the clonal cell lines derived from NIH-3T3 cell are the following: n-1, n-3, pMN transfected NIH-3T3 cell line; α_s-2, α_s-3, pMN-α_s (wt) transfected NIH-3T3 cell lines; α_s^*-1, α_s^*-3, α_s^*-12, α_s^*-14, pMN-α_s* transfected NIH-3T3 cell lines. Some clonal cell lines derived from RAT-1 cell are the following: R-n-1, pMN transfected RAT-1 cell line; R-

α_S-4, R-α_S-5, pMN-α_S transfected RAT-1 cell lines; R-α_S^*-1, R-α_S^*-2, R-α_S^*-7, pMN-α_S^* transfected RAT-1 cell lines.

2) Selection of clonal cell lines expressing high levels of adenylyl cyclase activity and showing enhanced basal levels of cAMP accumulation.

In order to select clonal cell lines which expressed α_S, the expression of the inserts was induced by addition of Dex and was monitored using two assays. It has been shown that activation of α_S stimulates adenylyl cyclase activity. First, the levels of adenylyl cyclase activity in transfected cells were measured and results are shown in Table 4-1. The cell lines (α_S-2 and α_S-3) transfected with the wild type construct showed the similar levels of adenylyl cyclase activity as the cell line (n-1) transfected with the vector alone. However, the cell lines (α_S^*-1 and α_S^*-3) transfected with the mutant activated showed more than 50% increase in adenylyl cyclase activity (Table 4-1).

Similarly the levels of adenylyl cyclase activity in RAT-1 cell lines were also determined and the results are summarized in Table 4-2. Again, it was observed that the levels of adenylyl cyclase activity in cell lines transfected with the wild type α_S (R-α_S-4 and R-α_S-5) were comparable to that in the cells transfected with the vector (R-n-1). The cell lines transfected with the mutant α_S (R-α_S^*-2 and R-α_S^*-7) again showed more than 50% increase in adenylyl cyclase activity (Table 4-2).

To establish that the expression of α_s in selected cell lines resulted in elevated cellular cAMP levels, I also directly determined accumulations of cAMP in clonal cells. The assay is based on the fact that activation of α_s increases adenylyl cyclase activity resulting in accumulation of cAMP levels in cells. The results of this study are shown in Table 4-3. In NIH-3T3 cells, the same level of cAMP accumulation was detected in cell lines α_s-2 and α_s-3 as well as in cell line n-1. The four cell lines (α_s^*-1, α_s^*-3, α_s^*-12 and α_s^*-14) containing α_s all showed higher levels of cAMP accumulation (Table 4-3). The data of the two assays were consistent in both NIH-3T3 and RAT-1 cells (Table 4-4). The increases in cAMP levels are similar to that reported by Wong et al. (1991) in α_s^*-transfected cells. The data showed that the expression of mutant α_s results in elevation of basal cAMP levels in these clonal cell lines.

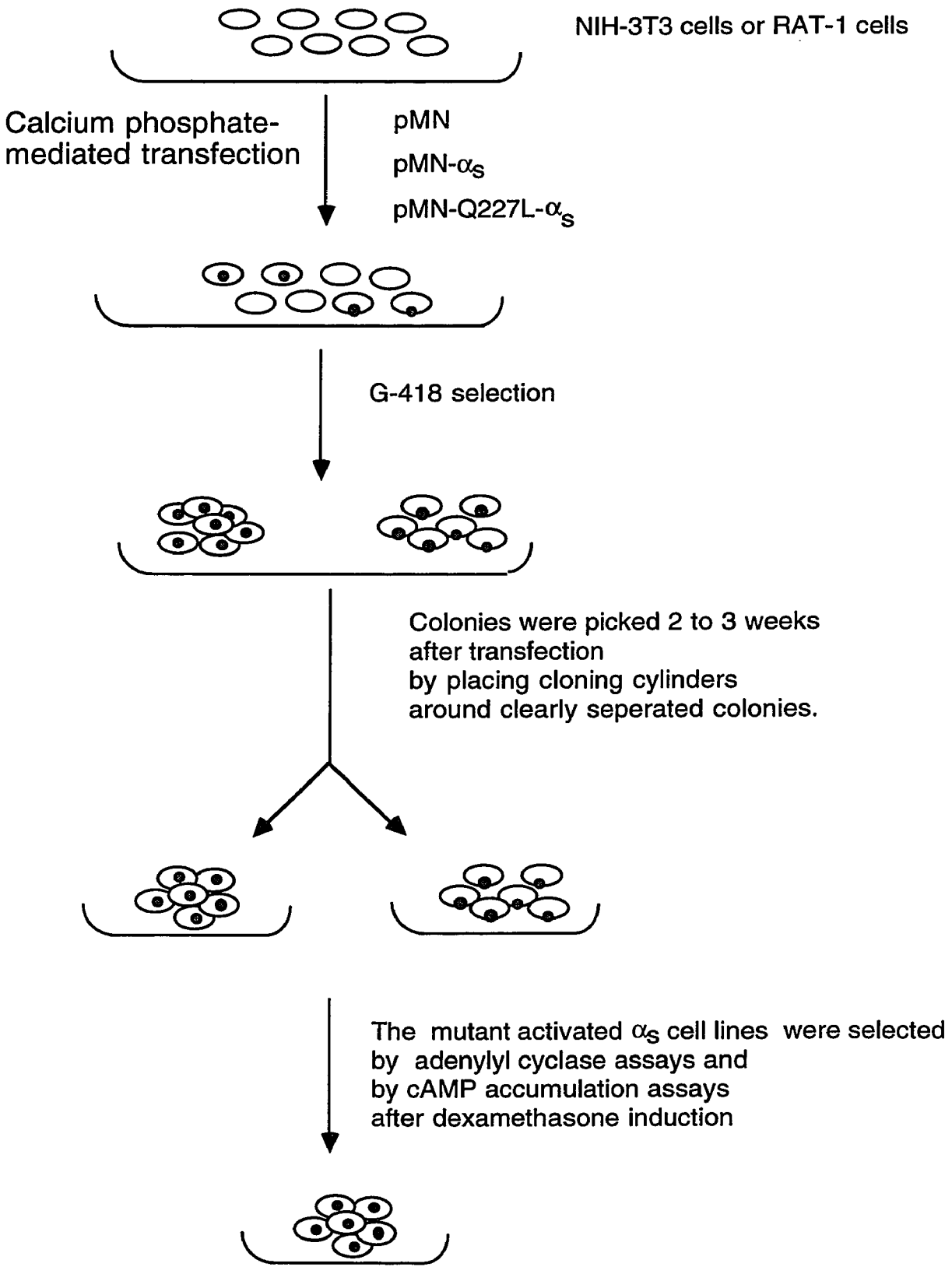

NIH-3T3 cells or RAT-1 cells
Calcium phosphate-mediated transfection
pMN
pMN-α_s
pMN-Q227L-α_s
G-418 selection
Colonies were picked 2 to 3 weeks after transfection by placing cloning cylinders around clearly seperated colonies.
The mutant activated α_s cell lines were selected by adenylyl cyclase assays and by cAMP accumulation assays after dexamethasone induction

Fig. 4-4. Generation of cell lines.

NIH-3T3 or RAT-1 cells were transfected with either pMN, or pMN-α_S or pMN-Q227L-α_S using calcium phosphate method (see chapter 2 for details). The transfected cells were then subjected to G-418 selection. Only cells which contained the plasmids survived. Individual colonies were picked two to three weeks after transfection by placing cloning cylinders around clearly separated colonies and colonies were subjected to further analysis. Two assays, adenylyl cyclase activity determination and cAMP accumulation measurement, were used to select the α_S^* expressing cell lines.

NIH-3T3 clonal cell lines	pmol cAMP/10^6 cells x min
n-1	1.53 ± 0.06
α_s-2	1.67 ± 0.10
α_s-3	1.51 ± 0.08
α_s^*-1	2.48 ± 0.11
α_s^*-3	2.25 ± 0.09

Table 4-1. Adenylyl cyclase activity in NIH-3T3 clonal cell lines.

Adenylyl cyclase activity in NIH-3T3 clonal cell lines was measured in individual clonal cell lines derived from NIH-3T3 cells transfected with pMN, pMN-α_S, and pMN-Q227L-α_S (pMN-α_S^*). Cells were treated with dexamethasone (DEX) to induce expression of α_S proteins for one week. Cells were lysed and incubated with reaction buffer containing [α-^{32}P]ATP. [2,8-^{3}H]cAMP was included as an internal control. cAMP was separated and [α-^{32}P]cAMP was measured. The enzymatic activity of adenylyl cyclase is represented as the amount of [α-^{32}P]cAMP synthesized. n-1, pMN transfected NIH-3T3 cell line; α_S-2, pMN-α_S transfected NIH-3T3 cell line; α_S^*-1, α_S^*-3, pMN-α_S^* transfected NIH-3T3 cell lines.

RAT-1 clonal cell lines	pmol cAMP/10^6 cells x min
R-n-1	1.29 ± 0.06
R-α_s-4	1.52 ± 0.12
R-α_s-5	1.58 ± 0.11
R-α_s^*-2	1.96 ± 0.12
R-α_s^*-7	2.23 ± 0.15

Table 4-2. Adenylyl cyclase activity in RAT-1 clonal cell lines.

Adenylyl cyclase activity in RAT-1 clonal cell lines was measured in individual clonal cell lines derived from RAT-1 cells transfected with pMN, pMN-α_S, and pMN-Q227L-α_S (pMN-α_S^*). Cells were treated with dexamethasone (DEX) to induce expression of wild type and mutant α_S proteins for one week. Cells were then lysed and incubated with reaction buffer containing the substrate [α-^{32}P]ATP. [2,8-^{3}H]cAMP was included in the reactions as internal control. cAMP was separated and [α-^{32}P]cAMP was measured. The enzymatic activity of adenylyl cyclase is represented as the amount of [α-^{32}P]cAMP synthesized. R-n-1, pMN transfected RAT-1 cell line; R-α_S-4, R-α_S-5, pMN-α_S transfected RAT-1 cell line; R-α_S^*-2, R-α_S^*-7, pMN-α_S^* transfected RAT-1 cell lines.

NIH-3T3 clonal cell lines	cAMP/(ATP+cAMP) ($x10^3$)
n-1	0.95 ± 0.05
α_s - 2	0.97 ± 0.01
α_s - 3	1.16 ± 0.08
α_s^* - 1	1.40 ± 0.29
α_s^* - 3	1.58 ± 0.16
α_s^* - 12	1.93 ± 0.23
α_s^* - 14	2.02 ± 0.21

Table 4-3. cAMP accumulation in NIH-3T3 clonal cell lines.

[^{3}H]cAMP accumulation was measured in individual clonal cell lines derived from NIH-3T3 cells transfected with pMN, pMN-α_s, and pMN-Q227L-α_s (pMN-α_s^*). Cells were treated with dexamethasone (DEX) for one week and then seeded subconfluently in 24-well plates. [^{3}H]adenine was used to label cells for 24 h. cAMP accumulation was measured in the presence of 1-methyl-3-isobutylxanthine as described in Chapter 2. cAMP level is presented by the ratio of [^{3}H]cAMP/([^{3}H]cAMP+[^{3}H]ATP)($x10^3$). Values are means ± SEM. n-1, pMN transfected NIH-3T3 cell line; α_s-2, α_s-3, pMN-α_s transfected NIH-3T3 cell lines; α_s^*-1, α_s^*-3, α_s^*-12, α_s^*-14, pMN-α_s^* transfected NIH-3T3 cell lines.

RAT-1 clonal cell lines	cAMP/(ATP+cAMP) ($x10^{3}$)
R-n-1	0.87 ± 0.06
R-α_s-4	1.01 ± 0.05
R-α_s-5	0.94 ± 0.05
R-α_s^*-1	1.29 ± 0.02
R-α_s^*-2	1.24 ± 0.01
R-α_s^*-7	1.30 ± 0.05

Table 4-4. cAMP accumulation in RAT-1 clonal cell lines.

[^{3}H]cAMP accumulation was measured in individual clonal cell lines derived from RAT-1 cells transfected with pMN, pMN-α_s, and pMN-Q227L-α_s (pMN-α_s^*). The methods used are the same as described in the legend to Table 4-3. Values are means ± SEM. R-n-1, pMN transfected RAT-1 cell line; R-α_s-4, R-α_s-5, pMN-α_s transfected RAT-1 cell lines; R-α_s^*-1, R-α_s^*-2, R-α_s^*-7, pMN-α_s^* transfected RAT-1 cell lines.

C. The effects of the expression of the mutant α_s on proliferation of the clonal NIH-3T3 and RAT-1 cell lines

To assess whether expression of α_s^* resulted in transformation, two assays were performed. First, the doubling times of the transfected cells were measured. The results are shown in Fig. 4-5. The presence of Dex has no effects on doubling times of clonal cell lines as these cell lines grew at the same rate in the presence or absence of Dex. The clonal cell lines containing the wild type α_s or the mutant α_s showed the same rate of growth as the clonal cell line containing the vector (Fig. 4-5). Similar observations were made in RAT-1 cell lines (Fig. 4-6). These results indicate that the expression of the mutant α_s has no effect on cell growth rate.

To examine if the cell lines containing the mutant α_s showed any characteristics of transformed cells, I also determined the ability of these cells to form colonies in soft agar. For both NIH-3T3 and RAT-1 cells, none of the clones expressing α_s^* showed any colonies in soft agar (Table 4-5 and Table 4-6). However, under the same conditions, expression of the pT-24 (H-*ras* gene) induced substantial levels of transformation as assessed by number of colonies formed (Table 4-5 and Table 4-6). The results suggested that α_s^* alone can not transform NIH-3T3 and RAT-1 cells, although the expression of a single oncogene is sufficient for its transformation.

NIH-3T3 clonal cell lines

	Doubling time
n-1	18hr
n-1 (1 μM DEX)	20hr
α_s-2	18hr
α_s-2 (1 μM DEX)	18hr
α_s^* -3	19hr
α_s^* -3 (1 μM DEX)	19hr

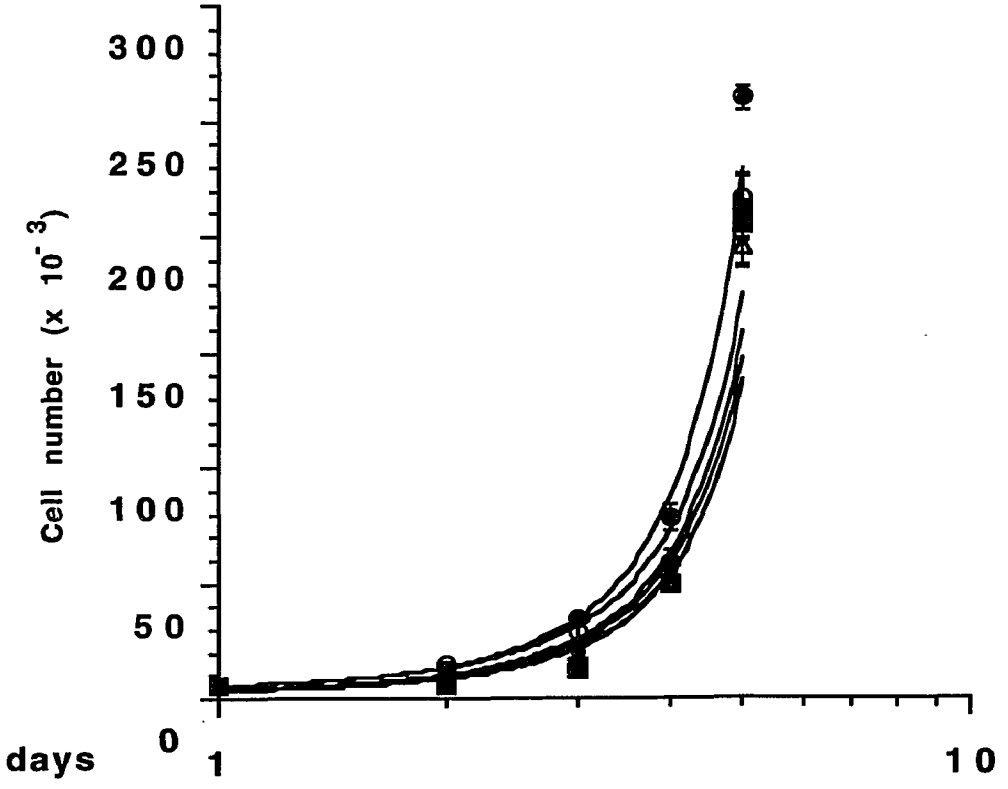


Fig. 4-5. Effect of expression of α_S^* on the proliferation of NIH-3T3 cells.

Cells were seeded into 24-well plates at a density of $6x10^3$ cells/well in the absence or presence of DEX. Each group had four replicate wells. The cell numbers were determined daily for 5 consecutive days. Doubling time was calculated according to equations described in Chapter 2. n-1, n-3, pMN transfected NIH-3T3 cell line; α_S-2, α_S-2, pMN-α_S transfected NIH-3T3 cell line; α_S^*-3, α_S^*-12, α_S^*-14, pMN-α_S^* transfected NIH-3T3 cell lines.

RAT-1 clonal cell lines

	Doubling time
R-n-1	16 hr
R-n-1 (1 μM DEX)	17 hr
R-α_s-5	15 hr
R-α_s-5 (1 μM DEX)	16 hr
R-α_s^*-7	16 hr
R-α_s^*-7 (1 μM DEX)	16 hr

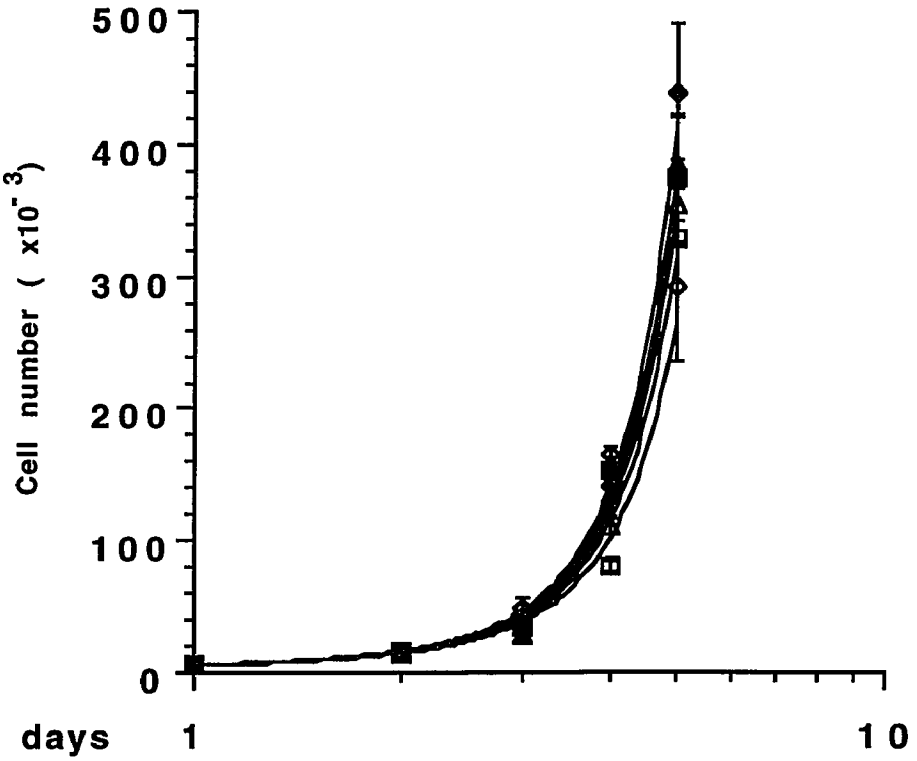


Fig. 4-6. Effect of expression of α_s^* on the proliferation of RAT-1 cells.

Cells were seeded into 24-well plates at a density of $6x10^3$ cells/well in the absence or presence of DEX. Each group had four replicate wells. The cell numbers were determined daily for 5 consecutive days. Doubling time was calculated according to equations described in Chapter 2. R-n-1, pMN transfected RAT-1 cell line; R-α_s-5, pMN-α_s transfected RAT-1 cell line; R-α_s^*-7, pMN-α_s^* transfected RAT-1 cell lines.

NIH-3T3 clonal cell lines	T24	colonies / 5 x 10^4 cells (0.15 mm-1.2 mm)
n-1	-	0
n-3	-	0
α_s -2	-	0
α_s^* -3	-	0
α_s^* -12	-	0
α_s^* -14	-	0
n-1	+	60 ± 1
n-3	+	55 ± 5

Table 4-5. Clonal NIH-3T3 cell lines expressing the mutant activated α_S are not transformed whereas pT24 H-*ras* DNA efficiently transforms NIH-3T3 cells.

Transformation was assessed by colony formation in soft agar. The method is described in chapter 2. n-1, n-3, pMN transfected NIH-3T3 cell line; α_S-2, α_S-2, pMN-α_S transfected NIH-3T3 cell line; α_S^*-3, α_S^*-12, α_S^*-14, pMN-α_S^* transfected NIH-3T3 cell lines.

RAT-1 clonal cell lines	T24	colonies / 5 x 10^4 cells (0.15 mm-1.2 mm)
R-n-1	-	0
R-α_s-5	-	0
R-α_s^*-1	-	0
R-α_s^*-2	-	0
R-α_s^*-7	-	0
R-n-1	+	89 ± 2

Table 4-6. Clonal RAT-1 cell lines expressing the mutant activated α_S are not transformed whereas pT24 H-*ras* DNA efficiently transforms RAT-1 cells.

The methods used are the same as described in the legend of Table 4-5. R-n-1, pMN transfected RAT-1 cell line; R-α_S-5, pMN-α_S transfected RAT-1 cell line; R-α_S^*-1, R-α_S^*-2, R-α_S^*-7, pMN-α_S^* transfected RAT-1 cell lines.

CHAPTER 5

THE MUTANT ACTIVATED α_s BLOCKS H-*ras*-INDUCED TRANSFORMATION

The mutation used to activate the α_s in this study is the same as that found in some pituitary tumors (Landis et al., 1989; Lyons et al., 1990), however, mutant activated α_s did not induce transformation by itself (Chapter 4). It is possible that α_s^* may be a weak oncogene and that its transforming potential might be observable in conjunction with another oncogene. Hence I examined this hypothesis by determining whether expression of α_s^* increased the frequency of transformation produced by low levels of H-ras DNA. Varying amounts of pT24 (H-*ras*) DNA were transfected into an α_s*-expressing clonal cell line (α_s*-3) and a clonal line (n-1) derived from vector (pMN)-transfected cells, and then the transfected cells were tested for anchorage-independent growth by measuring colony formation in soft agar. The results are shown in Fig. 5-1. Expression of α_s* resulted in an almost total blockade of *ras*-induced transformation at all concentrations of H-*ras* DNA tested (Fig. 5-1).

This result was rather unexpected. One possible explanation for this blocking of transformation by *ras* was that the expression of *ras* was inhibited in α_s^*-expressing cells resulting in no transformation. Thus the expression levels of *ras* gene were examined in α_s^*-expressing cell line transfected with *ras* DNAs. The same clonal cell lines (n-1 and α_s^*-3) used in the experiment shown in Figure 5-1 were employed in this set of experiments. Cells (α_s^*-3) were cotransfected with pT24 (H-*ras*) and pRSV1.1, a plasmid containing hygromycin B phosphotransferase gene (Krauss et al., 1992; Murphy and Efstratiadis, 1987), and grown for one week in hygromycin. The hygromycin selection enriched cells which contained *ras* DNA. Surviving cells were treated with DEX to induce

expression of α_s* and labeled with [^{35}S]methionine. The labeled cells were extracted in detergent and the extract immunoprecipitated with an anti-*ras* protein monoclonal antibody Y13-259 (Furth et al., 1982). The immunoprecipitate was dissolved in SDS-containing sample buffer, analyzed on SDS-polyacrylamide gels and visualized by autoradiography. A photograph of the autoradiogram is shown in Figure 5-2. Upon transfection with pT24 (H-*ras*) DNAs, there is an observable increase in the *ras* protein, and expression of α_s* does not affect this increase. This experiment indicates that the blockade of transfection is not due to a inhibition of the expression of the *ras* protein.

It is also possible that the expression of α_s* might make cells resistant to uptake of transfected DNAs. To determine whether expression of α_s* affected transfection efficiencies, clonal lines of NIH-3T3 cells expressing wild type α_s or mutant α_s* were transfected with pRSV1.1, and hygromycin resistant colonies were then counted. The results are shown in Table 5-1. Similar amounts of colonies were obtained from cells containing either vector (n-1, n-3), or α_s (α_s-2) or α_s* (α_s*-3). Thus it was concluded that the expression of α_s* did not affect transfection efficiencies of NIH-3T3 (Table 5-1). The result also implied that the inhibitory effects of the expression of α_s* on *ras*-induced transformation involves specific interactions in intracellular signaling.

To examine whether the suppression of transformation of NIH-3T3 cells by α_s* was unique to the one clonal line previously studied (Fig. 5-1), two additional clones expressing α_s* (α_s*-12, α_s*-14) were tested for their ability to block *ras*-induced transformation. Although there are

variations among these clonal cell lines when large amounts of pT24 (H-*ras*) DNAs were used in transfections, highly significant suppression of H-*ras*-induced transformation of NIH-3T3 cells was observed in all three clones (Fig. 5-3).

I extended the study to determine if the inhibitory effect of α_s* on *ras*-induced transformation was cell type specific. To answer this question, the effects of expression of α_s* on transformation of RAT-1 cells by H-*ras* DNA was investigated. Three independent clonal RAT-1 lines expressing α_s* were studied. The same assays described previously were performed and the results are shown in Figure 5-4. It was found that expression of α_s* significantly suppressed transformation of RAT-1 cells by H-*ras*, suggesting that the inhibitory effect of α_s* was not cell type specific (Fig. 5-4). Again it was confirmed that the expression of α_s* in RAT-1 did not affect the transfection efficiency of these cells (Table 5-2).

I also determined the effect of expression of α_s* on H-*ras*-stimulated mitogenesis in one of the clonal lines expressing α_s*. The cells were induced with dexamethasone and then transfected with 20 μg carrier DNA (purified from NIH-3T3 cells) with or without 1 μg of pT24 (H-*ras*) by the calcium phosphate method. The transfected cells were cultured for 2 weeks in the presence of dexamethasone on alternate days to induce the expression of α_s*. A typical experiment is shown in figure 5-5. Expression of α_s* did not significantly alter DNA synthesis as assessed by [^{3}H]thymidine incorporation. pT24 (H-*ras*) transfected cells showed a five-fold increase in DNA synthesis. Expression of α_s* significantly suppressed *ras*-induced DNA synthesis (Fig. 5-5).

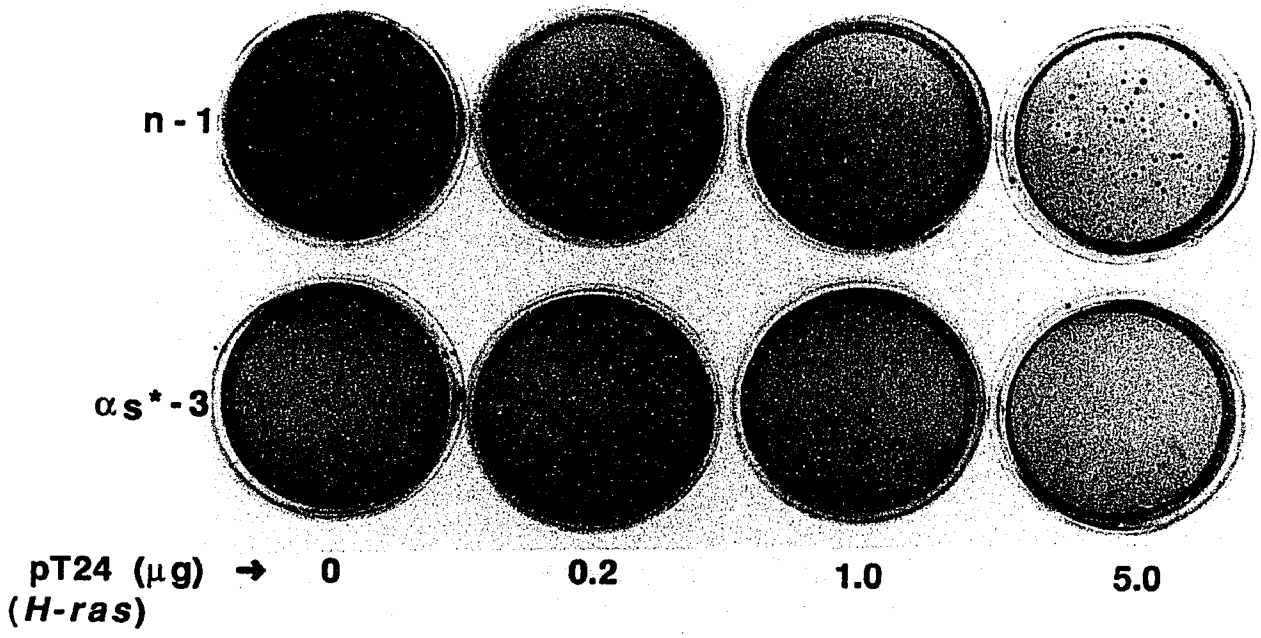


Fig. 5-1. Effect of the expression of α_S^* on the transformation of NIH-3T3 cells by H-*ras* DNA.

Transformation was assessed by colony formation in soft agar. n-1, a pMN-containing NIH-3T3 cell line and α_S^*-3, a NIH-3T3 clonal cell line, expressing mutant activated α_S (Q227L) were transfected by calcium phosphate method with 0 μg, 0.2 μg, 1 μg, 5 μg of pT24 DNA and 20 μg of genomic NIH-3T3 DNA as carrier. Cells were treated with DEX to induce expression of α_S^* and plated onto soft agar to assess colony formation. The procedures were described in details in Chapter 2.

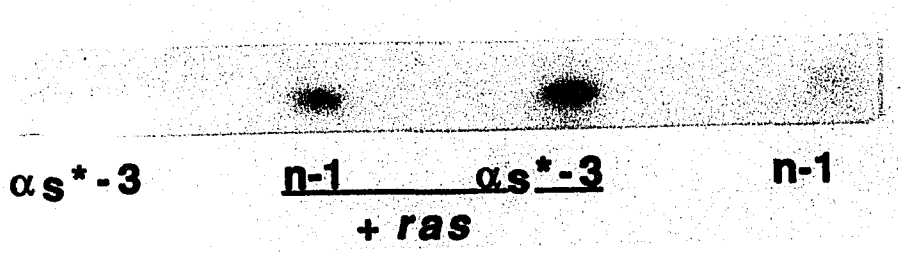


Fig. 5-2. Effect of α_s^* on the expression of *ras* protein in the α_s^*-expressing clonal NIH-3T3 cells transfected with H-*ras* DNA.

[^{35}S]methionine labeled H-*ras* p21 proteins were immunoprecipitated from extracts of NIH-3T3 cell lines, n-1 and α_s^*-3, which were transfected with 20 μg of genomic NIH-3T3 DNA, 1 μg of pRSV1.1 DNA and with or without 5 μg of pT24 DNA. After transfection as described in Chapter 2, cells were induced by DEX for one day and grown in media containing 400 μg/ml hygromycin B. Medium was changed every three days. After one week, cells were induced by DEX again. One week later, 3x10^6 cells were labeled with 0.25 mCi of [^{35}S]methionine for 3 h. Cells were then lysed and immunoprecipitated with anti-*ras* p21 monoclonal antibody Y13-259 according to the protocol provided by Oncogene Science Inc. The immunoprecipited products were resolved on 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and visualized by autoradiography.

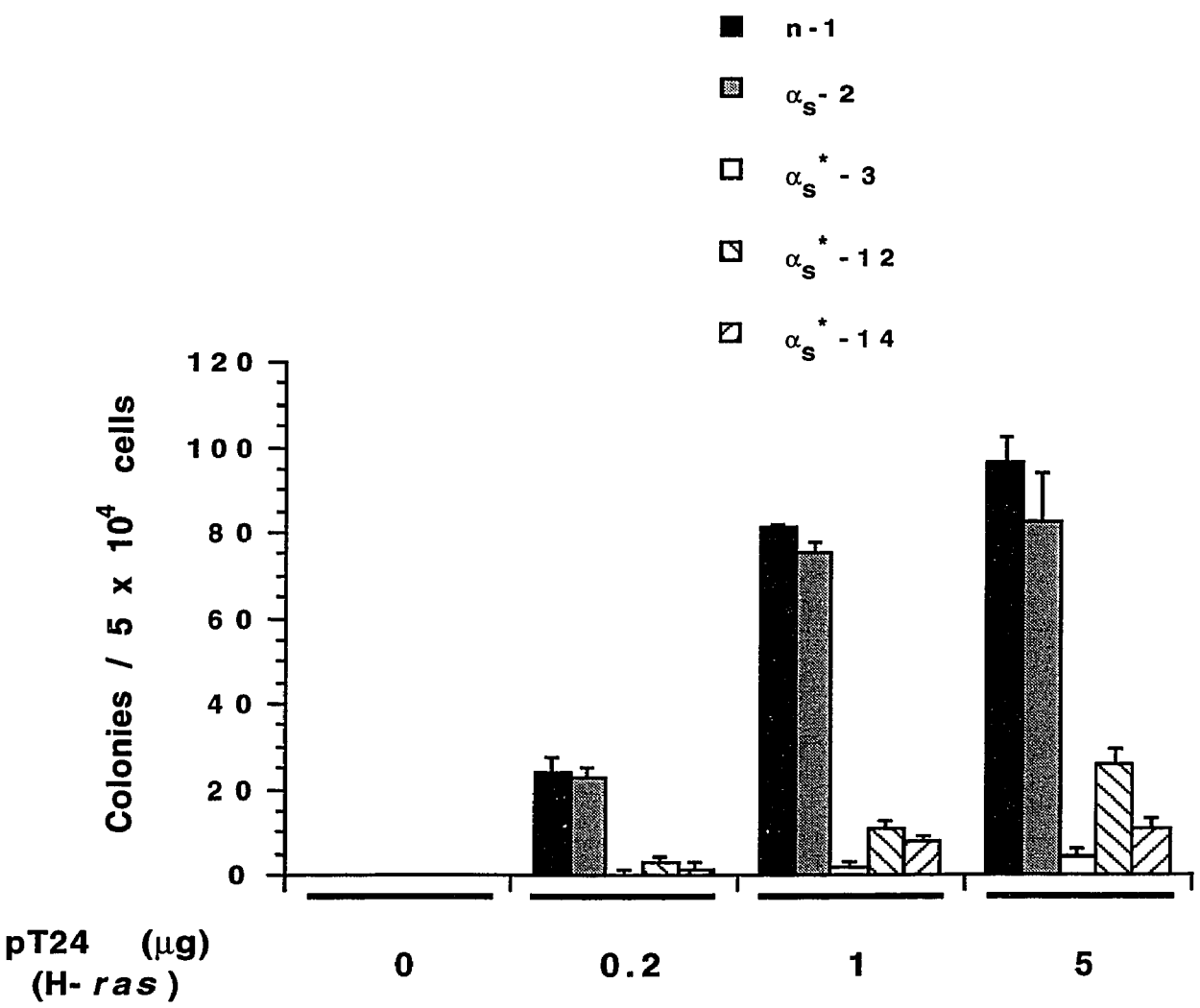

NIH-3T3 clonal cell lines
n-1
α_s-2
α_s^*-3
α_s^*-12
α_s^*-14
Colonies / 5 x 10^4 cells
120
100
80
60
40
20
0
pT24 (μg)
(H-ras)
0
0.2
1
5

Fig. 5-3. Effect of the expression of α_s^* on the transformation of NIH-3T3 clonal cells by H-*ras* DNA.

Three cell lines of NIH-3T3 origin expressing α_s^* as well as control lines were transfected with indicated amounts of pT24 DNA. The ability of transfected cells to form colonies in soft agar was used as a criterion for transformation. The methods of transfection and soft agar colony formation assay used in these experiments were same to that used in Fig. 5-1 as described in Chapter 2. NIH-3T3 cells: n-1, pMN-containing cell line; α_s-2, a cell line expressing wild type α_s; α_s^*-3, α_s^*-12, and α_s^*-14, cell lines expressing mutant activated α_s. Total number of colonies (0.15 mm - 1.2 mm) derived from $2x10^5$ cells transfected with or without various amounts of pT24 DNA in the presence of 20 μg of carrier DNA is indicated.

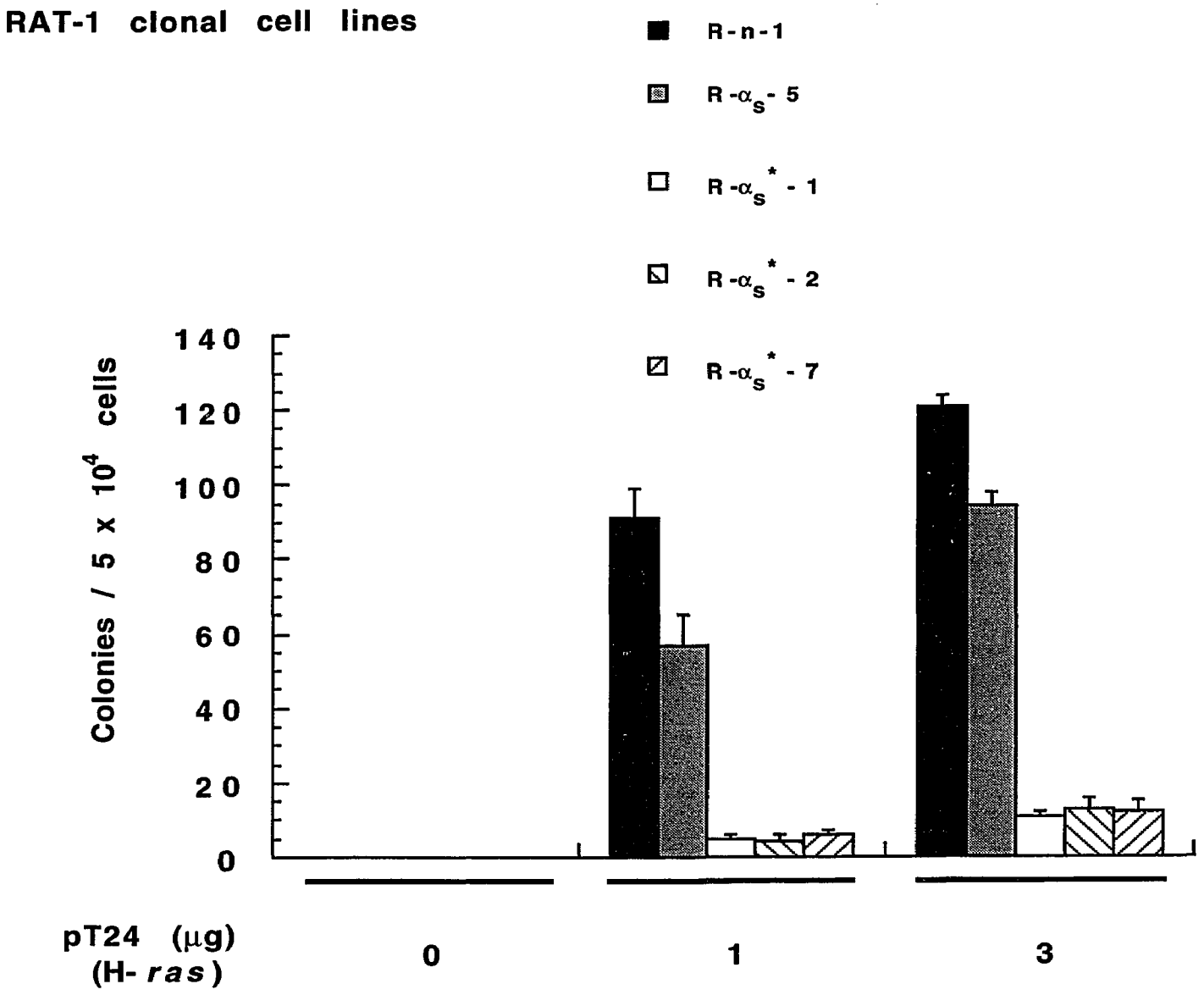

RAT-1 clonal cell lines
R-n-1
R-α_s-5
R-α_s^*-1
R-α_s^*-2
R-α_s^*-7
Colonies / 5 x 10^4 cells
140
120
100
80
60
40
20
0
pT24 (μg)
(H-*ras*)
0
1
3

Fig. 5-4. Effect of the expression of α_s^* on the transformation of RAT-1 clonal cells by H-*ras* DNA.

Three cell lines of RAT-1 origin expressing α_s^* as well as control lines were transfected with indicated amounts of pT24 DNA. The ability of transfected cells to form colonies in soft agar was used to represent transformation. The methods of transfection and soft agar colony formation assay used in these experiments were same to that used in Fig. 5-1 as described in Chapter 2. RAT-1 cell lines: R-n-1, pMN-containing cell line; R-α_s-5, a cell line expressing wild type α_s; R-α_s^*-1, R-α_s^*-2, and R-α_s^*-7, cell lines expressing mutant activated α_s. Total number of colonies (0.15 mm - 1.2 mm) derived from $2x10^5$ cells transfected with or without various amounts of pT24 DNA in the presence of 20 μg of carrier DNA is indicated.

NIH-3T3 clonal cell lines

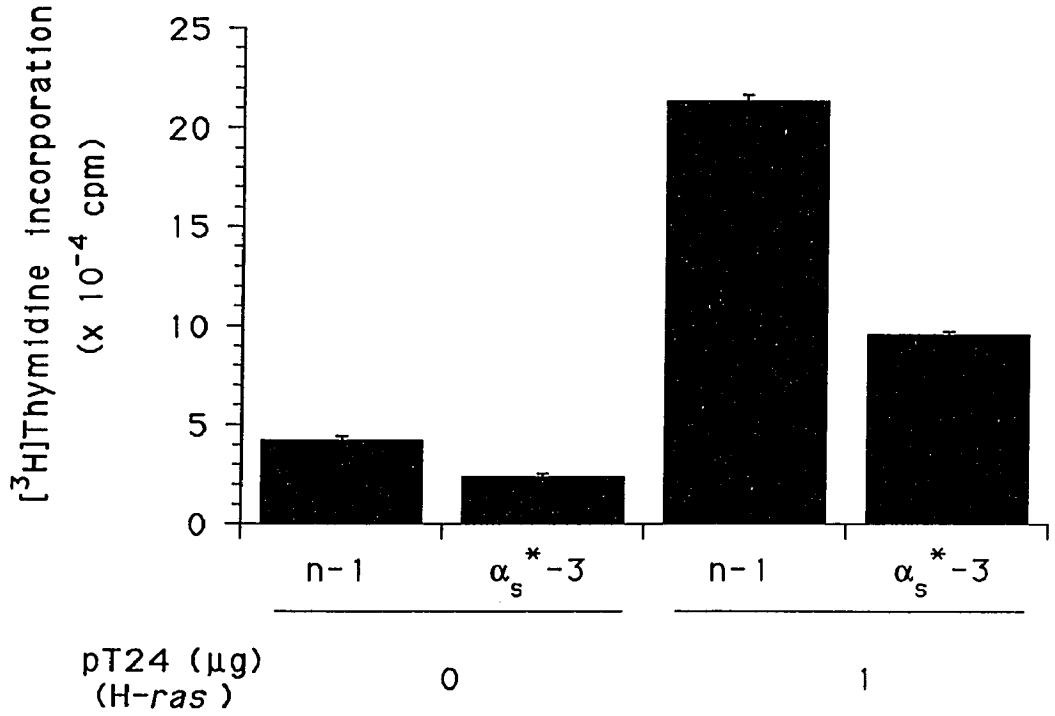


Fig. 5-5. Effect of the expression of the α_S* on H-*ras*-induced mitogenesis.

Clonal NIH-3T3 lines n-1 and α_S*-3 were transfected with or without pT24 (H-*ras*). The transfected cells were grown for two weeks in the presence of dexamethasone on alternate days. The cells were split on every third day. After the two week period, the cells were plated in 24 well plates and used in [^{3}H]thymidine incorporation experiments. Values are means of triplicate determinations. The results of one representative of four experiments are shown.

NIH-3T3 clonal cell lines	Transfection efficiency (colonies /1 μg pRSV 1.1/ 5 x 10^4 cells)
n-1	117 ± 4
n-3	110 ± 3
α_s -2	110 ± 2
α_s^* -3	128 ± 4

Table 5-1. Transfection efficiency of clonal NIH-3T3 cell lines expressing the wild type α_s and the mutant α_s^*.

Procedures for transfection were similar to that described in the Fig. 5-1 with some modifications. 1 μg of pRSV1.1 DNA (instead of 1 μg of pT24 DNA) was used in transfection. After transfection, the cells were split and grown in media containing 400 μg/ml hygromycin. After two weeks, the plates were stained in Hank's buffered saline containing 1.5% glutaraldehyde and 0.06% methylene blue. The number of Hygromycin B-resistant colonies was determined. The definitions for NIH-3T3 cell lines, n-1, n-3, α_s-2, and α_s^*-3 are same as that indicated in the legends of Fig. 5-1 and Fig. 5-3.

RAT-1 clonal cell lines	Transfection efficiency (colonies /1 μg pRSV 1.1 /5 x10^4 cells)
R -n-1	37 ± 1
R-α_s -5	30 ± 2
R-α_s^* -7	34 ± 1

Table 5-2. Transfection efficiency of clonal RAT-1 cell lines expressing the wild type α_s and the mutant α_s^*.

Procedures for transfection, soft agar assay and counting colonies were same to that described in Table 2. The number of Hygromycin B-resistant colonies was determined. The definitions for RAT-1 cell lines, R-n-1, R-α_s-5, and R-α_s^*-7 are same as that indicated in Fig. 5-4.

CHAPTER 6

α_s INHIBITS PROLIFERATE SIGNALS FROM *ras* BY STIMULATING cAMP PRODUCTION AND ACTIVATING PROTEIN KINASE A

It have been demonstrated that expression of α_s* did not affect the proliferation of both RAT-1 and NIH-3T3 cells (chapter 4). I also have shown that expression of α_s* can block H-*ras*-induced transformation in both cell types (chapter 5). However, it was not clear which signaling pathways were involved in these inhibitory effects. Lowering of cellular cAMP levels has been shown to be mitogenic in RAT-1 cells (van Corven et al., 1989), cAMP analogs are both antiproliferative and also block transformation of NRK cells (Tortora et al., 1989). As expression of α_s* results in increasing cellular cAMP levels in these cell lines (chapter 4), it is possible that the blockade of transformation by α_s* can be achieved by directly raising cellular cAMP levels in RAT-1 cells. To examine this hypothesis, I tested whether raising cAMP levels by adding 8 Br-cAMP could suppress mitogenesis in RAT-1 cells . 8 Br-cAMP is a cell permeable biologically active analog of cAMP (Chen et al., 1991). The results are shown in Fig. 6-1. It was found that addition of 8 Br-cAMP significantly suppressed the proliferation of RAT-1 cells (Fig. 6-1). Therefore I tested if addition of 8 Br-cAMP to a clonal RAT-1 line affected *ras*-induced transformation. The results are shown in Fig. 6-2. Addition of 8 Br-cAMP significantly decreased *ras*-induced transformation in *ras*-transfected RAT-1 cells (R-n-1) as assessed by the numbers of colonies formed on soft agar (Fig. 6-1). These data suggested that the cAMP analog can suppress transformation of RAT-1 cells by H-*ras* as well and implied that the α_s* might exert its role through this signaling pathway. It should be mentioned that although the expression of α_s* inhibits transformation of RAT-1 cells (Fig. 5-4), it is possible that the increase in

cAMP levels is not sufficient to inhibit mitogenesis of these cells (Fig. 4-6).

It has not been shown that lowering cAMP levels has the same antiproliferative effect on NIH-3T3 cells. I asked if it was possible that an increase in cAMP levels was responsible for inhibition of transformation of NIH-3T3 cells. To answer this question, I first determined if addition of 8 Br-cAMP affected proliferation of NIH-3T3 cells. The doubling times of two clonal cell lines of NIH-3T3 origin, n-1 and n-3, in the presence or absence of 8 Br-cAMP were measured. The results are shown in Fig. 6-3. In contrast to the effects on RAT-1 cells, addition of 8 Br-cAMP did not affect the proliferation of NIH-3T3 cells in two control clonal lines (Fig. 6-3). Then I tested if 8 Br-cAMP affected *ras*-induced transformation in NIH-3T3 cells. In these clonal lines, the presence of 8 Br-cAMP resulted in significant suppression of *ras*-induced transformation (Fig. 6-4). These results indicated that increasing cAMP levels do not affect the proliferation of NIH-3T3 cells, but can inhibit the transformation of these cells.

To ascertain if α_s* also used the cAMP and protein kinase A (PKA) pathway to suppress *ras*-induced transformation, I studied the effects of a dominant negative protein kinase A subunit on the transformation induced by *ras*. It is known that cAMP-dependent PKAs are the only cellular receptors for cAMP (Krebs and Beavo, 1979). The dominant negative regulatory subunit PKA blocks activation of this enzyme in NIH-3T3 cells (Clegg et al, 1989) and hence, if the effects of α_s* were mediated by the PKA pathway, the dominant negative PKA regulatory

subunit should relieve the α_s* suppression of *ras*-induced transformation. I cotransfected the dominant negative PKA regulatory subunit or wild type PKA regulatory subunit along with different amounts of pT24 (H-*ras*) into control and α_s* expressing cells. It was found that expression of dominant negative but not wild type PKA completely blocked the suppressive effect of the α_s* expression on H-*ras*-induced transformation (Fig. 6-5). These results indicated that the effects of α_s* may be mediated through the cAMP and PKA pathway.

RAT-1 clonal cell lines

	Doubling Time
R-n-1	15 hr
R-n-1 (1 μM 8 Br-cAMP)	23 hr

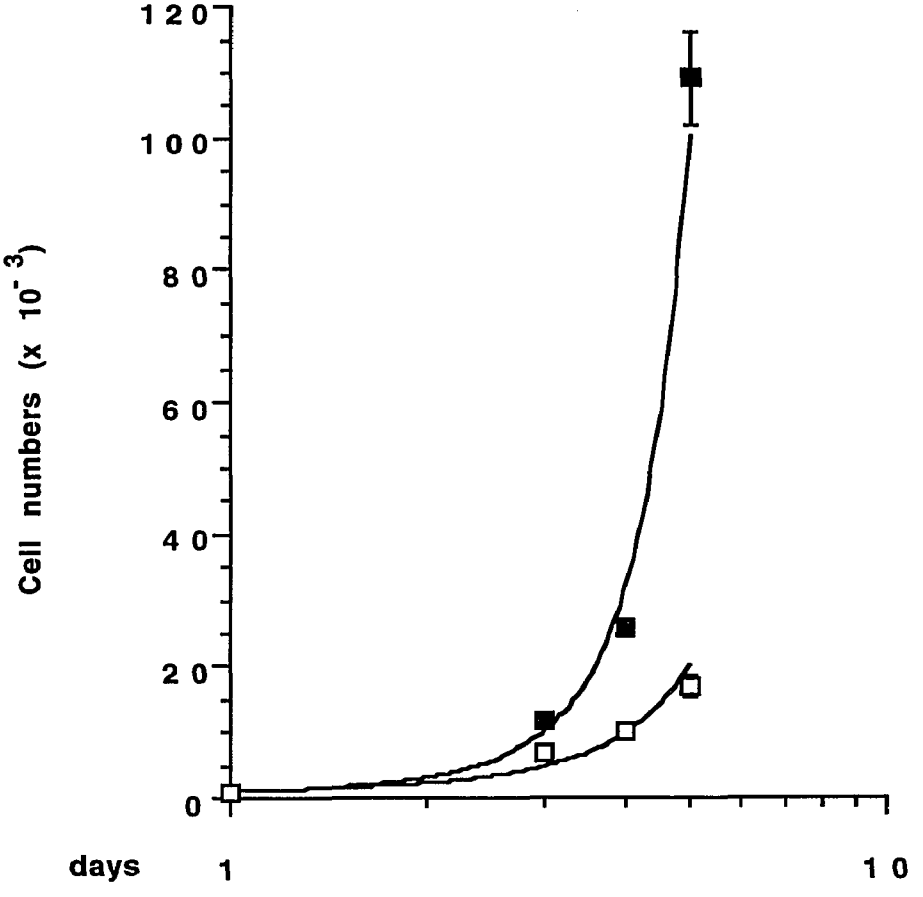


Fig. 6-1. Effect of the addition of 8 Br-cAMP on the proliferation of RAT-1 cells.

Equal number of RAT-1 cells were seeded into 24-well plates at a density of 1 X 10^3 cells per well in 1 ml of DMEM plus 10% bovine calf serum and were grown in the presence or absence of 1 μM 8 Br-cAMP. Each group included four wells. The cell numbers were determined on days 3, 4 and 5. Doubling time were calculated using the equations $Y=Y_0(e^{kt})$ and doubling time = (0.693/k)x24 h. R-n-1, pMN transfected RAT-1 cell line.

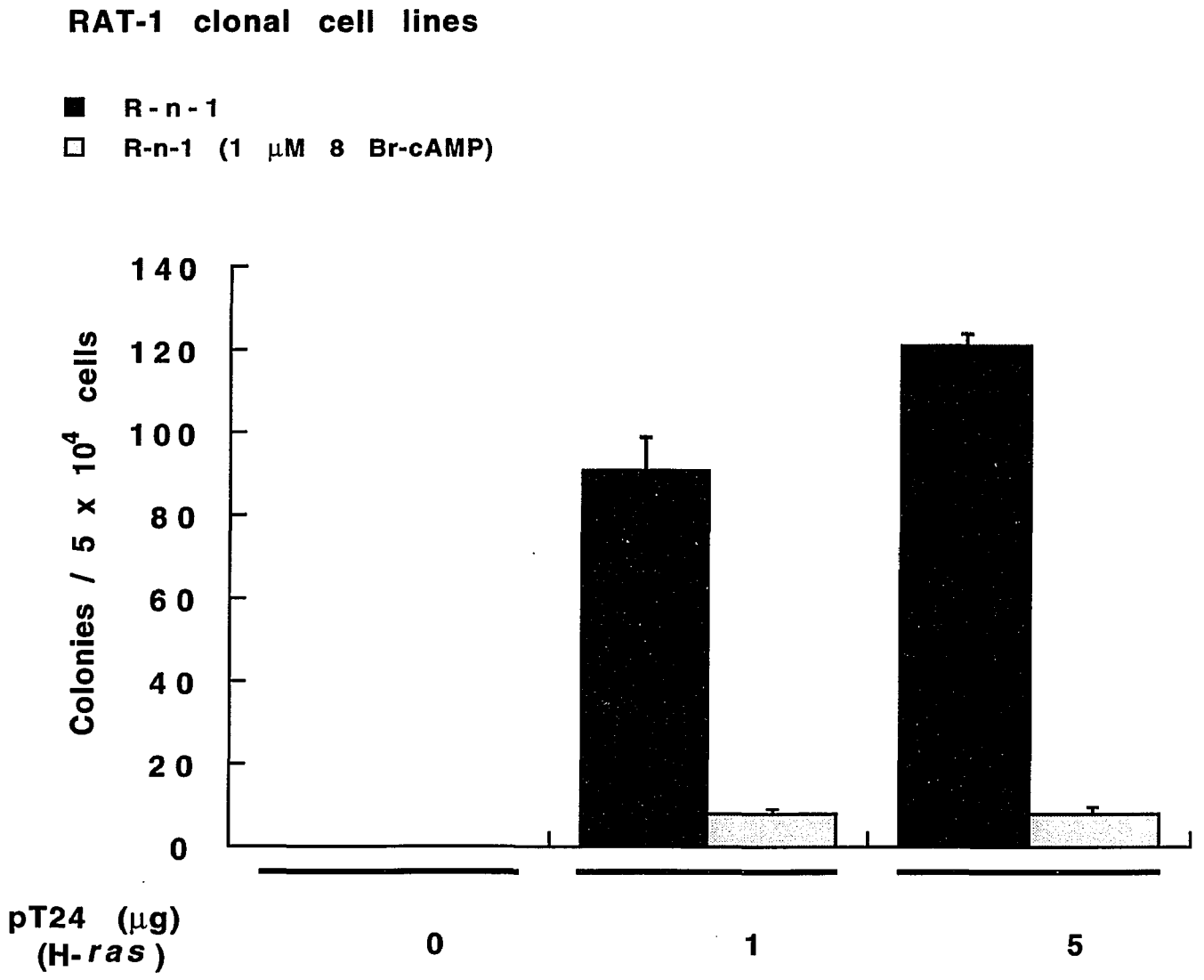

RAT-1 clonal cell lines
R-n-1
R-n-1 (1 μM 8 Br-cAMP)
Colonies / 5 x 10^4 cells
140
120
100
80
60
40
20
0
pT24 (μg)
(H-ras)
0
1
5

Figure 6-2. Effect of the addition of 8 Br-cAMP on H-*ras*-induced transformation of RAT-1 cells.

The clonal line R-n-1 was grown overnight in the presence of 1 μM 8 Br-cAMP and transfected without or with indicated concentrations of pT24 (H-*ras*) as described in chapter 2. After transfections, the cells were cultured for four days with dexamethasone treatment on alternative day prior to plating on soft agar plates. Then colonies formed on soft agar were scored and values are mean ± S.D. of triplicate plates. The experiments shown here are representatives of three experiments. R-n-1, a pMN vector-containing RAT-1 cell line.

NIH-3T3 clonal cell lines

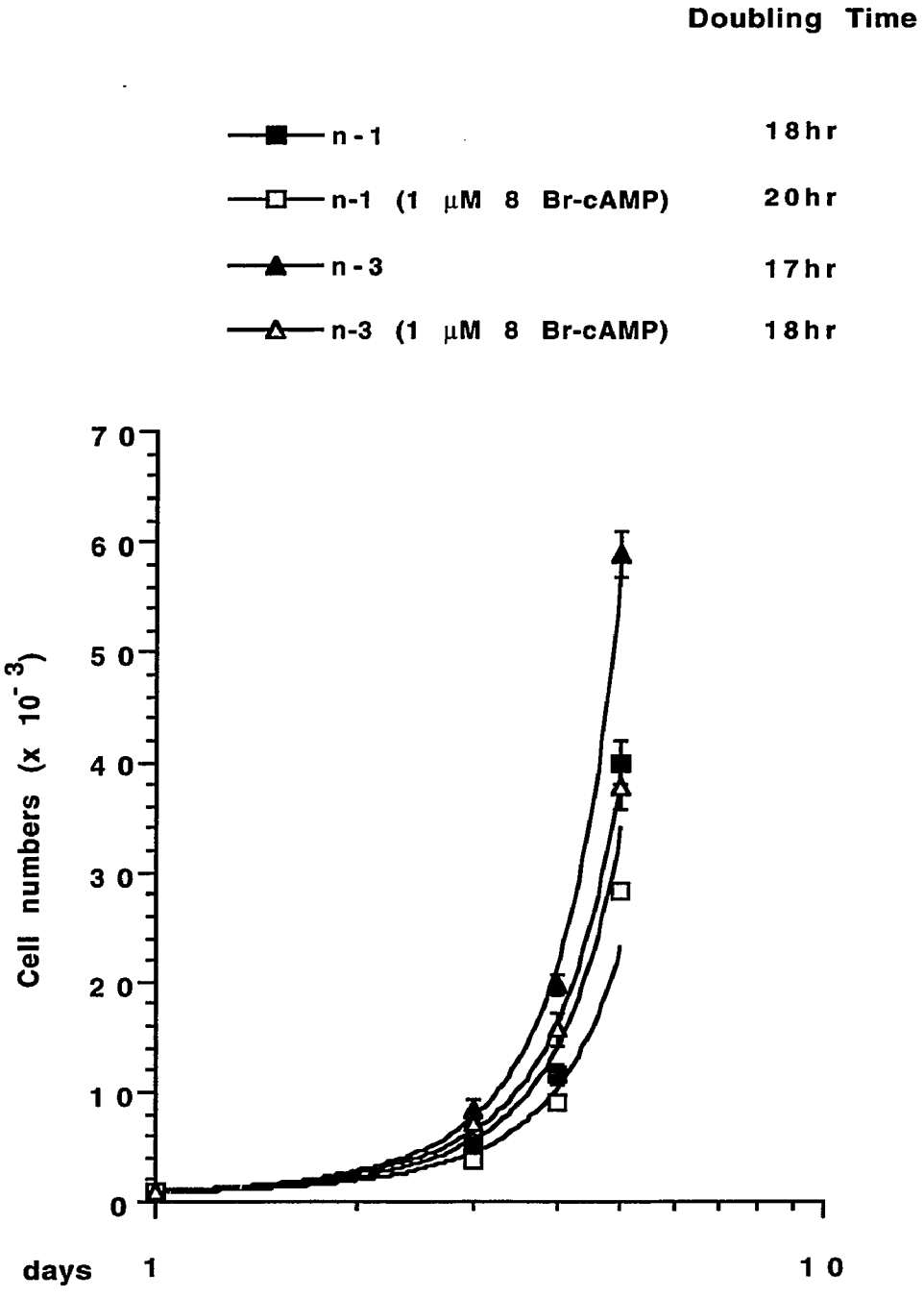


Fig. 6-3. Effect of the addition of 8 Br-cAMP on the proliferation of NIH-3T3 cells.

Equal number of cells from NIH-3T3 lines n-1 and n-3 were seeded into 24-well plates at a density of 1 x 10^3 cells per well in 1 ml of DMEM plus 10% bovine calf serum and were treated under the same conditions described in the legend of Fig. 6-1. Each group included four wells. The cell numbers were determined on days 3, 4 and 5. Doubling time were calculated using the equations described in the legend of Fig. 6-1. n-1, n-3, pMN-containing NIH-3T3 cell lines.

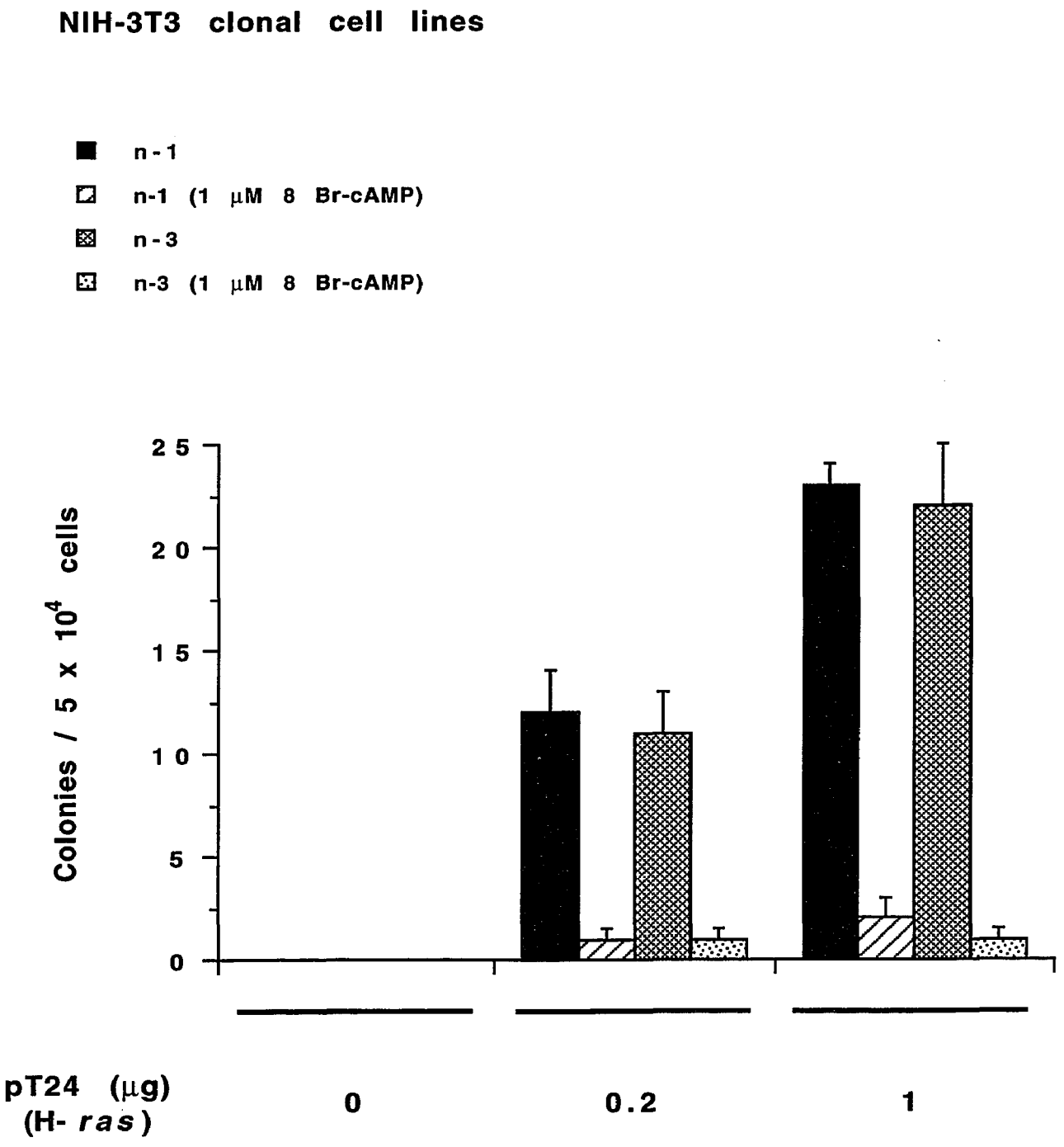

NIH-3T3 clonal cell lines
n-1
n-1 (1 μM 8 Br-cAMP)
n-3
n-3 (1 μM 8 Br-cAMP)
Colonies / 5 x 10^4 cells
25
20
15
10
5
0
pT24 (μg)
(H- ras)
0
0.2
1

Figure 6-4. Effect of the addition of 8 Br-cAMP on H-*ras*-induced transformation of NIH-3T3 cell lines.

The clonal lines n-1 and n-3 were grown overnight in the presence of 1 μM 8 Br-cAMP and transfected without or with indicated concentrations of pT24 (H-*ras*) as described in chapter 2.. After transfections the cells were cultured for one day in the presence of 1 μM dexamethasone to induce the expression of α_S* prior to plating on soft agar plates. Colonies were scored and values are mean ± S. D. of triplicate plates. n-1, n-3, pMN transfected NIH-3T3 cell lines. The experiments shown here are representatives of three independent experiments.

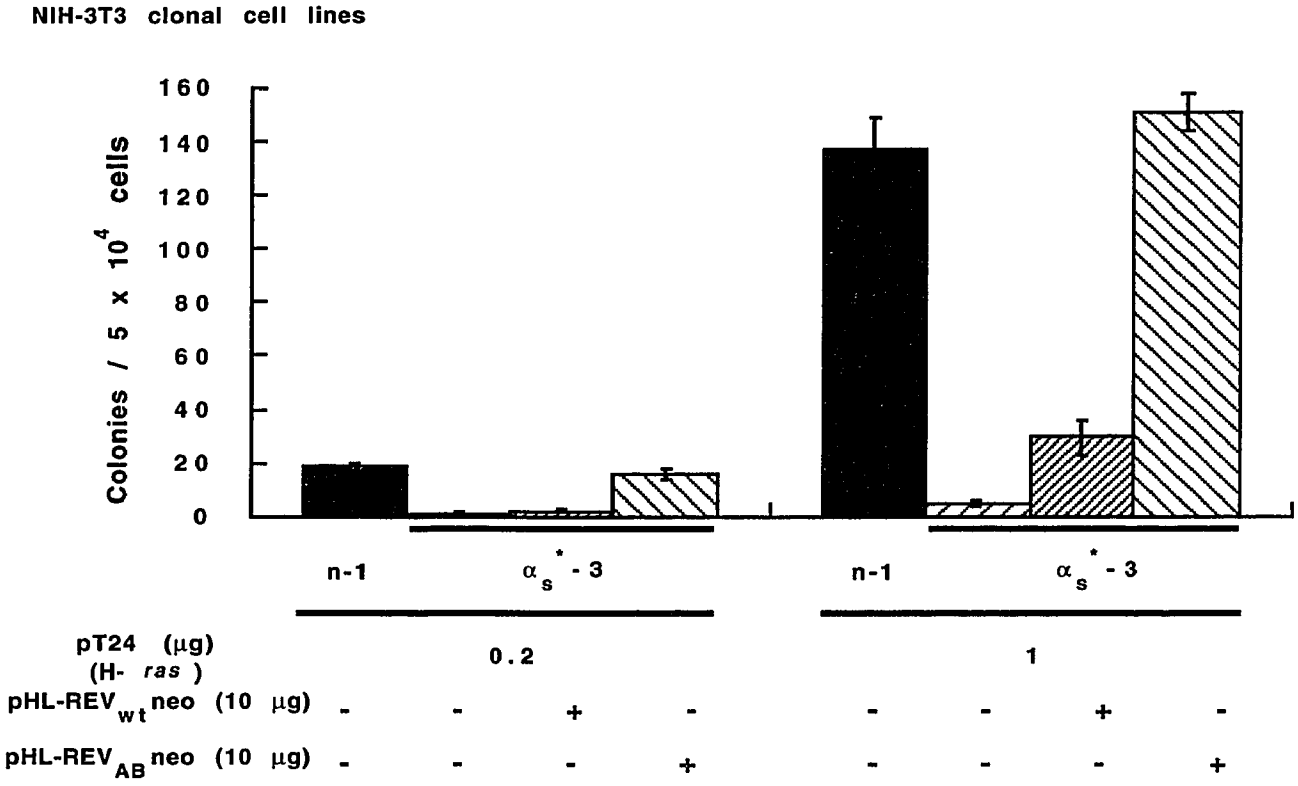

NIH-3T3 clonal cell lines
Colonies / 5 x 10^4 cells
160
140
120
100
80
60
40
20
0
n-1
αs* - 3
n-1
αs* - 3
pT24 (μg)
(H- ras)
0.2
1
pHL-REVwt neo (10 μg) - - + - - - + -
pHL-REVAB neo (10 μg) - - - + - - - +

Fig. 6-5. Effect of the expression of dominant negative PKA regulatory subunit (pHL-REV_{AB}neo) or control (wt PKA regulatory subunit in pHL-REV_{wt}neo) on H-*ras*-induced transformation of NIH-3T3 clonal lines.

The clonal cells were cotransfected with indicated amounts of pT24 without or with the vectors encoding the PKA regulatory subunits. After transfections, the cells were induced with 1 μM dexamethasone and plated onto soft agar plates to score for colony formation. Values are mean ± S. D. of triplicate determinations. The definition of n-1 is the same as that indicated in the legend of Fig. 3. α_s^*-3 is a clonal NIH-3T3 cell line containing the mutant α_s^*. The results shown here are representatives of three independent experiments.

CHAPTER 7

ACTIVATED α_s DECREASES H-*RAS*-STIMULATED MAP KINASE ACTIVITY

It is now well established that *ras* functions as a downstream element of several growth factor receptor tyrosine kinases (Lowy and Willumsen, 1993). It has also been shown that MAP kinase is one of the intermediates of the *ras* signaling pathway and that the mitogenic signal from *ras* is transmitted through the MAP kinase pathway (Davis, 1993; Marx, 1993). Both *ras* and α_s belong to the GTPase superfamily and can be converted to their active forms by mutations that block their intrinsic GTPase activities (Bourne et al., 1991). It is then possible that direct or indirect interactions between these pathways in the α_s^*-expressing cell lines resulting in blockade of transformation induced by *ras*. Thus I determined the effects of the expression of α_s^* on *ras*-induced MAP kinase activity in one of the clonal lines expressing α_s^*. MAP kinase activities resolved in Mono-Q column fractions of a cell line expressing α_s^* (α_s^*-3) were compared with that of one (n-1) which contains a vector (Fig. 7-1). The bar graph in Fig. 7-2 shows the data summarized from four separate experiments. Expression of α_s* by itself did not stimulate MAP kinase activity. Expression of H-*ras* stimulated MAP kinase activity extensively and this stimulation was reduced by about 50% in α_s* expressing cells (Fig. 7-2).

To establish that the observed reduction of H-*ras*-stimulated MAP kinase activity by α_s* was not due to clonal variation, I examined two other clonal lines expressing α_s*. The results are shown in Table 7-1. All these clonal lines (α_s*-3, α_s*-12, α_s*-14) expressing α_s* showed a about 50% reduction in H-*ras*-stimulated MAP kinase activity as compared to that of the control line (n-1) (Table 7-1).

I have shown previously that a cAMP analog, 8 Br-cAMP can inhibit the transformation induced by *ras* and it is likely that α_s* may function through this pathway (Chapter 6). To further examine this possibility, I determined if 8 Br-cAMP could inhibit the *ras*-induced MAP kinase activity. The NIH-3T3 cells (n-1) were treated with or without 8 Br-cAMP and were transfected with pT24 (H-*ras*) as described previously. Then MAP kinase activity was measured in these two kinds of cells. The results are shown in Fig. 7-3. The panel A of Fig. 7-3 shows a typical column profile. The panel B is a graph representing the results of two independent experiments. It was found that the addition of 8 Br-cAMP decreased MAP kinase activity significantly, suggesting that 8 Br-cAMP blocks *ras*-induced transformation via inhibition of MAP kinase.

I have shown that the expression of α_s* (by increasing cAMP levels) inhibits *ras*-induced MAP kinase activity. Since both the duration (Traverse et al., 1992) and amplitude of MAP kinase activity are probably deciding factors as to when MAP kinase activation gets converted into a biological response. My results and those reported by Cook et al. (1993) suggest that in certain situations partial inhibition of MAP kinase activity may translate into an almost total loss of biological response.

NIH-3T3 clonal cell lines

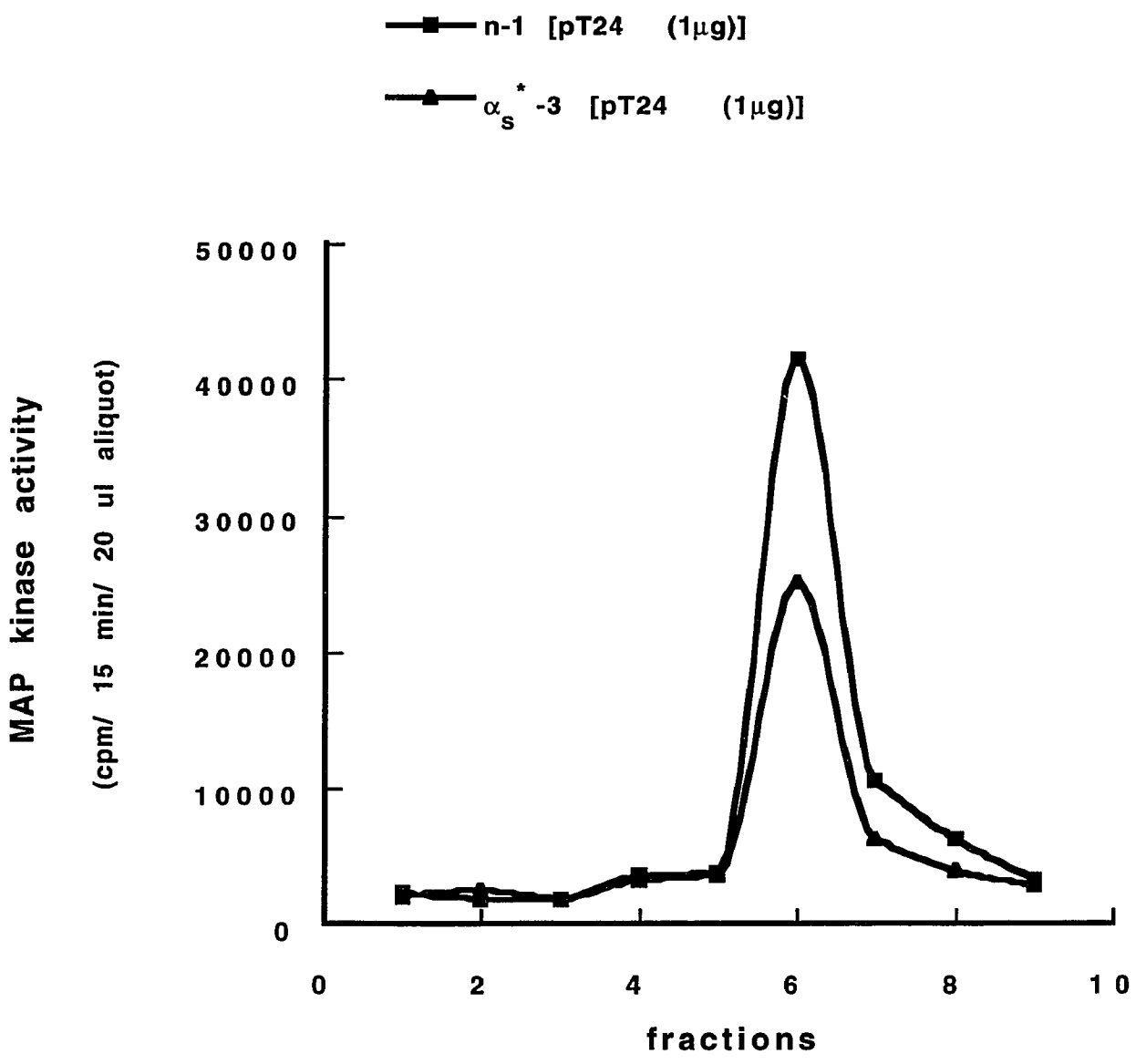


Figure 7-1. Effects of the expression of α_s* on H-*ras*-induced MAP kinase activity.

Clonal NIH-3T3 lines n-1 and α_s*-3 were derived from cells transfected with pMN and pMN-α_s*, respectively. The α_s*-3 line expressed α_s* in an inducible manner by treatment with dexamethasone. These cell lines were transfected with or without pT24 (H-*ras*). The transfected cells were grown for two weeks in the presence of dexamethasone on alternate days. 4 x 10^6 cells in 100-mm plates were incubated overnight (20 h) with DMEM without serum but with 0.1% BSA to achieve quiescence. The cells were then extracted and MAP kinase activity was measured using the method described in Chapter 2. The activity of each fractions eluted from a Mono Q HR 5/5 FPLC column was measured.

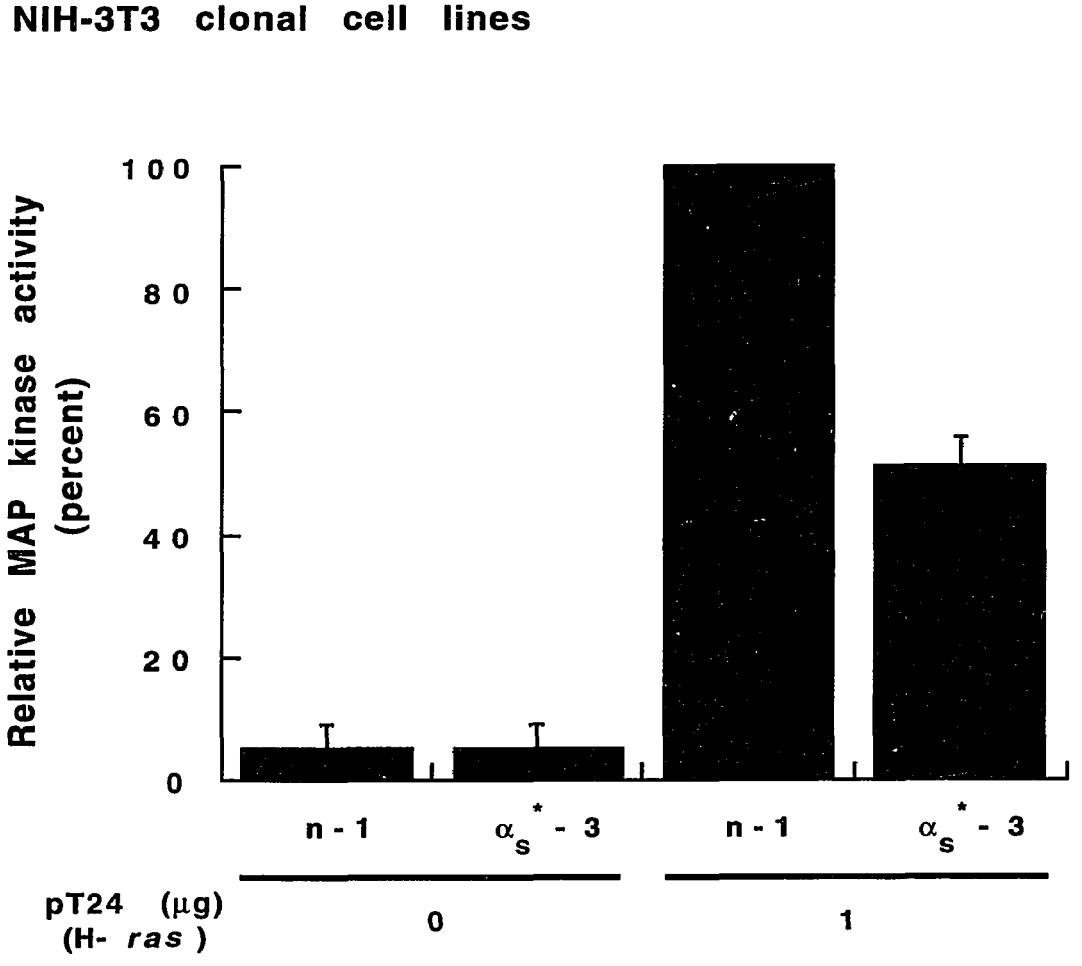
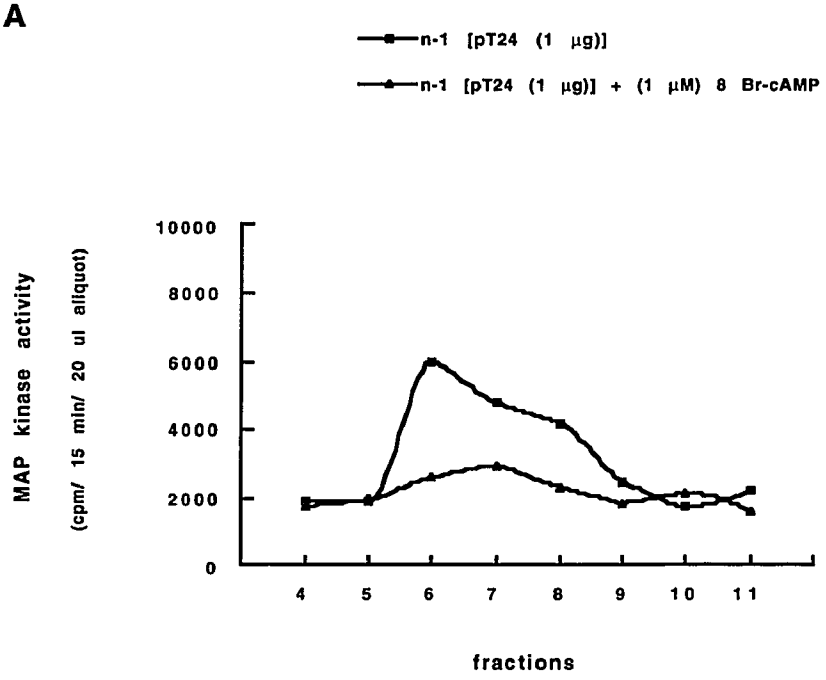

NIH-3T3 clonal cell lines
Relative MAP kinase activity (percent)
100
80
60
40
20
0
n - 1
α_s^* - 3
n - 1
α_s^* - 3
pT24 (μg)
(H- ras)
0
1

Figure 7-2. Effects of the expression of α_s^* on H-*ras*-induced MAP kinase activity.

This is a summary of four separate experiments which are similar to that described in Fig. 7-1. The MAP kinase activity of peak fractions were added together and the background activity was corrected. The MAP activity of the n-1 cells not transfected with pT24 or that of α_s^*-3 cell line transfected or not transfected with pT24 (H-*ras*) is represented relative to that of the fractions of n-1 cells transfected with pT24 (100%). Values are mean ± S. D.

NIH-3T3 clonal cell lines

A



B

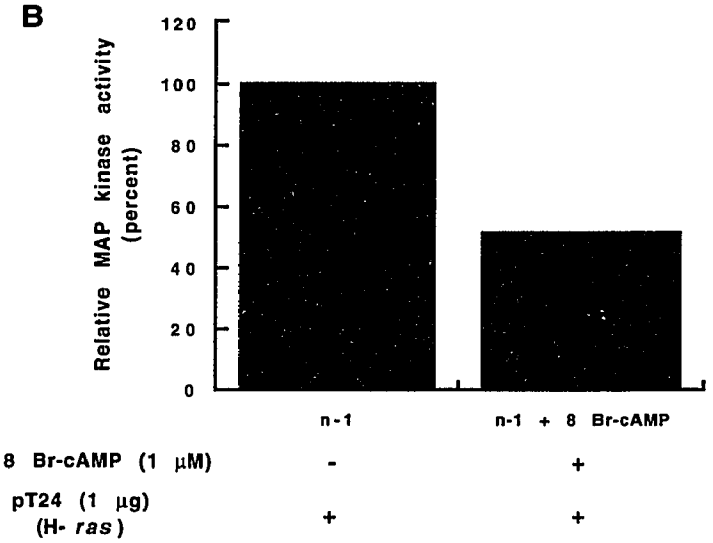


Figure 7-3. Effects of the addition of 8 Br-cAMP on H-*ras*-induced MAP kinase activity.

Panel A, a profile of one experiment showing activity of each fractions eluted from a Mono Q FPLC column. Panel B, a summary of two separated experiments. The methods used in these experiments were same to that described in Fig. 7-1. The MAP kinase activity is represented similarly to that in the Fig. 7-2. The MAP activity of the n-1 cells in the presence of 8 Br-cAMP and transfected with pT24 is represented relative to that of the sum of fractions of n-1 cells transfected with pT24 (100%). Values are averages of two experiments.

NIH-3T3 clonal cell lines	T24	MAP kinase activity CPM/ 15 min/ 1 μg protein ($\times 10^{-3}$)
n-1	+	4.80
α_s^*-3	+	2.84
α_s^*-12	+	3.26
α_s^*-14	+	2.47

Table 7-1. MAP kinase activity in NIH-3T3 clonal cell lines expressing the mutant α_s*.

MAP kinase activity was assayed after two weeks transfection with 1 μg of pT24. as described in Chapter 2. As the MAP kinase activities are the sum of several fractions that comprise the peak, no error estimates could be obtained. The lines used were n-1, a clonal line derived from cells transfected with pMN and α_s*-3 and α_s*-14 clonal lines derived from cells transfected with pMN-α_s*.

CHAPTER 8

DISCUSSION

1) Mutant Activated α-subunits of G proteins as Oncogenes.

Up to now two mutant activated α-subunits have been found in human tumors. These are mutated versions of G_s-α (α_s) and G_{i2}-α (α_{i2}) (Lyons et al., 1990, Landis et al., 1989). The occurrence of mutated α-subunits in the tumors led to the suggestion that these may be oncogenes. This is a testable hypothesis and it has been shown that mutant forms of α_i can function as an oncogene. α_i containing mutations that lower intrinsic GTPase activity were capable of inducing a transformed phenotype when expressed in Rat-1 fibroblasts (Gupta et al., 1992, Hermouet et al., 1991). Thus by several widely accepted criteria, mutant forms of α_{i2} can be classified as oncogenes.

Other α subunits such as mutant forms of α_q (De Vivo et al., 1992; Kalenic et al., 1992) and α_o (Kroll et al., 1992) are capable of transforming NIH-3T3 cells, though naturally occurring mutations in these α subunits have not been identified. Thus it seems that several G protein signaling pathways are involved in growth regulation.

Mutations in α_s had also been detected in some pituitary tumors (Landis et al., 1989; Lyons et al., 1990). To examine if the mutant activated α_s induced transformation, several α_s^*-expressing lines in RAT-1 and NIH-3T3 cells were established. I found that the mutant activated α_s did not affect mitogenesis of both RAT-1 cells and NIH-3T3 cells and that it can not induce transformation (Chapter 4). This observation is consistent with that reported by Zachary et al. (1990). However, I have found that α_s^* is able to suppress *ras*-induced transformation of both NIH-3T3 and Rat-1 fibroblasts. These results were

somewhat unexpected in the light of studies on several other types of α subunits.

My findings suggest that under certain experimental conditions α_s* may be able to suppress transformation of fibroblasts, induced by *ras*, especially in those cell types in which α_s* does not appear to inhibit proliferation by itself. As expression of α_s* results in only modestly increases in cellular cAMP levels, it is possible that the blockade of transformation by α_s* can be achieved without raising cellular cAMP levels to deleterious levels. It is well established that proliferative cells accumulate mutations as they progress towards the transformed state. Some mutations, such as those that occur in protooncogenes and tumor suppresser genes, accelerate the cell's progress towards the transformed state. My data raise the possibility that mutations such as those that activate α_s could halt the progression towards the transformed state. Thus it may be hypothesized that there exist genes that, when mutated, produce altered proteins that by themselves do not affect proliferation but can halt the progression towards the transformed state by other proliferative agents. Identification of such proteins may be useful in developing novel preventive and therapeutic approaches to cancer.

From the data presented here, it is not certain that α_s* will function as suppressor of transformation in all cell types. In some tissues, α_s* may function as a "cofactor " in promoting transformation. This may be especially true in cell types where elevation of cAMP leads to proliferation. Indeed, mutant activating forms of α_s have to date only

been found in cell types where cAMP is proliferative. Whereas this might argue that α_s* may promote transformation in those cells, there is at present no direct proof of this. Further, even the tumors that do harbor α_s* are non-malignant adenomas (Burton et al., 1991). At the current time, the role of α_s* in cells where elevation of cAMP levels is proliferative is not clear. It appears that α_s* or elevation of cAMP levels causes hyperplasia but not transformation. Further studies may be required to clarify this issue. However, it appears unlikely that α_s* functions as an oncogene even in well defined experimental conditions.

2) Sites of interactions between the α subunit of G_s and *ras* signaling pathways.

I have studied the possible mechanisms which underlie the inhibitory effects of α_s^* on *ras*-induced transformation. I used two approaches. First, I showed that a cAMP analog, 8 Br-cAMP can exert the same inhibitory effect as α_s^* on *ras*-induced transformation. Secondly, I demonstrated that a dominant negative regulatory subunit of PKA can block this inhibitory effect of α_s^* (Chapter 6). Thus it appears that α_s^* functions through cAMP and PKA pathway to suppress *ras*-induced transformation .

I have also attempted to determine where cAMP pathway interacts with *ras*-signaling pathway. Recently it has become clear that proliferative signals from *ras* are transmitted through the MAP kinase pathway. Hence I directly measured the MAP kinase activity in clonal

α_S^*-expressing cells transfected with H-*ras*. I found that H-*ras*-induced MAP kinase activity in these cells was significantly decreased. The suppression of H-*ras*-stimulated MAP-kinase activity by α_S^* was also obtained by using the cAMP analog 8 Br-cAMP (Chapter 7). Hence it appears reasonable to conclude that the cAMP pathway interacts with *ras* signaling pathway at or prior to MAP kinase activation and the resulting decrease in MAP kinase activity could be one of the causes for the inhibition of transformation induced by *ras*.

I have not determined exactly where the G_S pathway intersects with the *ras* signaling pathway. It is now known that *ras* through *raf* can activate MEK which in turn activates MAP kinases (Marx, 1993). Activation of the G_S pathway may directly affect MAP kinase or any of the proteins that regulate MAP kinase activity, such as MAP kinase kinase (MEK), *raf* or any of the putative proteins that regulate *raf* interactions with *ras*, or any combination of the above. MAP kinases, MAP kinase kinases and *raf* have putative protein kinase A phosphorylation sites. The location of the putative PKA consensus sites is shown in Figure 8-1.

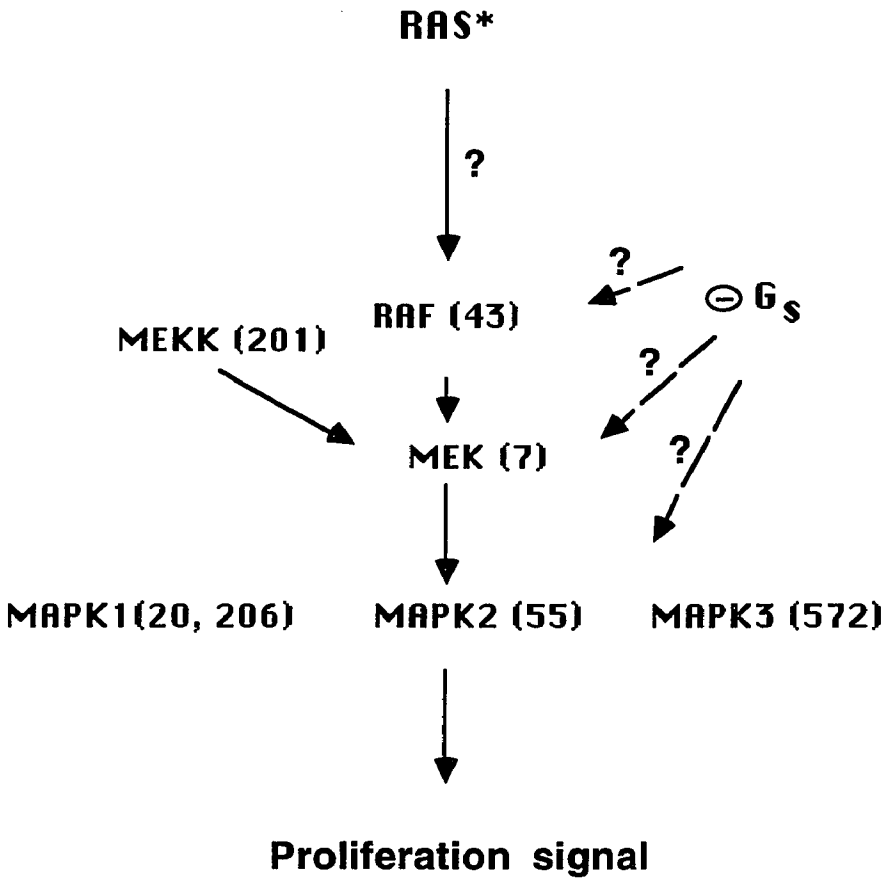

RAS*
?
?
⊖ Gs
RAF (43)
MEKK (201)
?
MEK (7)
?
MAPK1(20, 206)
MAPK2 (55)
MAPK3 (572)
Proliferation signal

Fig. 8-1. A schematic digram showing the regulation of MAP kinase activity by the *ras*-signaling pathway and G_S protein-mediated signal pathways.

Mutant activated *ras* (*ras**) can directly activates *raf* either alone or in cooperation with another unknown protein. *Raf* is a MEK kinase which can activate MEK. MEK in turn can activate MAP kinase. After several activation steps, the nuclear transcription factors are activated and the cell proliferation signal is sub-sequently mediated. The dotted lines represent possible connections of G_S protein-mediated signal pathways to the *ras* signaling pathway. MAP kinases, MAP kinase kinases and *raf* have putative protein kinase A phosphorylation sites whose positions in these proteins are shown in parenthesis.

Further studies are required to sort through these proteins to determine which of these are regulated by protein kinase A. Irrespective of the details of the regulation, the net effect of activating α_s is an inhibition of *ras*-stimulated MAP kinase activity and suppression of proliferation and transformation.

3) General Significance.

My results may be of general biological relevance for several reasons. Alterations in cellular cAMP by itself does not affect regulation of mitogenesis in NIH-3T3 cells. This lack of regulation is akin to what is seen in most mammalian cell types (Spada et al., 1992). The proliferative signal we have used, *ras,* is a very commonly occurring oncogene in human tumors (Barbacid, 1987). These experimental conditions suggest that activated α_s* may be able to suppress transformation of many cell types. As expression of α_s* results in only modestly increasing cellular cAMP levels, it is possible that the blockade of transformation by α_s* can be achieved without raising cellular cAMP levels to deleterious levels. It is known that NIH-3T3 cells are on the verge of transformation (Rigby, 1982) and are often transformed without the introduction of foreign oncogenes (Rubin and Xu, 1989). The use of such a system heightens the potential significance of our observations by indicating that α_s* can block the transformation of cells that are very close to being transformed. Taken together, all these considerations indicate

that targeted implantation of α_s* may be a useful strategy for preventing the development of cancers in some predisposed cells or tissues.

APPENDIX: PUBLICATIONS

1. **Chen, J.** and Iyengar, R. (1993) Activated G_S-α decreases H-*ras*-stimulated MAP kinase activity and blocks transformation of NIH-3T3 cells. submitted

2. Tian, J., **Chen, J.** and Bencroft, C. (1993) Expression of constitutively active G_S-α–subunits in GH_3 pituitary cells stimulates prolacting promoter activity. *J. Biol. Chem.* (in press)

3. De Vivo, M., **Chen, J.**, Codina, J. and Iyengar, R. (1992) Enhanced phospholipase C stimulation and transformation in NIH-3T3 cells expressing Q209L G_q-α subunits. *J. Biol. Chem.* **267,** 18263-18265

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Bourne, H. R., Sanders, D. A. and McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions *Nature* **348,** 125-132

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Chen, J. and Iyengar, R. (1993) Inhibition of cloned adenylyl cyclases by mutant activated $G_{i}\alpha$ and specific suppression of type 2 adenylyl cyclase inhibition of adenylyl cyclase by phorbol ester treatment. *J. Biol. Chem.* **268,** 12253-12256

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