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The growth effects of the G_0 protein

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The Growth Effects of The G_0 Protein

by

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Abstract

The Growth Effects of The G_o Protein

by

Spencer Daniel Kroll

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G proteins are cell surface GTP binding proteins that transduce extracellular signals. G proteins are activated by receptors and in turn, modulate intracellular effectors. G_o, a member of the G protein family, is especially abundant in brain. G_o specifically enhances muscarinic stimulation of phospholipase C-mediated Cl^- current in Xenopus laevis oocytes. Hence, G_o may promote release of intracellular calcium and stimulate protein kinase C (PKC).

Mitogenic signals are transduced by many receptors that stimulate phospholipase C through G proteins. Continuous stimulation of these receptors leads to desensitization and subsequent down-regulation of receptors. In contrast, G proteins do not appear to be subject to the same form of regulation. Persistent activation of G proteins may have important effects on cellular growth. Continuous cellular growth may be a contributory factor to the development of cellular transformation. For G_o, its involvement in signal transduction and, in particular, its ability to stimulate phospholipase C could be important in stimulating mitogenesis.

Maturation of Xenopus oocytes is a model system used to assess the capability of many substances (proteins, hormones, drugs) to trigger growth resumption. Experiments described herein show that persistently activated G_o protein induces cell cycle resumption in oocytes through the activation of PKC. The pathway by which maturation occurs is divergent from the presumed physiological activator progesterone, in that progesterone does not require PKC activation to induce oocyte maturation. Maturation induced by progesterone as well as G_o appears to converge at the requirement for the translation of the proto-oncogene c-*mos*, a ser/thr protein kinase. Synthesis of c-*mos* (p39) is necessary for maturation. Induction of c-*mos* by G_o is PKC dependent.

To assess whether activated G_o induced cell cycle resumption was specific to Xenopus oocytes, I studied the effect of activated G_o-α expression on the growth of mammalian cell lines. A plasmid expressed mutant G_o-α that could be persistently active was constructed. NIH-3T3 fibroblasts were transfected with vector containing WT α_o or mutant α_o. It was found that mutant G_o-α stimulated mitogenesis and subsequently induced transformation of NIH-3T3 cells. However, mutant G_o-α could not transform RAT-1 fibroblasts.

Hence, it appears that persistent activation of G_o protein can induce neoplasia when transfected into NIH-3T3 fibroblasts. Previous findings that G_o protein is linked to stimulation of phosphoinositide hydrolysis and my finding that G_o induces oocyte maturation through activation of PKC in Xenopus oocytes suggests that G_o can be an important intracellular growth control regulator.

Acknowledgements

This dissertation is dedicated to my family: my father, who taught me integrity; my mother who taught me sensitivity; my brothers, who taught me devotion; my grandparents Jack and Ruth Kroll, who never lost hope in me; and especially my grandmother Pauline Grossman, who gave me a love of learning and a love of life that I will take with me forever.

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The science of life is a superb and dazzling lighted hall which may be reached only by passing through a long and ghastly kitchen.

Claude Bernard,
Introduction to the Study of Experimental Medicine [1865]

He enjoyed long stretches of pure delight such as only a seaman may know, and moments of high, proud exultation that only a discoverer can experience.

Samuel Eliot Morison
Admiral of the Ocean Sea [1942]

Science is built up of facts, as a house is with stones. But a collection of facts is no more a science than a heap of stones is a house.

Jules Henri Poincare
Science and Method [1913]

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Introduction

Hormonal Signal Transduction and Cellular Growth

The transduction of hormonal signals across the cell surface is a ubiquitous process whereby an extracellular stimulus is translated into intracellular response. Physiological responses are elicited in response to the reception of specific signals which allows the cell to respond to a variety of different stimuli (Snyder 1985). Growth is one of the many important physiological processes that the cell undergoes. Cellular growth is controlled in response to extracellular signals. The reception of a mitogenic signal mobilizes the cellular components that orchestrate DNA synthesis and cell division. Hence, proper control of the signalling pathways that respond to mitogenic signals is imperative to maintaining cellular homeostasis.

The observation that the cancerous state involves unregulated proliferation and that cancer may be induced by specific DNA mutations, has led to a prolonged search during the past decade for proteins that induce cellular growth. This search has resulted in a convergence among many fields of scientific research including; endocrinology, cell biology, and oncology. Lesions involved in the transduction of extracellular signals are probably primary factors that contribute to neoplastic transformation. These lesions can result from congenital alterations as well as environmental exposure. Future pharmacological intervention in the treatment of cancers will probably involve agents that specifically "correct" abberant

growth pathways by possibly rectifying the genetic lesions by targeted gene therapy. Such future therapies based on the molecular mechanisms of signal transduction will probably be far more effective in blocking cancerous growth than the current pharmacological therapies based on the generalized prevention of cellular growth. As yet, however, the mechanisms by which these genetic lesions promote inappropriate growth remain to be determined.

Cell Surface Signalling And Regulation Of The Cell Cycle

Cellular growth is divided into four stages corresponding to the chromosome number of the cell (Lloyd et al. 1982). A newly divided cell undergoes a growth phase (G1), where a diploid chromosome number is found. Without external signals from mitogenic hormones or growth factors, the cell will remain in this stage indefinitely. Hormone binding elicits a cascade of events that lead to entry into the chromosomal synthesis phase (S). The cell then continues to grow and build up the necessary proteins that control mitosis. This second growth phase (G2) is normally where cells that are programmed to undergo meiosis are arrested. The final stage is mitosis or meiosis (M), where cells divide and begin the process again. The approximate times for these stages of the cell cycle for a growing cell are 11 hours, 7 hours, 3 hours and 1 hour, respectively. In developing stem cells, the cell cycle is arrested at a stage immediately preceeding meiosis (G2). After receiving the correct growth stimuli in the G1 phase of growth arrest for somatic cells, or G2 phase arrest for pre-meiotic cells,

the cells are committed to complete the entire cell cycle. The requirement of protein accumulation through *de novo* synthesis suggests that a threshold must be overcome for the cell to re-enter the cell cycle. The search for specific cellular components that elicit this switch from quiescence to growth is of central importance in understanding regulation of the cellular growth response. These cellular components are most likely to be the targets of modulation by extracellular signals. Thus an understanding of cellular signalling mechanisms and the temporal integration of these signals is important in elucidating the mechanisms that regulate cell cycle resumption.

Intercellular communication is achieved by the release of hormone (or growth factor) by one cell type and the reception of this signal by another. This hormone binds to a specific receptor that may reside on the target cell surface. Steroid hormone receptors are soluble receptor (possibly nuclear) proteins. Steroid hormones pass through the cell membrane to bind to their receptors. In contrast, the vast majority of peptide hormones and growth factors bind to cell surface receptors and the hormone binding signal is transduced to elicit a cellular response. One paradigm of signal transduction systems that respond to extracellular hormones is a multicomponent model consisting of a receptor which is specific for the hormone that binds to it and an effector enzyme, which typically catalyzes the production of an intracellular messenger. The second messenger goes on to alter other cellular targets, including direct activation or pathways leading to the activation of phosphorylating enzymes and

transcription factors. In many receptor signalling systems hormone/receptor activation of effector enzymes is guanine nucleotide dependent.

Receptor-activated second messenger systems are typically divided into three types: receptors that use multicomponent G protein mediated cell surface signalling systems such as adenylyl cyclase or phospholipase C; receptors that have intrinsic ion channel activities and receptors that have intrinsic tyrosine kinase activity.

G proteins

G proteins are a family of proteins that transduce cell surface receptor signals to the inside the cell (Iyengar and Birnbaumer 1990). The guanine nucleotide dependence of hormonal activation of adenylyl cyclase, was the first indication that an additional regulatory component was involved in signal transduction (Rodbell et al. 1971*a*) These studies, which utilized the glucagon sensitive adenylyl cyclase in rat liver plasma membranes, demonstrated a requirement of GTP for hormone induced activation of adenylyl cyclase. In addition, it was found that GTP affected hormone binding to receptors (Rodbell et al. 1971*b*). Non-hydrolyzable analogs of GTP were shown to persistently activate adenylyl cyclase activity (Londos et al. 1974) and hormones were shown to increase the rate at which this persistent activation occured (Salomon et al. 1975). Studies on the β-adrenergic receptor demonstrated that GTP alterations on hormone receptor binding were specific for isoproterenol (an

agonist) but not for propranalol (an antagonist) (Maguire et al. 1976). This specificity for agents that stimulated the second messenger system, rather than just binding to the receptor, suggested that guanine nucleotide dependent regulation of receptor binding was crucial in hormonal activation of adenylyl cyclase.

A multicomponent system was first suggested by cell fusion experiments (Orly and Schramm 1976, Schramm et al. 1977). The stimulation of adenylyl cyclase in catecholamine-sensitive cells was inactivated by N-ethylmaleimide treatment. These cells were fused with catecholamine-insensitive cells to receconstitute the catecholamine-sensitive response.

The demonstration that catecholamines, through the β-adrenergic receptor, stimulate GTP hydrolysis (Cassel and Selinger 1976) yielded many detailed analyses of the mechanism by which G proteins elicited their control on hormonal signal transduction including the fact that GDP dissociation serves as the rate limiting step in hormonal signal transduction (Cassel and Selinger 1978). In addition, it was also found that the mechanism of cholera toxin action was through the covalent modification of the guanine nucleotide component of the adenylyl cyclase system which resulted in blockade of agonist-stimulated GTPase activity (Cassel and Pfeuffer 1978). These observations explained the mechanisms by which cholera toxin persistently activates adenylyl cyclase (Sharp and Hymes 1971).

The first suggestion that a distinct GTP binding protein existed in the adenylyl cyclase system came from studies where

extracts of erythrocytes were passed over GTP affininity columns. It was found that some of the GTP stimulatable activity was lost in the fraction that did not bind the column (Pfeuffer 1976). The definitive demonstration of a GTP binding protein distinct from receptor or adenylyl cyclase came after the discovery of the cyc^- variant of the S49 murine lymphoma cell (Bourne et al. 1975). Communication between the receptor and effector is disrupted in the cyc^- murine lymphoma cell line. Adenylyl cyclase activity was restored by the addition of wild type S49 cells extracts to heat-inactivated adenylyl cyclase (Ross and Gilman 1977). The missing component of the cyc^- cells was soon purified and shown to reconstitute the receptor/effector communication and be responsible for the guanine nucleotide effects (Northup et al. 1980) This G protein, named G_S because of its stimulatory effect on adenylyl cyclase, was found to be heterotrimeric, consisting of α, β and γ subunits (Northup et al. 1980, Sternweis et al. 1981, Hildebrandt et al. 1984). The α-subunit has the GTP binding site which has intrinsic GTPase activity and is capable of stimulating the effector adenylyl cyclase (Northop et al. 1982, Ross 1984). The $\beta\gamma$ subunits are tightly associated and can be dissociated only under denaturing conditions (Hildebrandt et al. 1984). Hence G proteins behave as functional dimers of α and $\beta\gamma$ subunits.

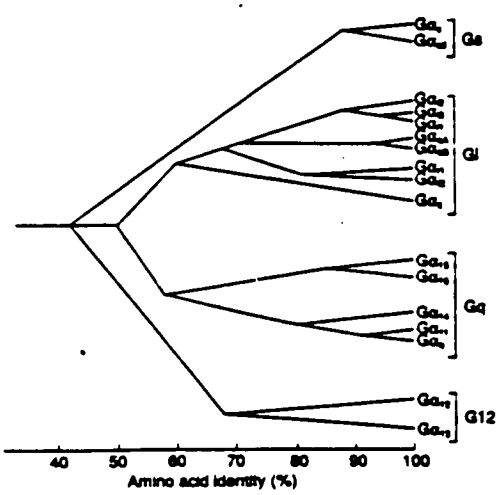
The retinal phototransduction system revealed the involvement of GTP as a regulatory factor in rhodopsin activation and transmission of the visual signal (Wheeler and Bitensky 1977). The retinal GTP-binding protein, which has been named transducin, is also a heterotrimeric protein composed of α,β and γ

subunits, similar to G_S, with the α subunit binding GTP (Godchaux and Zimmerman 1979, Kuhn 1980). The β subunits of transducin and G_S were found to be nearly identical (Manning and Gilman 1983). The overall structure of the retinal phototransduction system was found to be analogous to receptor-stimulated adenylyl cyclase in that the receptor, rhodopsin, activates its effector cGMP phosphodiesterase via the G protein transducin (Fung et al. 1981, Cassel and Selinger 1978). Although genes encoding several β and γ subunits have been cloned (Simon et al. 1991), it appears the unique characteristics of the individual G proteins are derived from the α-subunit. Hence, often the name of the G protein and α-subunit are used interchangeably.

Evidence for additional G protein types came originally from experiments that analyzed hormonal inhibition of adenylyl cyclase (Rodbell 1986, 1987). These ideas gained further support from studies analyzing the mechanism of pertussis toxin. It was known that pertussis vaccination supressed catecholamine induced hypertension (Sumi and Ui 1975). This action was localized to pancreatic islet cells, where it was observed that pertussis toxin inhibited α_2 adrenergic receptor-stimulated insulin secretion (Katada and Ui 1977). Pertussis toxin was found to stimulate the covalent addition of an ADP-ribose moiety to a 40-41 kDA membrane protein (Katada and Ui 1982). It was observed that somatostatin-induced inhibition of adenylyl cyclase was intact in cyc^- cells (Jakobs et al. 1983) and pertussis toxin disrupted this inhibition (Hildebrandt et al. 1983). Thus, a G protein that inhibited adenylyl cyclase was thought to exist. This protein was

purified and called G_i (Bokoch et al. 1983, Codina et al. 1983). Several substrates for pertussis toxin have now been isolated in the approximate size range of 39-42 kDa: These include, three forms of G_i (Jones and Reed 1987), two forms of G_t (the retinal phototransduction G protein, transducin) (Van Dop et al. 1984) and two forms of G_o, an additional pertussis toxin substrate of very high abundancy in brain (Sternweis and Robishaw 1984, Neer et al. 1984). Thus, for some time pertussis or cholera toxin sensitivity was thought to be an indicator of heterotrimeric G protein involvement.

Toxin sensitivity is now realized not to be an absolute discriminator of G protein involvement, as many newly isolated species of G protein α-subunits have been found that are not sensitive to cholera or pertussis toxin mediated ADP-ribosylation A similarity tree of the α-subunits by Simon et al. (1991) is shown in **Figure 1.**



Many individual G protein species have been purified. The G proteins possess similar apparent molecular weights (39-52 kDa) and contain conserved regions in their primary sequence, including highly conserved guanine nucleotide-binding regions. The individual G proteins serve unique functions as specified by the receptor and effector enzymes to which they couple (Johnson and Dhanashekaran 1989). For example, the four forms of the G_s protein (which arise from differential splicing of a single transcript) activate adenylyl cyclase. The G_s protein may also regulate Ca^{2+} channels (Mattera et al. 1988, Yatani et al. 1987). G_i proteins are presumably capable of inhibiting adenylyl cyclase as well as activating inward rectifying K^+ channels (Yatani et al. 1988). The G_t protein is found in retina and regulates cGMP phosphodiesterase in the transduction of the visual signal (Fung et al. 1981). The studied functions of the G_o protein will be explained in detail below. Briefly, the G_o protein appears to be capable of stimulating PIP_2 hydrolysis in Xenopus oocytes (Moriarty et al. 1990). G_o is also capable of inhibiting Ca^{2+} channels in neuroblastoma/glioma cells (Hescheler et al. 1987 1991).

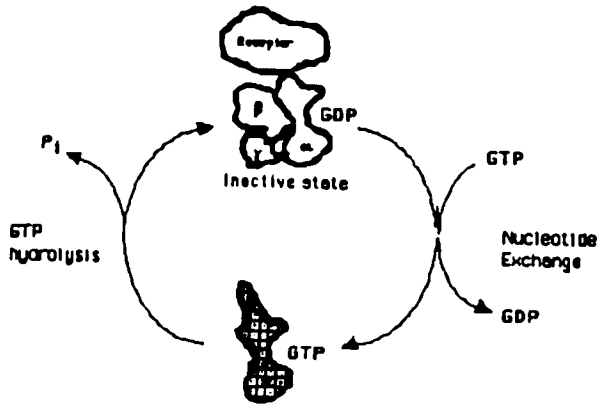
Pertussis toxin-sensitive pathways exist for stimulation of inositol phospholipid hydrolysis by phospholipase C and phospholipase A_2, opening of K^+ channels, inhibition of Ca^{2+} channels, as well as inhibition of adenylyl cyclase. The toxins of Bordetela pertussis and Vibrio cholera are ADP-ribosyl transferases that catalyze the transfer of ADP ribose to the α-subunit of G proteins. Pertussis toxin catalyzes ADP-ribosylation

of a cysteine in the fourth position from the carboxy terminus, found in G_i's, G_o's and G_t (West et al. 1985). This region is involved in contact with G protein coupled receptor (Sullivan et al. 1987), resulting in the observed uncoupling of the receptor from the signal transducing system. Cholera toxin ADP ribsosylates Arg-201 of G_s. This arginine is essential for GTP hydrolysis (Bourne et al. 1990). Hence, cholera toxin-mediated ADP ribosylation blocks GTP hydrolysis and locks G_s in a persistently activated state (Cassel and Sellinger 1978). This results in the observed stimulation of cAMP production. Even though all G protein α subunits identified thus far have this conserved Arg, only G_s appears to be the natural substrate for cholera toxin.

Structure and Function

As stated above, signal transducing, heterotrimeric G proteins consist of three subunits, designated α, β and γ. The α subunits have the ability to bind guanine nucleotides and contain an intrinsic GTPase (Heideman and Bourne 1990). The effector regulating activity of the α subunit is determined by the guanine nucleotide that is bound: GTP activates, while GDP inhibits G protein function. The intrinisic GTPase of the G protein hydrolyzes GTP to GDP, thereby deactivating the G protein. In the resting state, G proteins are found in the heterotrimeric state, with GDP tightly bound to the α subunit. Hormonal occupancy of the receptor stimulates GDP dissociation and GTP binding, promoting the dissociation of the $\beta\gamma$ subunits from the activated α-GTP subunit. It is this form of the α subunit that stimulates effector

enzymes. The GTP liganded α subunit directly regulates the activity of adenylyl cyclase (Northup et al. 1983), cGMP phosphodiesterase (Fung et al. 1981), K^+ channels (Codina et al. 1987) and Ca2+ channels (Mattera et al. 1988) and phospholipase C (Moriarty et al. 1990, Smrcka et al. 1991, Taylor et al. 1991) Subsequent hydrolysis of the bound GTP to GDP allows α subunit reassociation with the βγ subunits and a return to the resting state. Thus the dynamics of G protein activation involve a conformational change in the α-subunit whereupon GDP is released and GTP binding, thereby promoting its interaction with the effector enzyme. Hydrolysis resistant analogs of GTP activate G proteins persistently since they trap the α-subunit in the active state and have been used experimentally to enhance effector enzyme stimulation (Londos et al. 1974). The G protein α subunit maintains an inactive state by associating with βγ subunits. In fact, addition of an excess of βγ subunits is generally sufficient to inhibit communication between receptor and effector, presumably by shifting the equilibrium towards an inactive state (Katada et al. 1984, Moriarty et al. 1989). The dynamics of G protein activatation are shown in **Figure 2.**



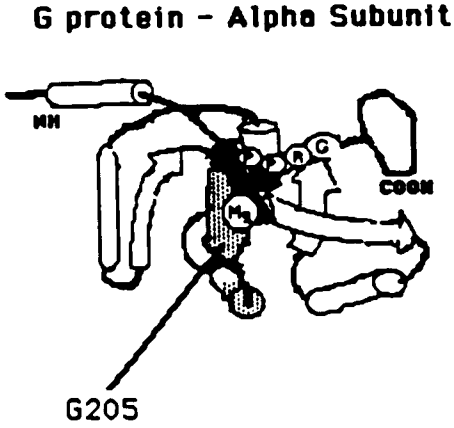
The mechanism of G-α protein function make the protein a likely candidate for mutations that could render it persistently active. As described in detail below, specific point mutations may inhibit the GTPase function of the α subunit, thereby locking the protein in a persistently active state. Similarly, other mutations have been defined that interfere with the function of the G-α protein: The Gly226Ala mutation in the G_s-α subunit inhibits subunit dissociation and prevents GTP activation of the G_s protein (Miller et al. 1988). Another interesting mutation was isolated from a S49 cell variant that was not capable of isoproterenol stimulated adenylyl cyclase activity (Haga et al. 1977). This cell line contains a mutant G_s-α subunit with a Arg389Pro mutation (Sullivan et al. 1987). This mutation uncouples the G protein from the receptor. This site is near (-6 position) the carboxy terminus of the G protein α subunit. The mapping of the pertussis toxin ADP ribosylation site to a cysteine near (-4 position) the carboxy terminus (West et al. 1985), as well as the observation that a synthetic decapeptide corresponding to the carboxy terminus of α-transducin blocks receptor interaction (Cerione et al. 1987) has led to the hypothesis that the α subunit carboxy terminal region is involved in G protein/receptor contact. Further evidence for this hypothesis comes from the retinal system where arrestin, a protein which binds to phosphorylated rhodopsin and competes with transducin for binding, contains a similar sequence to the α subunit carboxy terminus (Wistow et al. 1986).

GTPase Activity

The G proteins now appear to be a member of a large family of proteins displaying GTPase activity (Bourne et al. 1991). The GTPase family can be broadly characterized into two families: the G proteins, consisting of heterotrimeric structure with 38- 52 kDa α subunits; and the small molecular weight GTPases, which are monomeric in structure and possess a molecular weight 20-25 kDa. These small molecular weight GTPases consist of a variety of proteins which orchestrate a variety of cellular functions: the elongation and initiation factors, which function as a regulatory mechanism in protein synthesis; the Sec and Ypt1 proteins, which function in intracellular vesicular transport; the rho proteins, the rap proteins and the ARF proteins, which serve as cofactors in cholera toxin mediated ADP ribosylation (Kahn 1990). The *ras* gene, which was originally isolated from retrovirally induced rat sarcomas and subsequently found in human bladder, colon and pancreatic carcinomas, contains a point mutation that alters the function of its protein product. The p21ras protein contains an intrinsic GTPase and alternates between an active GTP bound form and an inactive GDP bound form (Barbacid 1987).

The elongation factor EF-Tu and p21ras have been crystallized and their crystal structure solved. These proteins as well as the α-subunits of other G proteins display regions of high similarity in the overall topology of the guanine nucleotide binding domain. Sequence alignment of the various GTPases allows for the visualization of the similarities among GTPases in

four seperate domains: as deduced from the crystallographic structure of p21ras, the G1 (A.A. 10-17) region is a loop where binding of the GDP α and β phosphates occur. The three dimensional shape of this region does not vary in the GTP or GDP bound form of p21ras. In contrast, the G2 (A.A. 32-40) region of p21ras does change conformation when GTP is bound rather than GDP. This region contains the site of Mg^{2+} binding. This magnesium ion, which is necessary for GTP hydrolysis, is coordinated with the β and γ phosphates of GTP. The G3 (A.A. 53-62) region is highly conserved between GTPases. In this region, hydrogen bonding to the γ phosphate of GTP occurs, which is a catalytic prerequisite to hydrolysis. This G3 region also dramatically changes conformation when GTP versus GDP is bound. The G4 (A.A. 112-119) region is also responsible for catalysis, forming hydrogen bonds with the guanine ring. One important residue defined in p21ras is Glu61 of the G3 region. The carbamoyl oxygen of this residue activates a water molecule (arbitrarily termed Wat-175), which acts as a nucleophile in the hydrolysis of GTP. Hence replacement of this glutamine residue will probably inactivate the GTPase function without altering the proteins ability to bind GTP or be converted into the active state. A comparison of G-α and p21 ras is shown in **Figure 3.**



	G-1	G-2	G-3	G-4
Consensus				
H/K/N-ras	5 KLVVVGAGGVGKSALT	32 YDPTIEDSY	53 LDILDTAGQE	112 VLVGNKCD
G_o · α	35 KLLL GAGESGKSTIV	174 DVLRTRVKTTGIVE	197 FKMFDVGGQR	266 ILFLNKKD

The GTPase function of the G protein may act as a timing mechanism and thus different G proteins may regulate effectors in a different temporal fashion. Unlike the intrinsic GTPase activity in G protein α subunits, the small molecular weight GTPases control their GTPase by association with GTPase-activating proteins (GAPs).

The G_o Protein

G_o was originally purified in attempts to purify G_i protein from bovine brain (Sternweis and Robishaw 1984, Neer et al. 1984). The procedure designed for isolating the pertussis toxin substrate from other tissues (cholate extraction, anion exchange

was further resolved from Gi by FPLC using anion exchange chromatography (Katada et al. 1986). The βγ subunits purified from G_o were found to be functionally identical to βγ from G_i (Sternweis and Robishaw 1984).

Attempts at raising antibodies to the G_o protein were troubled by the similarity of the G_o protein to G_i. Antibodies raised against the holo-G_o protein did not cross-react with G_i (Gierschik et al. 1986a), however, antibodies against the α_o subunit cross reacted with α_i (Huff et al. 1985, Katada et al. 1987, Mumby et al. 1986). Antisera raised to a peptide specific to the α_o subunit (NLKEDGISAAKDVK) did not cross react with G_i (Mumby et al. 1986). The G_o-α subunit is a major protein accounting for approximately 1% of membrane proteins in the central nervous system (Huff et al. 1985, Gierschik et al. 1986b). The G_o-α protein appears to be heterogeneously distributed in brain tissue and thus may account for even a higher percentage of membrane protein in some central nervous system structures. G_o appears to be more abundant in cortex, thalamus, hypothalamus and cerebellum and of lower abundance in the medullary and pons regions (Gierschik et al. 1986b, Asano et al. 1987). G_o-α immunoreactivity is observerd in brain tissue from many different species including human, pig, rat, chicken, frog, snail, locust (Homburger et al. 1987). It appears to be the only pertussis toxin substrate in invertebrate neural tissue (Harris-Warrick et al. 1988). G_o immunoreactivity was also observed in peripheral neuronal tissue including spinal cord (Gierschik et al. 1986b, sciatic nerve (Homburger et al. 1987) and adrenal medulla

peripheral neuronal tissue including spinal cord (Gierschik et al. 1986b, sciatic nerve (Homburger et al. 1987) and adrenal medulla and trachea (Mumby et al. 1986, Toutant et al. 1987). Other non-neuronal sites of G_o immunoreactivity have been observed in anterior pituitary (Homburger et al. 1987), heart and kidney medulla (Huff et al. 1985) and olfactory neuroepithelium (Anholt et al. 1987). The high abundance of G_o protein in neurons may confuse findings of peripheral distribution of G_o immunoreactivity because regional innervation may be co-purified. This is suggested by studies where it was observed that G_o-α immunoreactivity increased during cardiac parasympathetic innervation development (Luetje et al. 1987, Liang et al. 1986).

Cloning of a partial G_o-α was carried out from brain (Itoh et al. 1986). Sequence information was generated from tryptic degradation of the purified G_o-α and a full length clone was isolated from rat olfactory neuroepithelium (Jones and Reed 1987) and bovine retina (Van Meurs et al. 1987). The clones displayed a high homology with G_i-α (82%), 76% homology with transducin-α and 50% homology with G_s-α. The highly conserved regions have been mapped as the regions of GTP binding and hydrolysis (Dever et al. 1987). In addition, two immunologically distinct forms of the G_o protein have been isolated (Goldsmith et al. 1989, Padrell et al. 1991). These proteins display similar GDP release and GTPγs binding rates, although the two species possess different GTPγS binding kinetics in the absence of Mg^{2+}.

Multiple Forms of G_o

The function of G_o protein has been difficult to define. Reconstitution experiments have demonstrated that G_o can couple to muscarinic receptors (Kurose et al. 1986) and α_2-adrenergic receptors (Cerione et al. 1986). G_o was capable of reconstituting coupling of F-met-leu-phe receptors and phospholipase C activation (Kikuchi et al. 1986) in HL-60 cells, although G_o immunoreactivity was not found in these cells. Although the above interactions were also reported for G_i proteins, it appears that G_o is not part of an adenylyl cyclase inhibitory pathway in cyc^- membranes (Roof et al. 1985) or purified α_s-activated adenylyl cyclase (Katada et al. 1986). In neuroblastoma/glioma hybrid cells, G_o has been reported to inhibit Ca^{2+} channels (Hescheler et al. 1987, Harris-Warrick et al. 1988, Kleuss et al. 1991), though it is as yet uncertain whether this is a direct effect. Finally, it has been shown that G_o can reconstitute muscarinic stimulation of phospholipase C in Xenopus oocytes and that activated G_o-α subunit stimulates oocyte phospholipase C as assessed by the Ca^{2+} dependent Cl^- current in Xenopus oocytes. (Moriarty et al. 1990).

Toxin-Insensitive G Proteins That Stimulate Phospholipase C

Recently, it has been demonstrated that the G protein family is much larger than expected. Many new families of G proteins have been cloned (Strathmann and Simon 1990) including a class of pertussis toxin insensitive group known as the G_q family. This new class of G proteins was thought to be involved in pertussis-toxin insensitive regulation of phospholipase C, a function for which no other known G proteins had been designated. The G_q protein was purified from bovine liver (Taylor et al. 1990) and rat brain (Pang and Sternweis 1990) was found to stimulate phospholipase C (Taylor et al. 1990, Taylor et al. 1991). The G_q protein was subsequently found to stimulate the β1 isozyme of the phospholipase C enzyme (Taylor and Exton 1991, Taylor et al. 1991).

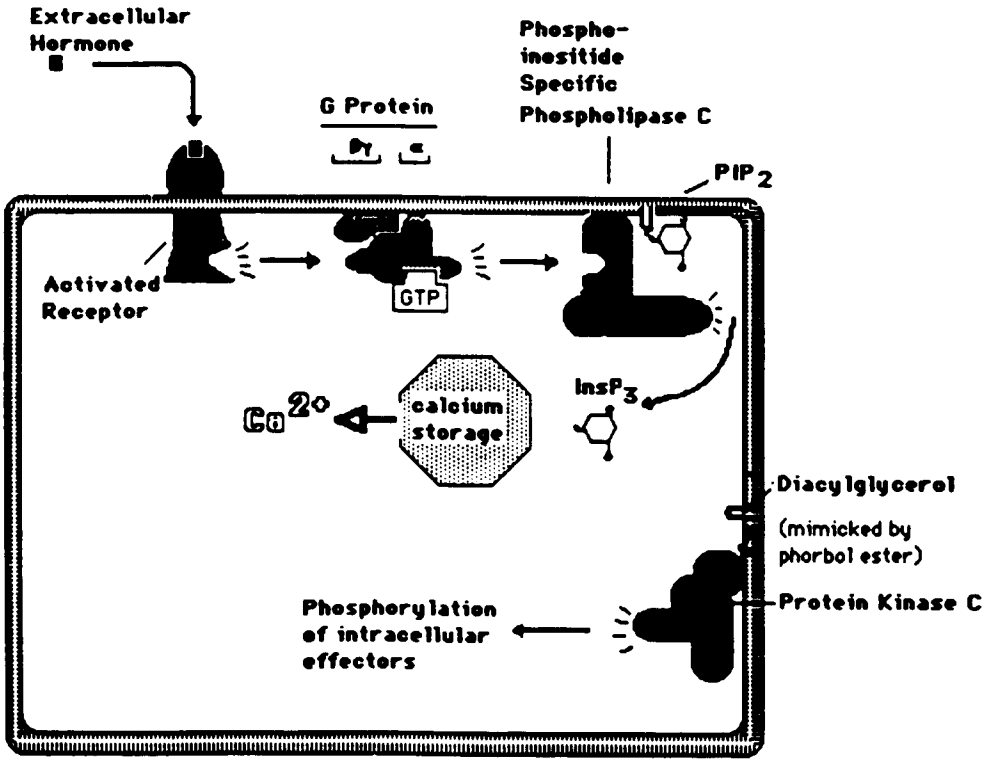
G proteins and Phosphatidylinositol Hydrolysis

The first indications that receptor mediated G protein regulated signalling systems existed independently of adenylyl cyclase activation came from the observations that GTPγS stimulated histamine release independently of cAMP (Gomperts 1983). Further, GTPγS stimulated serotonin release from platelets independently of cAMP and this release was blocked by GDPβS (Haslam and Davidson 1984). The existence of G proteins regulating phospholipid hydrolysis came from studies where Gpp(NH)p, but not GTP or ATP, stimulated inositol phosphate production in blowfly salivary tissue (Litosch et al. 1985). GTPγS was later shown to regulate inositol phospholipid hydrolysis in a variety of tissue while GDPβS blocked this effect (reviewed in

DeVivo and Gershengorn, 1988). Hormonal activation of many types of receptors leads to PIP_2 hydrolysis [(e.g. $\alpha 1$ adrenergic (Evans et al. 1985), M2 muscarinic (Putney 1986), $5HT_{1c}$ and $5HT_2$ serotonergic (Litosch et al. 1985), angiotensin, tachykinins (Vincenti and Villereal 1984)]. Many of these receptors display guanine nucleotide-dependent PIP_2 hydrolysis (Pouysseguir 1990, Cockcroft and Gomperts 1985). Thus, it is possible that a G protein may be responsible for coupling receptor activation to at least one form of the phospholipase C enzyme.

Many mitogenic hormones have been found to stimulate inositol phospholipid hydrolysis and specifically hydrolysis of phosphatydylinositol bisphosphate (PIP_2) (Hokin and Hokin 1957) This hydrolysis yields two intracellular second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to intracellular receptors on the endoplasmic reticulum, promoting the release of intracellular calcium (Berridge and Irvine 1984). DAG activates a cytoplasmic protein kinase with serine/threonine kinase activity, designated protein kinase C (Nishizuka 1984). Phospholipase C is the enzyme that catalyzes the hydrolysis of PIP_2 (Rebechi and Rosen 1987, Wilson et al. 1985) . This enzyme has been found to exist in several forms, suggesting various modes of regulation (Rhee et al. 1989). There are forms of the phospholipase C class of enzymes that are specific for the hydrolysis of phosphoinositides, although phosphatydylglycerol has been shown to be a poor substrate for the enzyme (Hofmann and Majerus 1982). Hydrolyzed inositol phosphates are recycled in the cell cytoplasm to reform PIP_2. Lithium, which is used

clinically for its anti-manic effects, blocks this recycling pathway, suggesting that clinical mania may correlate with a disfunction in the phosphoinositide signalling system (Goodman and Gilman 1991). Purified phospholipase C enzyme has been found to exist in many forms, with multiple subtypes being purified from the same tissue. Although there are many types, the phospholipases C can be categorized into three types: phospholipase C-α, phospholipase C-β, which is the presumable effector for G protein activation, and phospholipase C-γ which is phosphorylated by tyrosine kinase receptors. There also appears to exist other forms of the phospholipase C enzyme, phospholipase C-δ, phospholipase C-ε, as well as multiple sub-types of the phospholipase C-β enzyme (Rhee et al. 1989). Whether the action of all tyrosine kinase receptors is carried out by phospholipase C-γ remains to be determined, although receptor binding of PDGF promotes association with the phospholipase C-γ enzyme (Kumjian et al. 1989). Furthermore, in-vitro assays have demonstrated that phosphorylation of this enzyme by tyrosine kinase receptors increases the catalytic activity of the phospholipase C-γ enzyme (Nishibe et al. 1990). Tyrosine kinase receptor activation of DNA synthesis does not, however, require activation of phospholipase C (Hill et al. 1990). A schematic diagram of the receptor mediated activation of phospholipid hydrolysis and subsequent protein kinase C stimulation is shown in **Figure 4.**



Protein Kinase C: An Intracellular Ser-Thr Kinase Involved In Cell Growth Signals.

Protein kinase C is a soluble serine/threonine kinase that is activated and translocated to the plasma membrane by calcium and diacylglycerol (Nishizuka 1984). Intracellular calcium concentrations are typically maintained at sub-micromolar concentrations by sodium/calcium exchange across the plasma membrane (Carafoli and Zurini 1982). Additionally, calcium is stored in intracellular vesicles where it is released in response to hormonally induced production of inositol trisphosphate, as stated above (Spat et al. 1986). This calcium release is transient as free

calcium is quickly sequestered or pumped out of the cell. The diacylglycerol produced by PIP2 metabolism is quickly reconverted to inositol phospholipid by means of conversion to phosphatidic acid. Phosphatidic acid is converted to CDP-diacylglycerol and inositol is added to form phosphatydyl inositol (Majerus 1986). Thus, hormonal stimulation of protein kinase C by means of enzymatic production of inositol trisphosphate and diacylglycerol is transient, as these activating factors are quickly metabolized.

Experiments which describe the multistep causation of the malignant state have demonstrated that when small amounts of carcinogens are painted on mouse skin, mutations are initiated but cancers do not form. Additionally, the mouse skin must be treated with a tumor promoter. These agents, while not inducing malignant transformation or growth by themselves, act in conjunction with other primary signals to induce transformation.

The tumor promoting phorbol esters stimulate protein kinase C (Castagna et al. 1982). The activation of protein kinase C appears to be sufficient to promote cell cycle progression. Long term activation of protein kinase C can be achieved by permeant phorbol esters. These agents mimic the secondary structure of diacylglycerol in activating the protein kinase C enzyme (Parker et al. 1984). The phorbol esters are known as tumor promoters because of their tumor promoting effects on intact cells (Berenblum and Shubik 1947). Since phorbol esters stimulate protein kinase C activity, and since protein kinase C is presumably activated by many hormone signalling pathways, it would appear

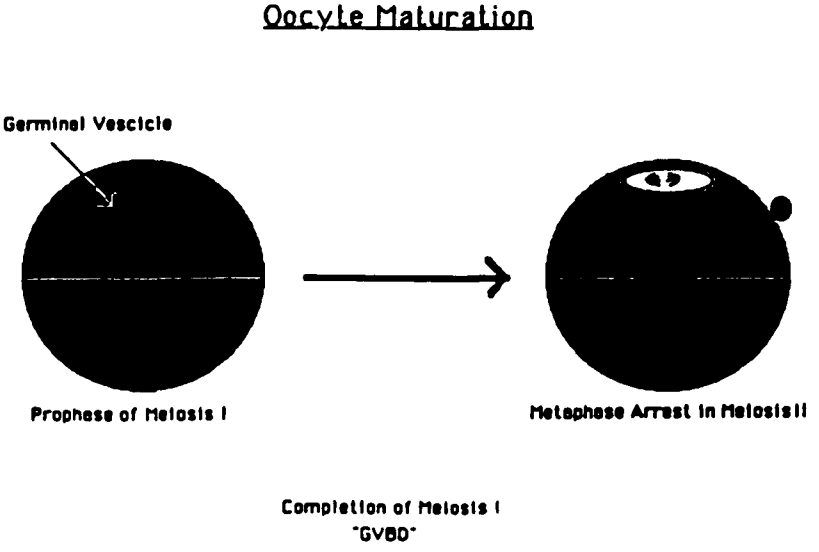
that there should be unregulated proliferation induced in response to these hormones. However, a feedback pathway has been characterized in which activated protein kinase C phosphorylates receptors, down regulating their activity (Corvera and Garcia-Sainz 1984, Leeb-Lundberg et al. 1985). Therefore, activation of protein kinase C by hormone receptors induces a subsequent loss of hormone responsiveness. Additionally, activation of protein kinase C by phorbol esters leads to a rapid down regulation of protein kinase C activity, due to proteolysis of the enzyme (Wickremasinghe et al. 1985).

Although many substrates for the protein kinase C enzyme have been characterized in vitro, no specific regulatory pathways related to growth have been elucidated that are controlled by the protein kinase C enzyme. The enzyme is inhibited by a self contained pseudosubstrate region which has been mapped to amino acids 19 through 36 (House and Kemp 1989). A synthetic peptide to this sequence is an excellent substrate for the enzyme. Substitution of alanine for the phosphorylated serine of the pseudosubstrate peptide creates an inhibitor of the enzyme. An antibody to the pseudosubstrate peptide activates the protein kinase C enzyme (Makowske and Rosen 1989), presumably by preventing the pseudosubstrate region from binding to the active site of the enzyme. Thus protein kinase C activation can be experimentally determined using the serine pseudosubstrate peptide while specific inhibition can be achieved with the alanine pseudosubstrate peptide. Several different forms of the protein kinase C enzyme have been cloned (Nishizuka 1988), including

two distinct types in Xenopus Laevis oocytes (Chen et al. 1989). Both the oocyte isozymes contain the pseudosubstrate sequence.

Xenopus Oocyte And Growth Regulation

The Xenopus oocyte has emerged as a model system for testing substances that promote cellular growth (reviewed by Smith, 1989). Fully grown amphibian oocytes are growth arrested in the late G2 phase prior to meiosis I. These cells must progress to the second meiotic metaphase for fertilization by sperm to proceed. For the maturation event to occur, in vivo, gonadotropic releasing hormone stimulates the release of progesterone from ovarian follicular cells. This progesterone directly stimulates the maturation of the oocyte, causing the oocyte nuclear membrane to breakdown. This allows for migration of the nuclear contents to the animal pole of the oocyte as shown in **Figure 5.**



This migration displaces pigment on the oocyte surface causing a white spot to form in the darkly pigmented animal pole. This white spot formation is a readily discernable feature of the mature oocyte and has been termed germinal vesicle breakdown (GVBD) (Dumont 1972). Surgically removed oocytes can undergo the maturation process by application of progesterone to the culture bath (Maller and Krebs 1977). Maturation is typically observed to occur about 6 to 18 hours after progesterone addition. In addition to the ease with which growth control can be measured, the Xenopus oocyte is extremely useful because of its large size. Xenopus oocytes are routinely used for microinjection studies. Thus the growth promoting effects of various cellular entities can be directly measured by microinjection into the oocyte by assaying for oocyte maturation.

There are many different agents that stimulate oocyte maturation (reviewed in Smith, 1989). This has led to the hypothesis that oocytes may contain separate pathways by which growth resumption can be stimulated. Phorbol esters induce oocyte maturation (Stith and Maller 1987) suggesting that activation of the protein kinase C pathway may play a role in oocyte maturation induction. Microinjection of the catalytic fragment of the protein kinase C enzyme induces germinal vesicle migration, however disolution of the vesicle does not occur (Muramatsu et al. 1989). Phorbol ester induced maturation is inhibited by forskolin, exogenous cAMP or 3-isobutyl-1-methyl-xanthine (IBMX, a cAMP phosphodiesterase inhibitor) in Rana pipiens oocytes. Several different oncogene products have been

shown to stimulate oocyte maturation, including the p21ras protein (Birchmeier et al. 1985), truncated forms of the EGF receptor (Opresko and Wiley 1990) and p39mos (Sagata et al. 1988). Other oncogenes either fail to induce oocyte maturation, as in the case of *src* kinase (Spivack et al. 1984). Injection of protein kinase C potentiates the effect of p21ras induced maturation (Kamata and Kung 1990).

The Mos Protein and Oocyte Maturation

Progesterone induced oocyte maturation requires protein synthesis, as demonstrated by cycloheximide inhibition of progesterone induced oocyte maturation (Wasserman and Masui 1975). Kinetic analysis of cycloheximide treatment has allowed the identification and characterization of a specifically translated and phosphorylated protein from maturing oocytes. This protein has been found to be the cellular homolog of the viral (*v-mos*) oncogene product (Sagata et al. 1989a). This oncogene is overexpressed in cellular transformation by the Moloney Murine Sarcoma Virus. Microinjection of antisense oligonucleotides to the *c-mos* transcript inhibit progesterone induced maturation (Sagata et al. 1989b). Immature Xenopus oocytes contain a large amount of the *mos* mRNA species which is translated upon progesterone treatment (Sagata et al. 1988). This translational control mechanism is specific for meiotic maturation, rather than a generalized mechanism in that mos protein is typically found physiologically in germ cells. Microinjection of excess *c-mos* mRNA into oocytes is sufficient to induce maturation (Sagata et al.

1989a). Thus, *mos* appears to be at least one of the specifically translated mRNA species that elicits oocyte maturation. Mature oocytes contain a cytoplasmic factor whose activity correlates with entry into M phase. The factor, termed maturation promoting factor (MPF) has been purified. It contains a ubiquitous Ser-Thr kinase called cdc2 kinase. The regulatory component of MPF is called cyclin, a protein essential for the cell cycle (Murray and Kirschner 1989). MPF is also phosphorylated during the cell cycle. Hence, it appeared possible that mos protein may regulate the activity of MPF through phosphorylation. However, MPF does not appear to be the direct target of mos dependent phosphorylation.

Oncogenes and Cellular Transformation

A significant discovery in the research of neoplastic development is the elucidation of various mutated forms of cellular genes that induce cancer (Shih et al. 1979). Up to now, at least sixty individual oncogenes have been isolated. Genes mutated during carcinogenesis can be categorized into two broad groups: dominant oncogenes, whereby a change in the activity of the gene, either by mutation or amplification induces neoplastic transformation; or tumor suppressor genes, where loss of function induces neoplastic transformation. Gene transfer experiments have elucidated many of these genes and have allowed for the characterization of some of their functions. These genes code for proteins that are typically involved in various modes of cellular signal transduction. In the case of most dominant oncogenes, a mutation in the gene typically destroys the regulatory ability of

the individual protein for which it codes, rendering the protein persistently active. Alternatively, overproduction of the cellular forms of several oncogenes leads to transformation.

Oncogenes were originally identified as the causative agents for animal transforming retroviruses (Duesberg and Vogt 1970). The first insights into the molecular genetics of cancer were gained by studying the genome of these retroviruses. It was found that the retroviruses had aquired portions of normal cellular genes and incorporated them into their own genome. This was found by comparing the viral genome to that of the host, which led to the finding of proto-oncogenes, or genes that did not cause cancer except after retroviral insertion. The proto-oncogenes were found to code for various proteins which are important to cellular signal transduction. These proto-oncogenes were converted to oncogenes either by over-expression by the retrovirus or mutation, typically by truncation of a regulatory region of the gene. Thus these viruses may exert their effect by integrating cellular proto-oncogenes into their genome and affecting the regulation of this gene so that some abberation in cellular signal transduction takes place.

Subsequent to finding oncogenic insertion in retroviruses, oncogenes were characterized in other ways as well. By searching for the specific genetic alterations that induce cancer in human cells, many of the same oncogenes found in retroviruses were found (Bishop 1987). Some of these oncogenes had undergone many of the same specific mutations and had thus gained ability to induce transformation (Shih et al. 1981). These experiments

were performed by gene transfer. The mouse fibroblast cell line, NIH-3T3, is commonly used for transfection with the cellular DNA of various tumor cell types. This assay system has been used to identify discrete genes that induce transformation.

Chromosomal karyotyping has also led to the finding of several oncogenes (Nowell and Hungerford 1960). In the case of various leukemias, chromosomal translocation serves to position genes involved in signal transduction next to powerful promoters which drive their transcription. Chromosomal karyotyping has become a routine clinical test for staging and prognosis of various hematologic malignancies (Koeffler and Golde 1981).

Oncogene products found in the above ways have been classified into several categories, based upon the presumed function of their protein products: growth factors, growth factor receptors, tyrosine kinases, serine/threonine kinases, transcription factors, and GTPases. Broadly, the oncogenes can be classified based upon the site of action of their protein products: either cell surface, cytoplasmic or nuclear. These oncogenes are activated either by point mutation, deletion, insertion or gene amplification. Thus the genetic basis for neoplastic transformation appears to reside in the reproducible mutation of discrete genes that evidently play a crucial role in cellular homeostasis. Oncogenes are often identically activated in similar tumor types, allowing for a genetic basis for tumor classification.

In addition to testing growth promotion in Xenopus oocytes, many oncogenes have been characterized by gene transfer experiments. Primary cells cannot be transformed by a single

oncogene. Rather, two or more oncogenes must be transfected in order for transformation to occur (Land et al. 1983, Hunter 1991). This suggests that more than one mutation must occur before a cell may become transformed (Weinstein 1990). This notion is supported by the observation of an exponential rise in cancer incidence with age. Futhermore, examination of isolated human tumors has revealed that there are many genetic changes in both proto-oncogenes and growth suppressing genes isolated from a single tumor (Hunter 1991). Thus, a mutation in any one pathway may be "pre-cancerous". It is impossible to determine, in these tumors, which mutation is the primary initiating event in carcinogenesis and which mutation is a secondary event. Indeed, treatment of cells with phorbol esters does not transform cells. Rather, pre-treatment with a carcinogenic agent is additionally required for transformation. Historically, testing for oncogenicity has been performed using established cell lines. Cultured cells typically undergo a finite number of generations before dying. Cells can become immortalized by selecting for those cells that survive this dying period (Todaro and Green 1963). These established cells will undergo an infinite number of generations, require normal growth factors for survival, yet can become transformed with only one oncogene. Thus, in the immortalization process, these cells may have lost a gene that programs their mortality. Alternatively, these cells may have acquired some mutation, perhaps in a nuclear oncogene, that promotes immortalization. This latter assumption is supported by the observation that in most cases, nuclear oncogenes immortalize

cells, while not altering cell morphology, while cytoplasmic oncogenes have no effect on immortalization, yet alter cell morphology (Weinberg 1985).

Cooperation of oncogene protein products may lead to transformation in many ways: it is likely that some oncogenes are going to be on intersecting or parallel pathways leading to growth and immortalization. Becuase, these oncogenes may be mutated independently and over time, an accumulation of enough mutations may lead to transformation. However, in some cases, mutation of one proto-oncogene may affect either the mutational state of another oncogene, or affect the abundance of cells possessing another mutation. It is unclear whether oncogenes increase mutation frequency or increase the number of cells at risk for a secondary mutation. If a mutation occurs which is not transforming on its own, clonal expansion of the cell harboring this mutation by other mutations in growth signalling pathways could lead to transformation. This appears to be the case for transformation by Epstein-Barr virus (reveiwed in Sawyers et al. 1991). This virus immortalizes B cells in culture, yet does not lead to malignancy during human infection except in some geographic areas, where populations may harbor some other mutation. The latency of lymphoma formation after EB viral infection suggests that EBV may expand a cell population prone to subsequent oncogenic mutations (such as *c-myc* rearrangement by chromosomal translocation).

Another mechanism by which one one oncogene may effect the action of another is through cell differentiation as well as

apoptosis. Some oncogenes may act as inhibitors of differentiation. Other genes which stimulate growth may stimulate the expansion of these non-differentiated cells leading to tumors. For example, in erythroid leukemias, erb-B protein, which is a constitutively active form of the tyrosine kinase EGF-receptor, stimulates continuous growth while erb-A protein, a mutant form of the thyroxine receptor, a member of the steroid receptor superfamily, blocks differentiation. The avian erythroblastosis virus carries activated forms of both of these oncogenes (reviewed in Sawyers et al. 1991).

Another example of mutations which may affect subsequent mutational events is the activation of autocrine growth factor loops. In the case of *v-sis* (PDGF), overstimulation of the PDGF receptor, both extracellularly and intracellularly, may result in the subsequent mutation of other genes or the clonal expansion of a pre-existing mutation. Further, a mutation which acts analogously to phorbol esters in stimulating protein kinase C may act to stimulate further mutation or clonal expansion.

Overall, the cooperation between oncogenes is difficult to decipher. The development of an in vivo tumor probably involves many different mechanisms, which are likely to be cell- or tissue-type specific and is probably the reason why so many oncogenes/proteins have been found.

There are many tumor suppressor gene products which inhibit cellular growth, the loss or mutation of which is detrimental. Additionally, proto-oncogene products may stimulate the clonal expansion of pre-existing mutations, or

stimulate further mutation. Also, gene products exist which prevent apoptosis, that promote autocrine growth or promote paracrine growth, leading to transforming effects in other cells. Many of these genes would not fit the definition of an oncogene which must cause transformation. Yet in the proper situations, alterations in these genes may eventually lead to transformation. Thus it is important to utilize defined systems to define which category a gene and its product fits into in order to discriminate between the various contributing factors to carcinogenesis.

Model Systems For The Study Of Oncogenes.

The immortalized fibroblast is a system where genes and their proteins may be tested for their transforming ability. Immortalized fibroblasts grow similarly to primary fibroblasts in culture, displaying a morphological phenotype of anchorage dependence (Macpherson and Montaigner, 1964) and contact inhibition (density dependent) growth (Holley and Keirnan 1968). These immortalized cells can be transfected with oncogenic DNA to induce transformation. Additionally, some non-mutated cellular genes, when expressed in high quantity, can also be transforming (Chang et al. 1982). Upon transformation, these cells lose the phenotypic traits associated with the non-transformed state. The cells round up, form foci and grow in soft agar. The oncogenicity of a gene can thus be tested using these criteria for transformation (Shih et al. 1981). Additionally, transformed fibroblasts can be injected into athymic mice (nude mice). These

of a gene can thus be tested using these criteria for transformation (Shih et al. 1981). Additionally, transformed fibroblasts can be injected into athymic mice (nude mice). These mice do not reject such grafts and will grow tumors in response to transformed cells (Freedman and Shih 1974).

The immortalized fibroblast assay is thus an important model for determining the transforming action of a gene. The interplay of previously activated (or deactivated) genes or subsequent genetic alterations is difficult to interpret. However, the identification of these cooperating events will allow for an understanding of the unique functions of the various oncogenes and their contributions to cellular transformation.

Regulation of Mitogenesis By G Protein Dependent Pathways

Experimental evidence from several systems suggest that G proteins can act as oncogenic proteins (Landis et al. 1989, Lyons et al. 1990). The GTPase mutants isolated from tumors are shown in **Table 1**.

Gene	Amino Acid	Source of Tumor
c-*ras* (H,K,N)	Gly12, Gln61	wild type
H-ras	Gly12, Leu61	lung carcinoma
	Val12, Gln61	bladder carcinoma
K-ras	Val12, Gln61	lung carcinoma
	Gly12, Leu61	lung carcinoma colon carcinoma pancreatic carcinoma
N-ras	Gly12, Gln61	neuroblastoma lung carcinoma
G_s-α	Arg201*, Gln 227	wild type
gsp	His201, Gln227	pituitary adenoma
	Cys201, Gln227	pituitary adenoma
	Arg201, Arg227	pituitary adenoma
	Arg201, Leu227	pituitary adenoma
G_{i2}-α	Arg179, Gln205	wild type
gip	His179, Gln205	adrenal adenoma
	Cys179, Gln205	adrenal adenoma

*G_s-α Residue 201 is the site of ADP ribosylation by cholera toxin

	201											227										
α_s	D	L	L	R	C	R	V	L	T	S	(205)	F	D	V	G	G	Q	R	D	E	R	R (232)
$\alpha_{i(1-3)}$	-	V	-	-	T	-	-	K	-	T	(182)	-	-	-	-	-	-	-	-	S	-	- K (209)
α_t	-	V	-	-	S	-	-	K	-	T	(177)	-	-	-	-	-	-	-	-	S	-	- K (205)
α_o	-	I	-	-	T	-	-	K	-	T	(183)	-	-	-	-	-	-	-	-	S	-	- K (210)

By understanding the molecular regulation of G protein function, it is possible to identify sites by which the G protein could exist in a persistently activated state as a result of genetic mutation (Masters et al. 1989). A mutation that slows or blocks the intrinsic GTPase of the G protein will create a persistently

active species capable of unregulated effector enzyme stimulation. Single point mutations have been found to persistently activate the G_S α subunit and these point mutations have been found to be analogous to activating point mutations in the p21ras oncogene product: Gly49Val, Gly225Leu and Gln227Leu (the equivalents of Gly12, Gly59 and Gln61 in p21ras) serve to activate the G_S α subunit in the presence of GTP resulting in elevated levels of adenylyl cyclase activity and cAMP concentrations in cells transfected with these mutants (Graziano and Gilman 1988, Woon et al. 1989). The mutation Gly225Thr, which is activating in p21ras protein, does not serve to persistently activate G_S α subunit. Rather, this mutation is inhibitory to adenylyl cyclase. Mutation of Gly226Ala inhibits βγ subunit dissociation from G_S α subunit (Miller et al. 1988). Thus it is possible that, because of the heterotrimeric nature of G_S protein, position 226 or 225 may be inhibitory. p21ras protein is not regulated by heterotrimeric association and thus, does not have analogous residues.

The p21ras protein and G protein α subunits share a highly conserved structure in the GTP binding/hydrolysis regions (Hurley et al. 1984). The mutations that activate p21ras proteins alter its interaction with guanine nucleotides. All the mutations studied in p21ras either decrease the GTPase of the p21ras protein or increase the rate of exchange of bound GDP for GTP. The alterations found in the p21ras protein isolated from cancer cells cause the protein to possess a decreased k_{cat} for its GTPase, resulting in a protein "locked" in a persistently activated state (Gibbs et al. 1984, Manne et al. 1985). The mutations observed in

the *ras* oncogene are conserved over a large sample of tumor types in both "spontaneous" and chemically induced tumors (Sigal et al. 1986). These mutations occur as single point mutations in regions encoding the guanine nucleotide binding region of the protein. The most common mutations found in *ras* oncogenes are Gly12Val, Ala59Thr and Gln61Leu. These mutations serve to lower the k_{cat} of the p21ras protein GTPase such that the protein is no longer regulated by the GTPase activating protein (GAP). As stated above some of these homologous mutations induce persistent activation of G_s-α subunit. Similar alterations have been found in the genes coding for G proteins isolated from various human endocrine tumors (Vallar et al. 1987, Clementi et al. 1990). These mutations suggest that the mutated G proteins may also remain in a persistently activated state due to a slower k_{cat} for their intrinsic GTPase. It might be expected that similar mutations in other G protein α subunits may induce persistent activation of the effector with which the G protein normally interacts. A mutant α_{i2}, with reduced GTPase, transforms Rat-1 cells in culture upon cDNA transfection (Pace et al. 1991). Hence, it is possible that similar mutations in the G_o protein will inhibit its GTPase activity and persistently activate phospholipase C and consequently stimulate cell growth. These residues, along with similar flanking regions, are conserved in the α_o subunit.

G_o as a signal transducer in Xenopus Oocyte Phospholipase C Pathways.

In addition to its use as model for cellular growth regulation, the Xenopus oocyte is an important assay system for the study of hormone stimulated second messenger production by the phospholipase C pathway (Dascal 1987). Additionally, the size of the oocyte makes it an excellent site in which to express exogenous RNA transcripts (Gurdon et al. 1971, Lane et al. 1971). The oocyte can be used to express various receptor proteins and their activation can be measured by the indirect measurement of phospholipase C activity. The oocyte has been extensively used because of its Ca^{2+} activated Cl^- conductance that is evoked by receptor agonists. Receptor stimulation of phospholipase C produces inositol trisphosphate that induces intracellular release of Ca^{2+}. This Ca^{2+} opens the Cl^- channels, resulting in an inward Cl^- flux. This channel is sensitive to inositol trisphosphate which liberates intracellular calcium. Thus, the oocyte can be used as a system in which to study hormonal signalling systems linked to phosphoinositide hydrolysis (Moriarty and Landau 1990).

Oocytes are typically removed surgically from female toad ovaries. The oocytes are detached from the surrounding follicular cells by collagenase treatment and surgical manipulation. There appears to be heterogeneity among the intact muscarinic response in isolated Xenopus oocytes. After collagenase treatment, some Xenopus oocytes, from some frogs, display native muscarinic receptor invoked Ca^{2+} release after collagenase treatment (Kusana et al. 1977). This muscarinic receptor responds to agonists by

promoting the release of intracellular Ca^{2+}. IP_3 can induce this Ca^{2+} rise and pre-incubation with IP_3 abolishes the muscarinic response, suggesting that the Xenopus oocyte muscarinic receptor is coupled to phosphoinositide hydrolysis. On the basis of antagonist binding, it was found that the oocyte muscarinic receptor is of the M3 subtype, with an antagonist potency of 4-DAMP>pirenzipine>AF-DX 16 (VanWezenbeck et al. 1988). The muscarinic receptor induced Cl^- current in the oocyte is pertussis toxin sensitive (Moriarty et al. 1989). This receptor has has been shown to couple to phosphoinositide hydrolysis (Peralta et al. (1987).

By use of the IP_3 dependent, Ca^{2+} activated Cl^- current as an assay system, It was found that the bovine brain G_o protein can function as a signal transducer in the muscarinic receptor regulated phosphoinositide pathway of Xenopus oocytes (Moriarty et al. 1990). A cDNA encoding an α_o-type protein has also been cloned from the Xenopus oocyte, suggesting that a G_o- like protein may be involved in the native pathway.

Many growth factors and oncogenes that activate entry into the G1 phase of the cell cycle alter phosphoinositide metabolism. Hence it appeared feasible that activated G_o could regulate growth. Because of the possible function of this protein in coupling receptor stimulation to protein kinase C activation, a genetic lesion that results in a mutated form of the G protein that is continuously active may have effects on growth control.

Statement of Problem

The objective of these studies is to define the growth promoting potential of a persistently activated G protein. At the time these studies were initiated (summer, 1988), no demonstration of cellular growth control being affected by a persistently activated G protein had been reported. Although mutated G proteins (G_s and G_i) had been identified in various tumors, it was not clear whether these mutated proteins had a causal role in tumor formation. Although the G_o protein had been implicated in phospholipase C coupling in the Xenopus oocytes, no evidence for G_o affecting cellular growth had been obtained. Since phosphoinositide hydrolysis is activated by many mitogenic hormone receptors, it appeared feasible that G_o protein could regulate cellular growth.

The Xenopus oocyte has been used as an assay system to measure cell cycle resumption. Agents that trigger oocyte maturation are often growth promoting factors. We used the oocyte to measure the growth promoting effects of the G_o protein. The oocyte can also be used to examine the downstream effects of G_o protein mediated cell cycle resumption. Hence, protein kinase C and $pp39^{mos}$ activation by G_o in oocytes was studied.

In order to determine whether the growth promoting effects of G_o were unique to the oocyte, or generally applicable to mammalian cells, an activating mutation was introduced into G_o-α subunit cDNA and transfected into NIH-3T3 cells. The effect of

this activating mutation was studied, in terms of mitogenesis and transformation, in the NIH-3T3 cell system.

The resting state of the G protein is inactive in terms of effector enzyme stimulation. Thus, the G protein must be altered in order to "lock" the protein into a persistently activated state. Furthermore, this activated protein (or gene encoding it) must be introduced into a system in which growth control can be measured. These problems can be overcome by taking advantage of G protein purification and activation with non-hydrolyzable GTP analogs, or site-directed in-vitro mutagenesis of the G protein cDNA at a site which will decrease intrinsic GTPase activity. Reconstitution of this altered G protein can be performed using either the activated protein, by injection into Xenopus oocytes, or using the altered cDNA, by transfection into NIH-3T3 fibroblasts. The maturation of Xenopus oocytes or the stimulation of mitogenesis and the subsequent transformation of NIH-3T3 fibroblasts, as well as the tumor formation in athymic mice, is indicative of alterations in growth control. Although within the NIH-3T3 fibroblast assay the other events which may be contributory to cell growth or transformation are difficult to elucidate, the Xenopus oocyte represents a useful system to test the possible downstream effects of persistent G protein activation. Hence, some of the downstream elements involved in the G_o signalling pathway were analyzed in Xenopus oocytes.

Methods and Experimental Procedures

Materials

Female Xenopus Laevis were purchased from NASCO (Fort Atkinson, WI). A peptide containing residues 19-36 of protein kinase C [PKC(19-36)] was from Bachem and the protein kinase C assay kit was from Amersham. Oligonucleotides were synethesized at the Brookdale Oligonucleotide Synthesis Facility of the Mount Sinai School of Medicine. G_o was purified from bovine brain by Drs. Carty and Padrell. The rat heart a_o-cDNA was obtained from Drs. Codina and Birnbaumer. NIH-3T3 stock cells were from the University of California at San Francisco Cell Culture Facility. Rat-1 fibroblasts were obtained from Dr Alan Frey, NYU Medical School. *H-ras* was the kind gift of Dr Jay Gibbs (Merck Research Labs). Cell culture reagents were obtained from Hyclone. Geneticin (G418) was from Gibco. *Nu/Nu* mice were obtained from the National Cancer Institute. pMN (pMAM-neo) vector was obtained from Clontech and pRC/CMV vector from Invitrogen.

Activation of G protein α-subunits

G_o used for my experiments was purified from bovine brain by either Drs. Elena Padrell or Donna Carty of this laboratory. This purified protein consists of the heterotrimeric complex (α,β,γ subunits). The α subunit retains GDP bound to its guanine nucleotide binding domain. Incubation of G proteins with high Mg^{2+} concentrations and GTP promotes the release of the bound

GDP and the binding of GTP. This GTP is subsequently hydrolyzed to GDP as described above. Using the non-hydrolyzable GTP analog, GTPγS, the G protein can be locked into a persistently activated state. The binding of GTP promotes subunit dissociation, so that incubation of holo-G protein with Mg^{2+} and GTPγS allows for the resolution of the α subunit from the βγ duplex. βγ subunits were purified from human erythrocytes by Drs. Padrell and Carty (Codina et al. 1984)

For a typical G_o protein activation, a purified solution of holo-G_o (~ 30 μg) was added to 25 mM Hepes-Na, pH 7.5, 1 mM EDTA, 20 mM β-mercaptoethanol, 30 % v/v ethylene glycol, 5 mM $MgCl_2$, 2 μM GTPγS and ~2 x 10^6 cpm of [^{35}S]GTPγS (specific activity = ~5000 cpm/pmol). This mixture is incubated at room temperature for 60 minutes. The incubation is then loaded on a hydroxylapatite chromatography column at 4°C and washed overnight with 10 mM $MgCl_2$, 20 mM Hepes-Na pH 7.4, 100 mM NaCl, 20 mM β-mercaptoethanol. The α subunit is eluted from the column in low (~50 mM) phosphate solution containing 10 mM $MgCl_2$, 20 mM Hepes-Na pH 7.4, 100 mM NaCl, 20 mM β-mercaptoethanol. The peak fractions, as assessed by [^{35}S] radioactivity, were pooled. This sample was concentrated and diluted on a centricon spin column to remove excess unbound GTPγS. The eluted protein is concentrated and diluted by centrifugation to exchange its buffer. The final dilution buffer contained 10 mM β-mercaptoethanol, 100 mM NaCl, 2 mM $MgCl_2$, 25 mM HEPES pH 7.5. The GTPγS activated form of the G_o-α subunit is referred to as α_o*.

Oocyte Extraction

Oocytes were surgically removed from Xenopus laevis ovary under tricaine anesthesia. All wounds were sutured and the animals were reused after one month. Defolliculation of oocytes was carried out by slowly shaking the oocytes in a solution containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5, 2.5 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml collagenase. The collagenase enzyme was inactivated by transferring the oocytes to a similar bathing medium containing 1.8 mM $CaCl_2$ with no collagenase. Stage VI oocytes were selected and additional follicular cells were peeled away with jeweler's forceps. Microinjection experiments were performed at least 24 hours after surgical extraction.

Oocyte Maturation: Intracellular Microinjection Studies

All oocytes were microinjected in the equatorial region between the animal and vegital poles. The appearance of a white spot in the animal pole was characteristic of GVBD. Oocytes that aberrantly displayed white spots or matured early were not removed. To verify maturation, the oocytes were fixed in 10% trichloroacetic acid, sliced and visualized under a low power (10X) dissecting microscope. α subunits were typically injected in 50 nl to yield a final concentration of 3 nM, assuming a 20 fold dilution into the oocyte. Final concentration of βγ dimer was 20 nM. For coinjecion of α and βγ subunits, the subunits were mixed together

before injection. Progesterone induction of maturation was carried out by incubating the oocytes in bathing media containing 10 μM progesterone.

The ras protein used in these studies was derived from *H-ras*, with a valine substitution for Gly12. For ras protein induced maturation, the oocytes were microinjected with 10 nanograms ras protein per oocyte.

For experiments that analyzed the requirement for protein kinase C activity for oocyte maturation, a peptide corresponding to Xenopus oocyte protein kinase C amino acids 19-36 (RFARKGSLRQKNVHEVKN), except that serine 25 was replaced by alanine was used. The peptide PKC(19-36) was injected into the oocyte at a final concentration of 1 μM, either as a coinjection with α subunit or immediately prior to progesterone addition to the bath. Peptide was stored as a stock solution of 1 mM in distilled water.

For cycloheximide treatment of oocytes, the cells were placed in 100 μg/ml cycloheximide immediately prior to microinjection or progesterone addition.

For all oocyte microinjection experiments performed, fifteen to thirty oocytes were used per group.

Chromosome Condensation

To visualize condensed chromosomes, oocytes were incubated in 10 mM progesterone or microinjected with α_o^* (3 nM final concentration). After 16 hours, the matured oocytes were fixed in 10% formaldehyde solution containing 60 mM Hepes-Na

pH 7.4, for two hours. The DNA chromophore Hoechst 33258 was added (5 μg/ml final concentration) and the oocytes were stained for two hours. The white spot in the animal pole was dissected with a jeweler's forceps in a solution containing 100 mM Tris-HCl pH 7.5, 2% n-propyl gallate and 10% ethanol. The patch was mounted on a slide and the condensed chromosomes were visualized by flourescent epi-illumination (Gerhart et al. 1984).

In-Vitro Assay Of Protein Kinase C Activity

For the measurement of protein kinase C activity, 50 nl of vehicle or α_o* were injected into the oocyte (3nM final concentration). Fifteen minutes after the injection, groups of twenty oocytes were homogenized in a medium containing 50 mM Tris-HCl, 5 mM EDTA, 10 mM EGTA, 0.3% β-mercaptoethanol, 1 mM phenylmethylsulfonyl flouride (freshly prepared), 10 mM benzamidine and 5 μg/ml leupeptin, pH 7.5. Typically each oocyte was homogenized in 17 vol (17 μl) of homogenization medium. 25 μl of pooled homogenates were assayed for protein kinase activity. The assay was performed essentially as described in the manual provided with the Amersham protein kinase C enzyme assay system (code RPN77). This kit uses the pseudosubstrate peptide, except Ala-25 is substituted with serine. This makes the peptide a very specific substrate for the protein kinase C enzyme. The assay mixture contained 50 mM Tris-HCl, 1mM calcium acetate, 15 mM magnesium acetate, 50 μM [γ-^{32}P]ATP (specific activity = 3000 cpm/pmol), 2.5 mM DTT and 75 μM peptide

substrate in a final volume of 75 µl. For stimulation of enzyme activity during the assay, 0.7 mole% phosphatidylserine and 2 µg/ml phorbol-12-myristate-13-acetate was used. The enzyme activity was measured for 15 minutes at 22-24°C (RT). At the end of the reaction, the enzyme activity was stopped by acidification. The reaction mixture was spotted to peptide binding paper. The paper was washed in 5% acetic acid and counted in a liquid scintillation counter.

Mos Protein Antisense Experiments

To assay for mos protein translation, specific sense and antisense oligonucleotides were synthesized corresponding to the mos sequence by the Brookdale Oligonucleotide Synthesis Facility, Mount Sinai School of Medicine. Oligonucleotides were purified after synthesis by sodium acetate/ethanol precipitation. The final pellets were resuspended in 100 mM KCl to achieve a concentration of 4 mg/ml. When mixtures of oligonucleotides were used, the final concentration of the individual oligonucleotides was 2 mg/ml. 50 nl of this solution was injected per oocyte. ASM1 and ASM2 correspond to antisense oligonucleotides F^- and G^- in the article by Sagata et al., 1988. ASM1 is an oligonucleotide complementary to nucleotides +178 to +198 in the coding region of c-*mos* . ASM2 is complementary to nucleotides -21 to +6 and spans the ATG initiation codon. MOS1 and MOS2 are the oligonucleotides encoding the corresponding sense sequence in the *mos*-mRNA. Oligonucleotides were

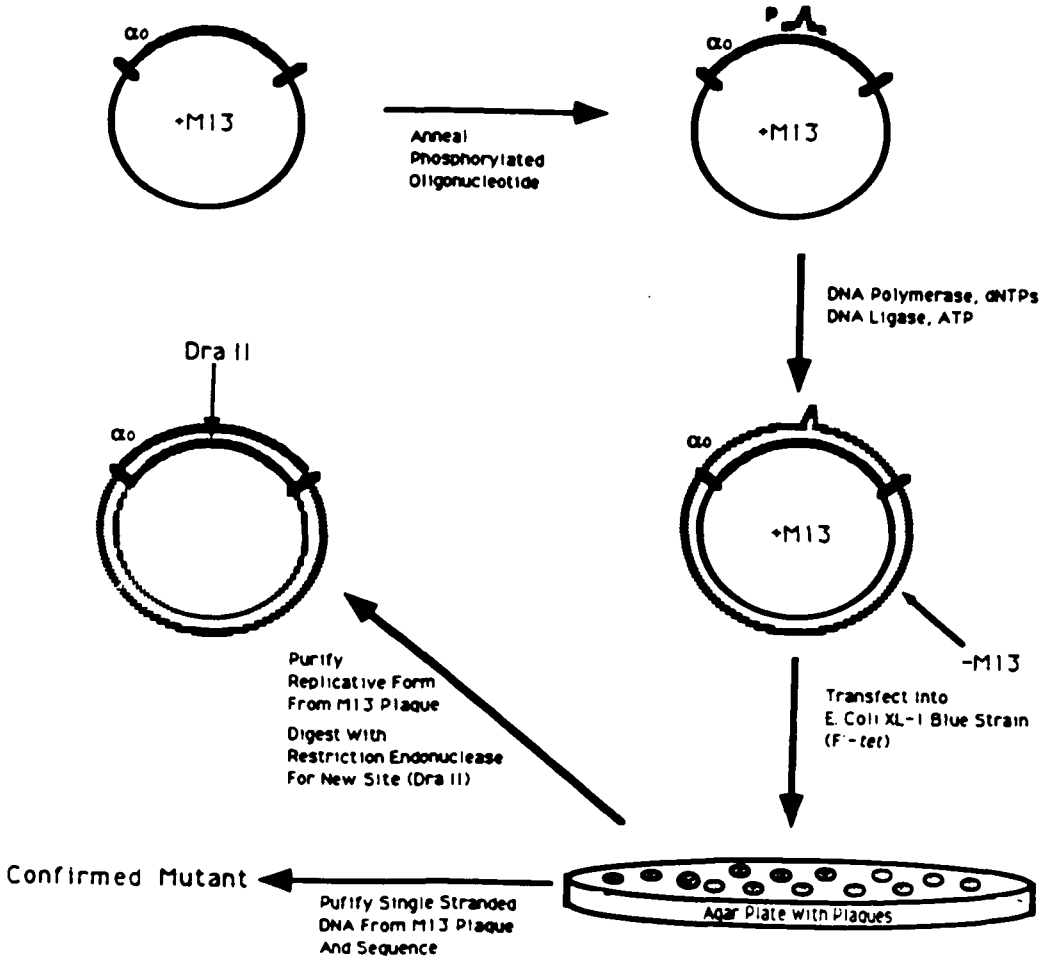
microinjected four hours prior to progesterone treatment or α_0* injection.

Mos Protein Immunoprecipitation

Oocytes were incubated for 4 hrs in bathing medium with 25 mCi ^{32}P. Typically, one hundred oocytes were incubated in 5 ml of radioactive medium. After four hours, the groups of 25 oocytes were injected with either vehicle, α_0*, or α_0*+ PKC(19-36) peptide. After six hours the oocytes were homogenized in buffer (100 µl per oocyte) (Watanabe et al. 1989). The pp39*mos* protein was immunoprecipitated with the S5 monoclonal antibody raised against Xenopus *mos* protein expressed in *E. Coli*. (Sagata et al. 1988). For this procedure the clarified lysates (2.5 ml) were incubated with 25 µl of the antibody for 16 hr at 4° C. 300 µl of 1:3 slurry of protein A Sepharose was added and the incubation was continued for another 16 hr at 4°. The protein A Sepharose was then washed repeatedly and the bound protein extracted in 2% SDS and 10% β-mercaptoethanol. Aliquots of these extracts, normalized with respect to total number of counts in each oocyte homogenate prior to the start of the immunoprecipitation, were resolved by SDS-PAGE and visualized by autoradiography. Since the α_0*-injected lanes have a much higher background, contact positives with varying exposure times were made of the autoradiograms. The progesterone, vehicle-injected, and α_0* + PKC(19-36)-injected lanes had a 20 sec exposures, while the α_0*-injected lane had a 90 sec exposure to obtain contact positives with Kodak EDF film.

In Vitro Mutagenesis Of α_0 cDNA

Genetic lesions can be reproduced experimentally by utilizing the techniques of molecular biology. Many experiments described within this thesis involve the manipulation of DNA by mutation, recombination and cellular transfection. For the creation of specific mutations within a particular gene, the method of site-directed in-vitro mutagenesis has emerged as a powerful means of creating defined genetic alterations (Messing et al. 1977) as shown in **Figure 6**.



This system uses a filamentous phage, which is a virus particle, capable of infecting bacterial cells. The phage particle consists of a single-stranded DNA circle surrounded by a protein coat. The phage infects E. Coli bacteria by absorbing to the F' pillus, which is a mating factor for bacterial conjugation. Upon infection, the single stranded DNA is replicated in a double stranded form within the cell. After a critical limit is reached, the bacterial cell replicates only the single stranded DNA, which it packages into a protein coat and secretes into the culture medium. The M13 genome consists of genes for the various proteins of the viral coat, the proteins necessary for attachment and entry to the bacterial pillus as well as a nicking protein which cleaves the single stranded DNA at its origin of replication. The host bacteria provides the enzymes necessary for replication, including DNA and RNA polymerases.

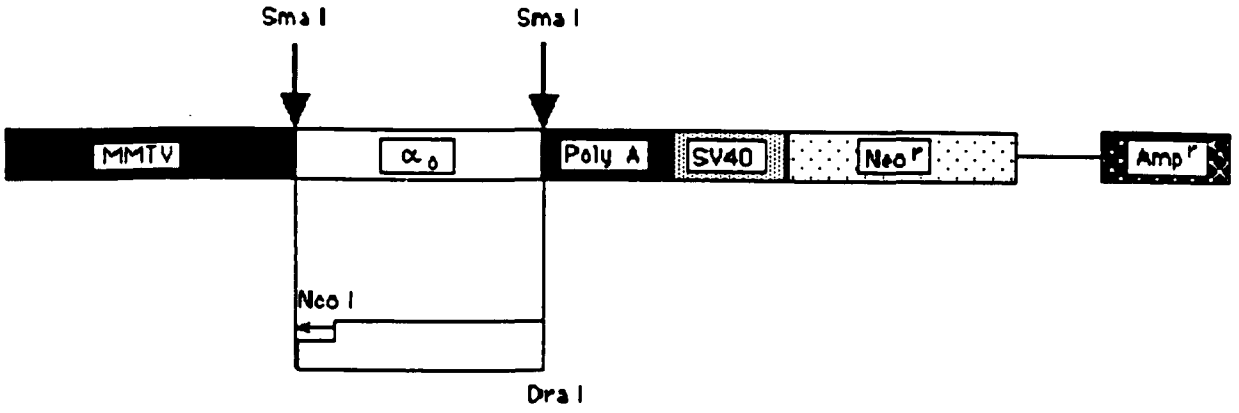
The advantage of the M13 phage system comes from its ability to replicate as both single stranded and double stranded DNA, and unlike other viral phages, its ability not to kill the host bacteria. The M13 genome has been engineered to create a multiple cloning site in which a foreign gene may be cloned (Yanisch-Peron et al. 1985). Insertion of an exogenous gene disrupts the beta-galactosidase gene, which is integrated into the M13 genome (Miller 1978). This allows the selection of recombinant clones by plating M13 infected bacteria on a lawn containing an inducer of the beta-galactosidase gene (isopropyl-1-thio-β-D-galactoside) and a substrate for the beta-galactosidase enzyme (5-bromo-4-chloro-3-indoyl-β-D-galactoside).

Subcloning of the desired gene for mutation into the M13 cloning site and transfection into bacterial cells allows the purification of single stranded M13 DNA containing the exogenous gene. An oligonucleotide can be synthesized which is complementary to a portion of the inserted gene. The oligonucleotide, which contains a mismatch at the desired site of mutation is annealed onto the M13 recombinant DNA. By adding purified DNA polymerase, deoxynucleotides and DNA ligase, the second strand can be completed. This double stranded DNA containing a defined mismatch is introduced back into E. Coli and infected bacteria. Since M13 can replicate from both strands (although this is not the way in which the normal virus infects cells), cells can be selected and analyzed for the presense of the wild-type or mutant sequences. The purification of single stranded DNA is used for dideoxy DNA sequencing to detect the presence of a mutation. Additionally, the creation or destruction of a sequence that is recognized by a restriction enzyme allows rapid screening of many samples of putative mutants. When a bacterial cell is found to contain the mutant gene, the double stranded DNA is purified, the DNA is excised and ligated into other vectors for eukaryotic transfection, in vitro transcription or other uses.

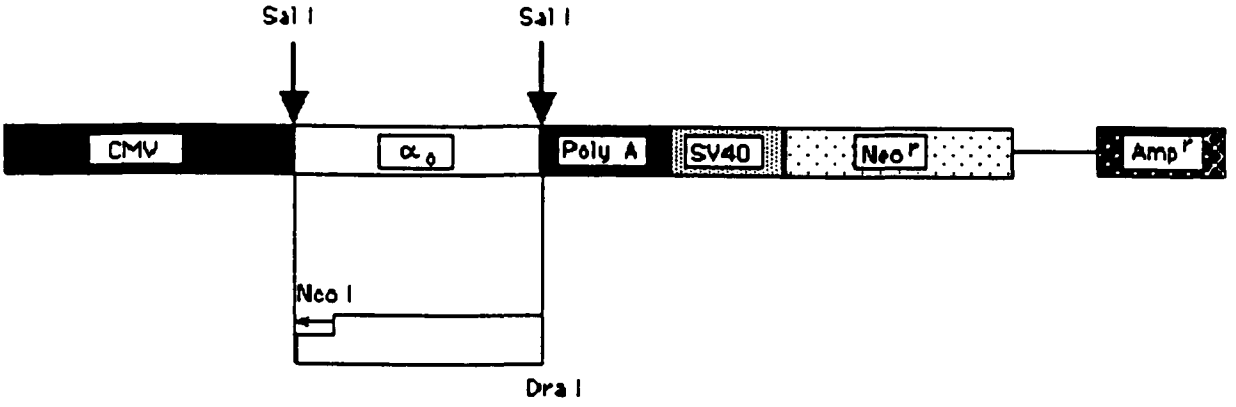
Insertion Into Mammalian Transfection Vectors

The method of insertion of wild type α_o and α_o Q205L is shown in **Figure 7.**

pMAM



pRC/CMV



The wild type α_o and α_o Q205L DNA were excised from M13mp19 using the restriction enzymes NcoI and DraI. These enzymes cut the insert out of the vector in its entirety. The "sticky ends" of the

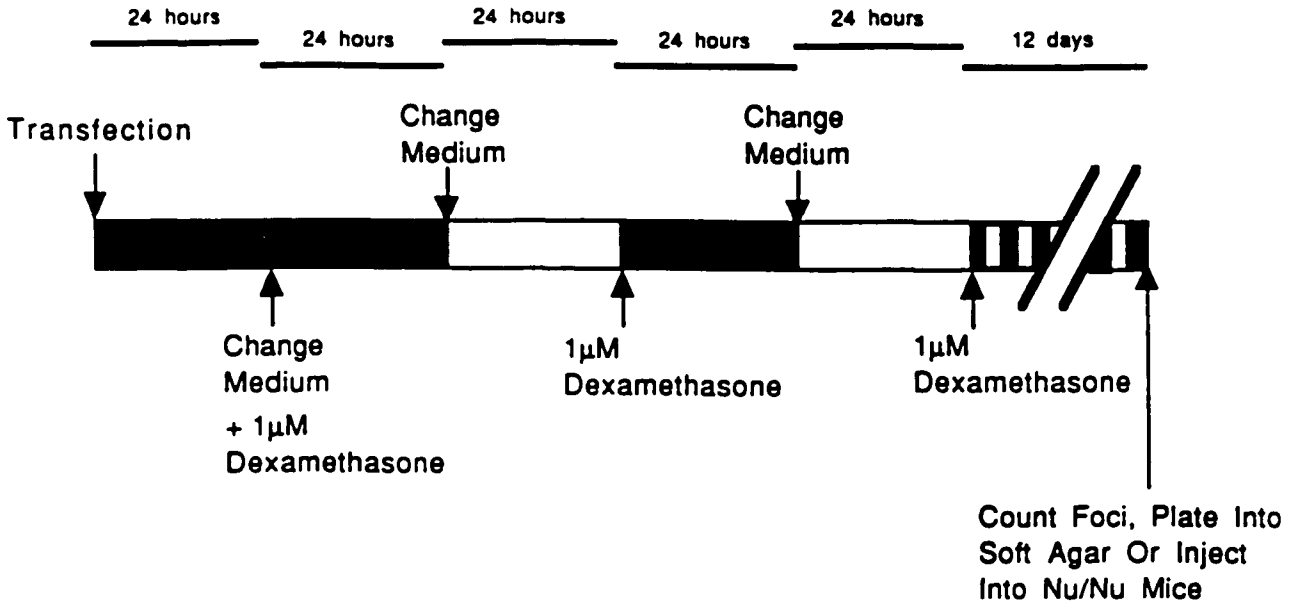
insert were filled in with deoxyribonucleotide and Klenow fragment. The DNA was then subcloned into the Sma I restriction site of pMAM-neo vector. This vector was purchased from Clontech, Inc. H-ras DNA was also subcloned into this vector. Proper orientation of the inserts was confirmed by restriction mapping.

The wild type α_o and α_o-Q205L DNA was also inserted into the constitutive expression vector pRC/CMV. The wild type α_o and α_o Q205L DNA was excised from M13mp19 using the restriction enzymes NcoI and DraI. The ends were filled in with Klenow fragment and deoxynucleotides. The constructs were then inserted into the Sal I site of pRC/CMV. This vector contains a cytomegalovirus promoter upstream from a multiple cloning site and a bovine growth hormone poly A sequence.

Transfection Of Fibroblasts

For eukaryotic transfection, alkaline lysis minipreps were made of the individual pMAM-neo constructs as well as pMAM containing no insert. The DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol/sodium acetate twice. The DNA was resuspended in sterile water and precipitated with 2.5 mM calcium phosphate (Sambrook et al. 1989). This DNA (20 µg per transfection) was added to sub-confluent NIH-3T3 or Rat-1 fibroblasts. After 24 hours, the media was changed. When transfection efficiency was being measured, cells resistant to G418 were selected for by adding geneticin at a final concentration of 400 µg/ml. The

transfection and subsequent feeding/dexamethasone treatment protocol is shown in **Figure 8**.



Selection Of Cells For Neomycin Resistance

Positive selection of transfected eukaryotic cells can be acheived by transfecting a gene for a selectable marker enzyme. This gene can either be co-transfected as a seperate plasmid, or as an integrated sequence in the transfected construct. One of the most commonly used eukaryotic selection markers is that of neomycin resistance. The drug geneticin (G418 sulfate) is an aminoglycoside antibiotic similar to gentamycin which is toxic in

high doses to eukaryotic cells (bacterial potency = ~500 μg/ml ED_{50} in cultured mammalian cells = ~2200 μg/ml). The resistance gene is an aminoglycoside phosphotransferase. It is bacterial in origin but can be expressed in eukaryotic cells. Transcription of the resistance gene can be achieved when it is linked to eukaryotic DNA sequences that permit mRNA synthesis in host cells.

Transfection efficiency was characterized by ability to survive selection in G418 (geneticin) at a concentration of 400 μg/ml (effective concentration). Cells were plated at 100,000 cells per plate and treated with G418 for 10 days. After this period, the number of surviving colonies were counted and compared to the original number of cells.

Protein Concentration Determination

Protein concentrations were determined by the Lowry method (Lowry et al. 1951). 100 ml of 1N NaOH, 1.8% SDS were added to samples and vortexed. 900 μl of 2% Na_2CO_3, 0.01% $CuSO_4$, 0.02% Na-K-tartrate was added to the mixture and vortexed. After 15 minutes, 100 μl of 33% Folin-Coulcateau was added and the color was allowed to develop for 30 minutes. Absorbance was read at 660 nanometers and compared to a standard curve of BSA concentrations.

PCR Based mRNA Detection

The pMN constructs (pMN, pMN-α_o, pMN-Q205Lα_o and pMN-ras) were purified and transfected by calcium phosphate

precipitation into sub-confluent fibroblasts (10^6 cells per bottle). Twenty four hours after transfection, the cells were selected for DNA incorporation with G418 (0.4 g/l). After the selection, the media was changed and the cells were allowed to grow for 2-3 days to confluency in DME containing 5% bovine calf serum. After 16h treatment with 1 μM dexamethasone, the cells were harvested and total RNA was prepared. cDNA was prepared using Abelson murine leukemia virus reverse transcriptase. PCR amplification of this cDNA was carried out using PCR primers 5'-GGGGAAGCTTACIATAGTIAARCARATGAA (corresponding to nucleotides 144-160 of the α_o cDNA) and 5'GGGGGAATTCRTTCGTGGTTTCRTCYTCRTG (corresponding to nucleotides 711-726 of the α_o cDNA). These regions encode sequences unique to G_o-α and these oligonucleotides were designed to amplify G_o-α like cDNA from different tissues. The PCR reaction was carried out using TAQ DNA polymerase for 30 cycles with 95°C melting temperature, 50°C annealing temperature and 72°C extension temperature. Dra II enzyme digestion was carried out directly on the PCR amplified DNA in the appropriate enzyme buffer for two hours at 37°C. DNA was electrophoretically resolved on a 1.5% agarose gel.

Immunological Detection of α_o

For detection of α_o, the transfected cells were selected with 0.4 g/l G418 for 10 days and treated with 1 μM dexamethasone for 16h on alternate days. After three such treatments, the cells

were washed with phosphate buffered saline and purified plasma membranes were prepared by sucrose density gradient ultracentrifugation. For immunoblotting experiments, 100 μg of purified membranes were resolved by SDS electrophorhesis, transferred to nitrocellulose, and probed with a 1:1000 dilution of α_o-specific antiserum. The secondary antibody (1:3000) is conjugated to horseradish peroxidase. The immunoreactive bands are detected by use of the ECL reagent and hyperfilm. The α_o-specific antiserum was generated in a rabbit by immunization with a peptide encoding the sequence used for U-46 , an α_o specific antisera previously generated (Mumby et al. 1986).

Mitogenesis Assays

To analyze cellular rate of growth, cells were transfected with cDNA by calcium phosphate transfection for 2 days. The cells were treated for one day with 1 μM dexamethasone. Cells were dispersed and plated at a concentration of 1 x 10^4 cells per well in a Falcon six well dish. Cells were cultured in DMEM plus 5% calf serum. Alternating dexamethasone/feeding protocol was used as above. The cells in one well were dispersed in trypsin each day and counted in a hemocytometer. For G418 (Geneticin) inhibition of phospholipase activity, cells were incubated in 100 μM G418 antibiotic. Growth kinetics were determined by fitting the data to an exponential growth equation:

$$y=y_o(e^{kt})$$

where y_0=initial cell number, k=growth rate constant and t=time. Doubling time was calculated by dividing the natural logarithm of 2 by the growth rate constant.

Focus Assays

For focus formation assays, transfected cells were allowed to grow to confluency and split with trypsin/EDTA into four duplicate plates. The cells were grown for two weeks and then counted for foci formation under a medium power (100X) microscope.

Soft Agar Colony Formation

To test the ability of cells to grow in soft agar, thereby displaying anchorage independence, the following procedure was utilized: 5 ml of molten 1% agarose was poured into a tissue culture dish and allowed to solidify. Transfected cells were lifted with trypsin/EDTA as above and counted. The cells were plated at a concentration of 10,000 cells per plate in 1X DMEM media, 5% calf serum and 0.56% agarose. The cells were fed once a week with 1X DMEM media, 5% calf serum and 0.56% agarose. Colony formation was counted after fourteen days.

Tumorigenicity In Athymic Mice

For animal injection assays, transfected cells were selected for G418 resistance as above and grown in bottles for three weeks with similar alternating feeding/dexamethasome schedules. Cells were lifted from plates with trypsin EDTA and counted. Cells

were washed with DMEM media without serum and 1 x 10^6 cells in 0.1 ml were injected into each animal subcutaneously. The animals were in groups of five and duplicates were performed for wild type α_o , Q205Lα_o, *H-ras* and vector alone transfected cells.

Results

Persistently Activated α_o* Induces Oocyte Maturation

The heterotrimeric GTP binding protein G_o was purified from bovine brain and activated with Mg^{2+} ions and GTPγS. Activated α_o subunit was purified and injected into oocytes. The oocytes were observed for germinal vesicle breakdown (GVBD) which manifests itself as a white spot in the dark animal pole that can be seen under a dissecting microscope. Activated G_o-α (α_o*), induced GVBD (Figure 9) like progesterone. Background response in vehicle injected eggs may be due to β-mercaptoethanol which causes GVBD (Smith 1989). Since G protein α–subunits are labile without sulfhydryl group reducing agents, vehicle was not altered.

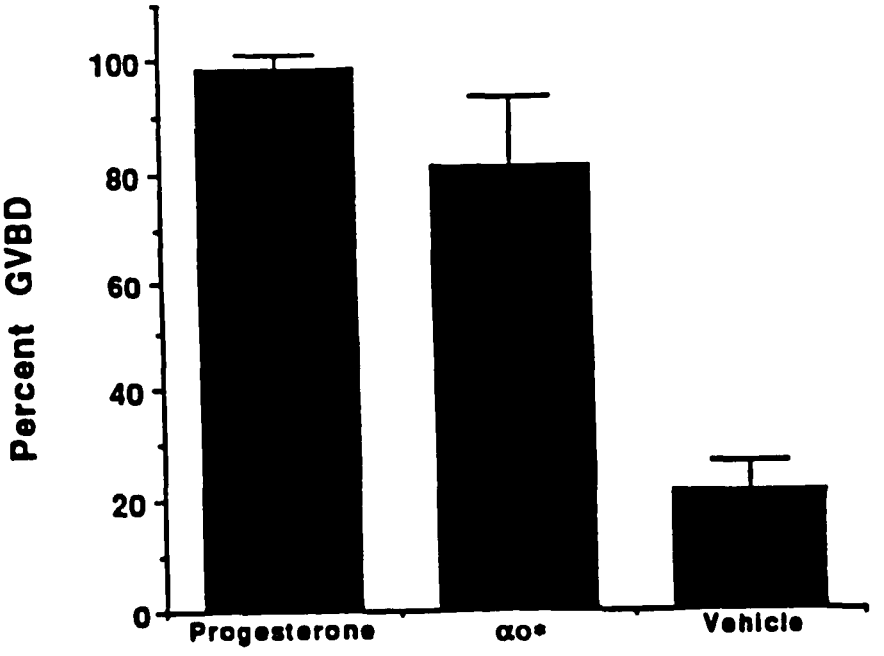
Activated G_i2-α subunits (Figure 10) did not induce substantial GVBD. Activated G_i1-α and G_i3-α subunits did not appear to induce GVBD (Figure 10). Heterotrimeric G_o (holo-G_o) protein also did not induce GVBD (Figure 11). Although α_o* induced maturation was observed to be slower than that induced by progesterone (Figure 12), temporal differences may not be biologically significant since the time course of progesterone induction is variable.

After the initial experiments in oocytes had been carried out, a new G protein from bovine brain, G_q, which is capable of stimulating phospholipase C, was identified. This led me to determine if G_o-α was the active element in activated α_o preparations. In order to ascertain this, I immunodepleted the preparation of G_o-α subunit with a G_o-α subunit specific antibody (against the carboxy terminus decapeptide of G_o-α, ANNLRGCGLY).

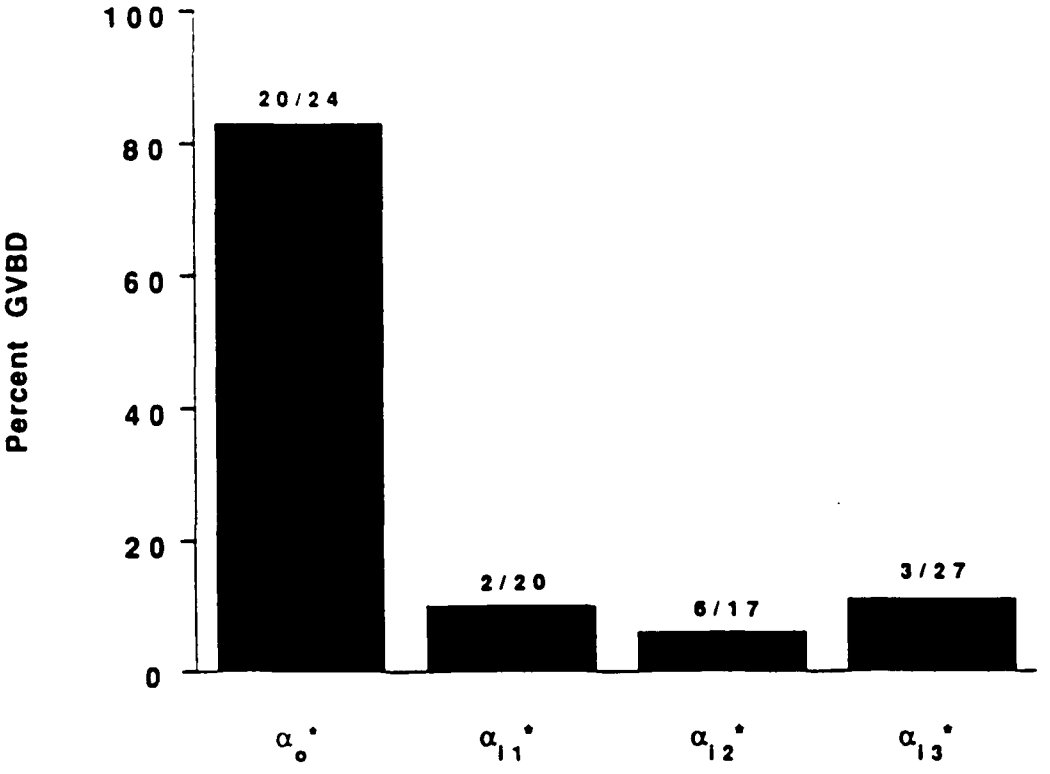
As a control for this experiment, the antibody was pre-blocked with the specific peptide to which it was raised. Immunodepletion of G_o-α subunit demonstrated a marked reduction of oocyte maturation capability (Figure 13) and indicates that the maturation effect is specific for the G_o-α subunit. The activated α_o effect was concentration dependent. Half maximal effects were observed at about 150 pM assuming α_o* equilibrates fully within the cell (Figure 14).

Prolonged exposure to muscarinic receptor agonists did not induce maturation (observations of Dr. Gila Omri). This could be due to desensitization of the receptor signal. In contrast, when G_o is activated with GTPγS, the Go-α subunit should be persistently activated. Hence, I tested the effect of co-injected G protein $\beta\gamma$ subunits which preferentially associate with the GDP bound form of α_o and should not affect α_o*. Coinjection of $\beta\gamma$ subunits did not affect α_o* induced GVBD (Figure 15). This observation indicates that the GTPγS liganded G_o-α stays persistently activated in the oocyte.

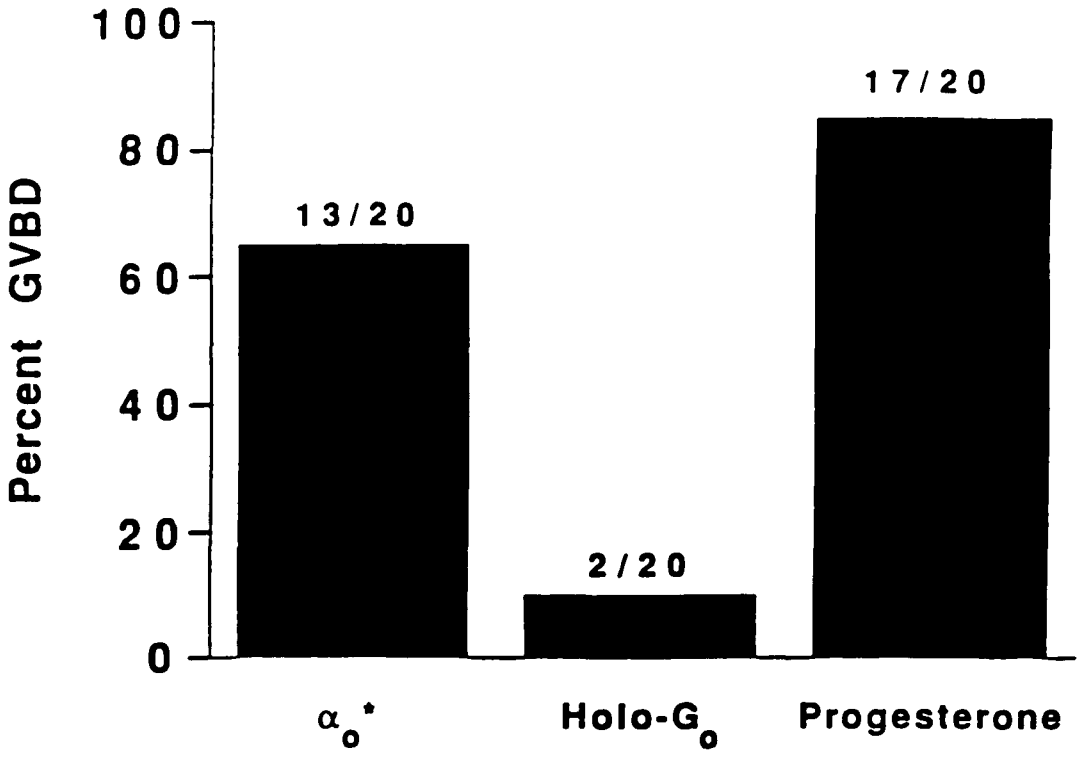
To ascertain that α_o* injected oocytes had completed the first meiotic cycle, I looked for condensed and clustered chromosomes. Oocytes that had undergone GVBD were fixed in 10% formaldehyde, stained with Hoechst dye 33258 and dissected. Chromosomes were visualized by fluorescent epi-illumination. Injection of α_o* resulted in chromosome condensation similar to that seen in progesterone treated oocytes (compare Figures 16 A and B), indicative of the completion of at least meiosis I.



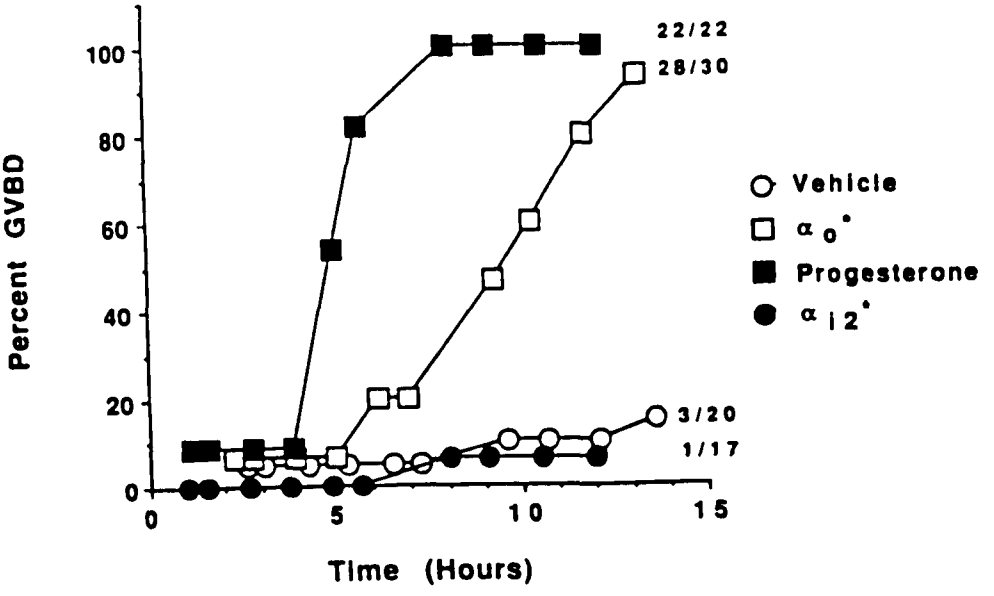
9. The induction of Xenopus oocyte maturation by activated G_o-α subunit. Oocyte Maturation was assessed by germinal vesicle breakdown (GVBD) sixteen hours after progesterone incubation (10 μM final concentration), α_o* microinjection (3 nM final intracellular concentration) or vehicle microinjection. α_o* induced GVBD was measured at the earliest time when progesterone induced GVBD was complete (~100%). (N=10 +/- S.D.).



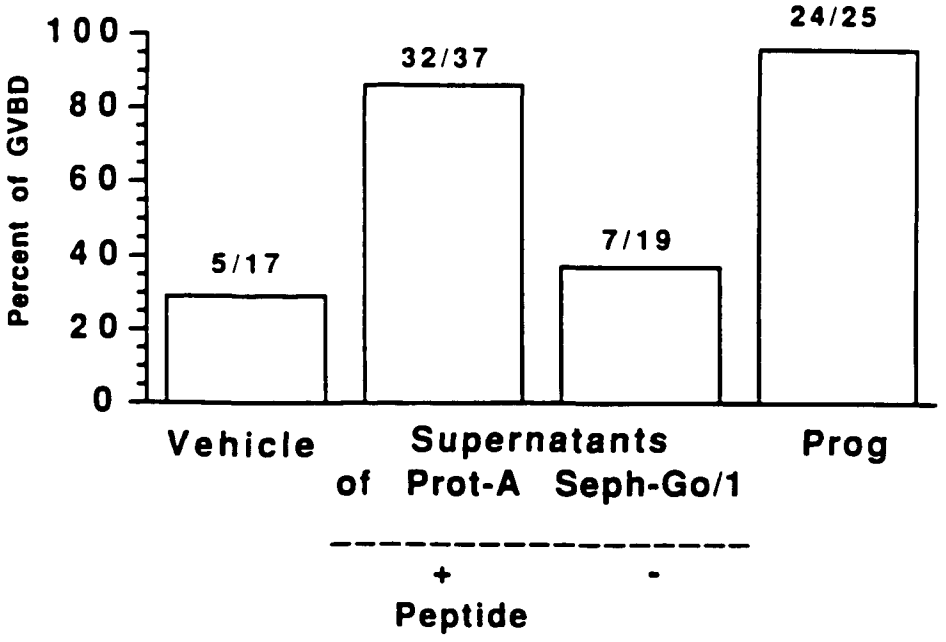
10. Failure of activated G_i-α subunits to induce oocyte maturation. G_i-α subunits were purified from human erythrocytes and activated with GTPγS in the same manner as activation of G_o-α. Microinjection of 50 nl of 50 nM purified α_i* subunits failed to elicit oocyte maturation similar to maturation induced by 50 nl of 63 nM α_o* subunit sixteen hours after oocyte microinjection.



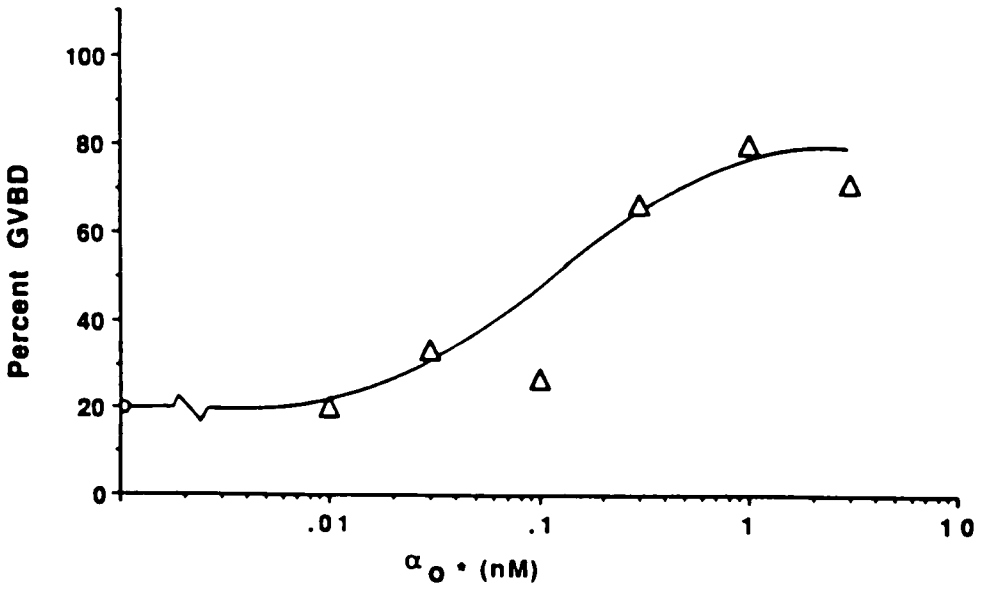
11. Failure of holo-G_o protein to induce oocyte maturation. Microinjection of 50 nl of 50 nM purified G_o protein failed to induce oocyte maturation similar to maturation induced by 50 nl of 63 nM α_o* subunit sixteen hours after oocyte microinjection.



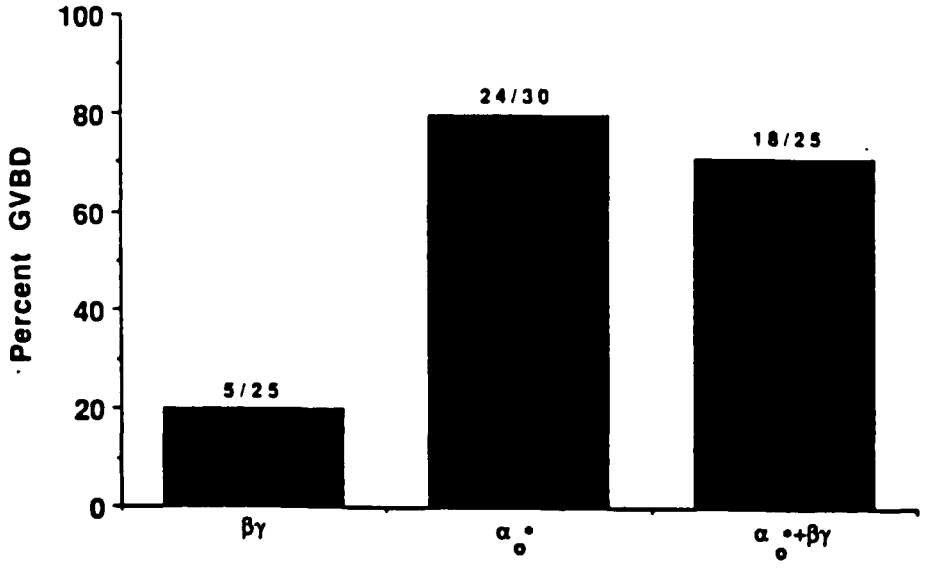
12. Time course profile of activated G_o-α subunit induced oocyte maturation. Time course of maturation for progesterone incubation (10 μM final concentration), α_o* microinjection (3 nM final intracellular concentration), α_{i2}* (2.5 nM final intracellular concentration or vehicle microinjection. Numbers at the end of the curve represent number of oocytes that display GVBD/total number of oocytes.



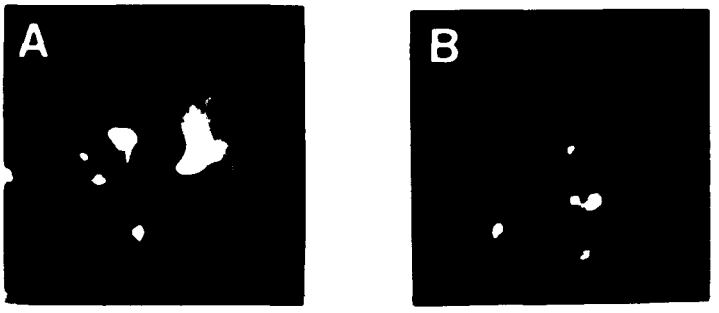
13. Effect of specific immunodepletion of activated G_o-α subunit on oocyte maturation. G_o-α subunit was specifically immunodepleted from a purified solution of the activated subunit. A stock solution was divided and incubated in the presence (+) or absense (-) of G_o/1 antibody precoupled to protein A sepharose. After overnight incubation at 4 °C, the sepharose was separated by centrifugation and the supernatant was injected into oocytes. Germinal vesicle breakdown was compared to that elicited by vehicle injection or progesterone (10 μM) incubation sixteen hours after oocyte microinjection.



14. Dose response profile of injection of varying amounts of G_o-α subunit into oocyte. Microinjection of 50 nl of varying concentrations of G_o-α induced oocyte maturation with a half maximal effect at 150 pM. GVBD was measured 16 hours after microinjection.



15. Failure of G_o-βγ subunits to inhibit activated G_o-α subunit maturation induction.. Effect of βγ dimer on maturation and on α_o* induced maturation. βγ dimer was microinjected at a final concentration of 6 nM by itself or with α_o* (3 nM final intracellular concentration). Germinal vesicle breakdown was recorded after 16 hours.



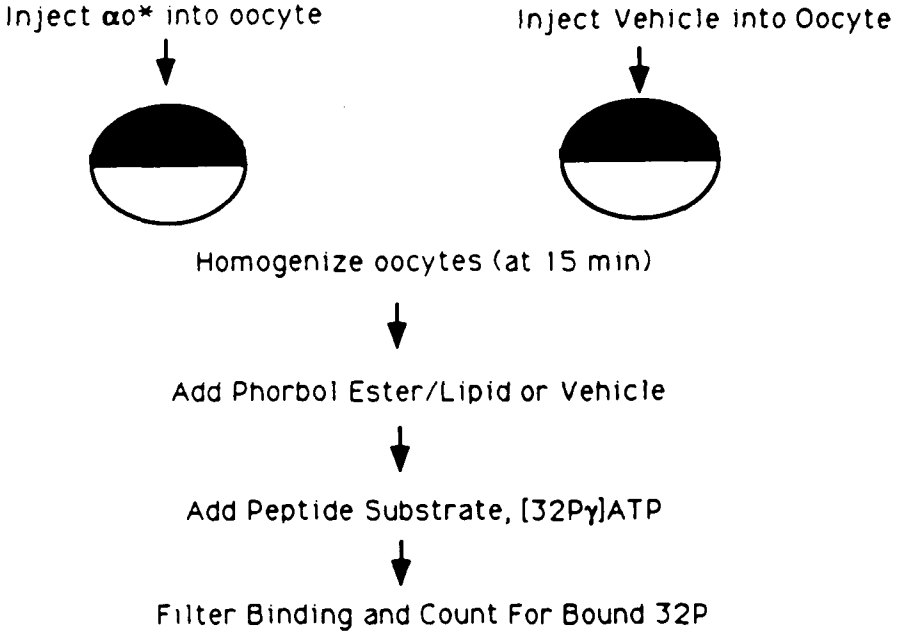
16. Xenopus oocyte chromosome condesation induced by progestererone and activated G_o-α subunit. Condensed chromosomes in progesterone treated (10 mM final concentration, A) or α_o* microinjected (3 nM final intracellular concentration, B) oocytes and followed to mature up to the appearance of the white spot in the animal pole. At this stage, the oocytes were fixed in 10% formaldehyde. The chromosomes were visualized from single matured oocytes by epi-illumination after staining with the Hoechst dye 33258, dissection by a jeweler's forcep and photographed onto Kodak Pan X black and white film. The bar represents 10 μ.

Protein Kinase C In α_0* Induced Maturation

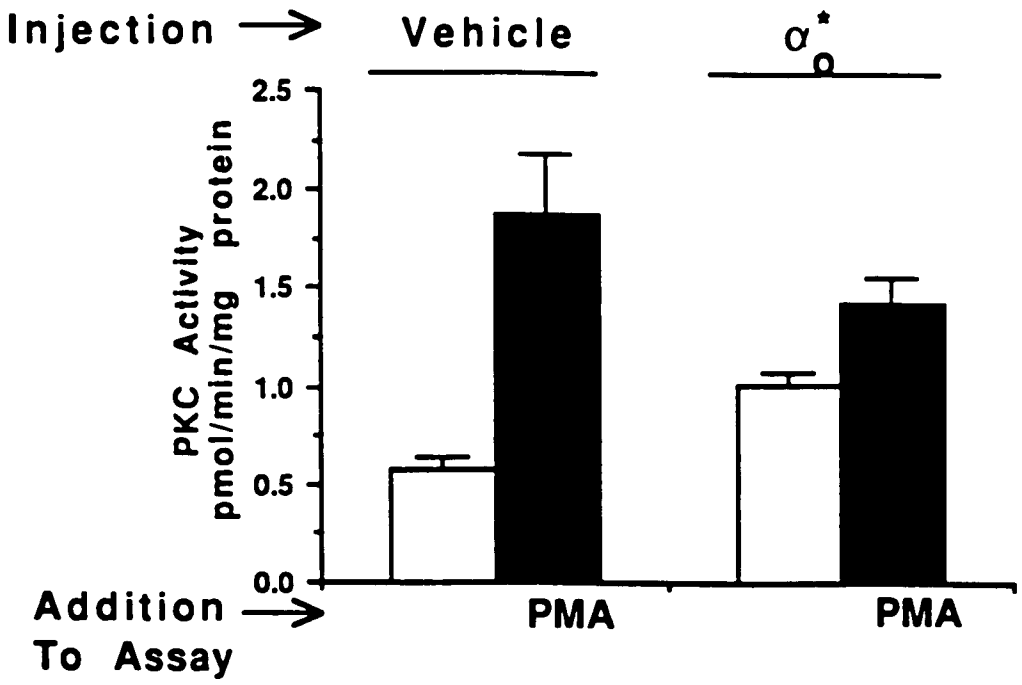
Since α_0* is thought to stimulate phospholipase C, it should activate protein kinase C through diacylglycerol. I measured protein kinase C activity in homogenates from vehicle and α_0* injected cells using a specific peptide substrate PKC(19-36ΔThr 25) (Figure 17). I found at least a 2.5 fold stimulation of protein kinase C in α_0* injected cells (Figure 18) by two criteria. First, protein kinase C specific activity in homogenates of α_0* injected oocytes was 2 - 2.5 fold higher than that of vehicle injected oocytes. Second, the protein kinase C activity in homogenates of α_0* injected oocytes was elevated and further stimulation by phorbol esters was only forty percent, in contrast, to vehicle injected homogenates where phorbol esters stimulated protein kinase C activity 3.5 fold.

To determine if increased protein kinase C activity was required for the α_0* effect, I co-injected α_0* with a specific peptide inhibitor PKC (19-36) which encodes the pseudosubstrate region in the regulatory domain of protein kinase C. Co-injection of the PKC (19-36) peptide blocked α_0* induced maturation (Figure 19), but by itself did not induce maturation in excess over vehicle injection. PKC (19-36) peptide did not affect progesterone induced GVBD (Figure 20), indicating that protein kinase C activation was not essential for the progesterone induced pathway. PKC (19-36) inhibition of α_0* induced maturation was concentration dependent with an approximate half maximal effect in the 10-20 nM range (Figure 21).

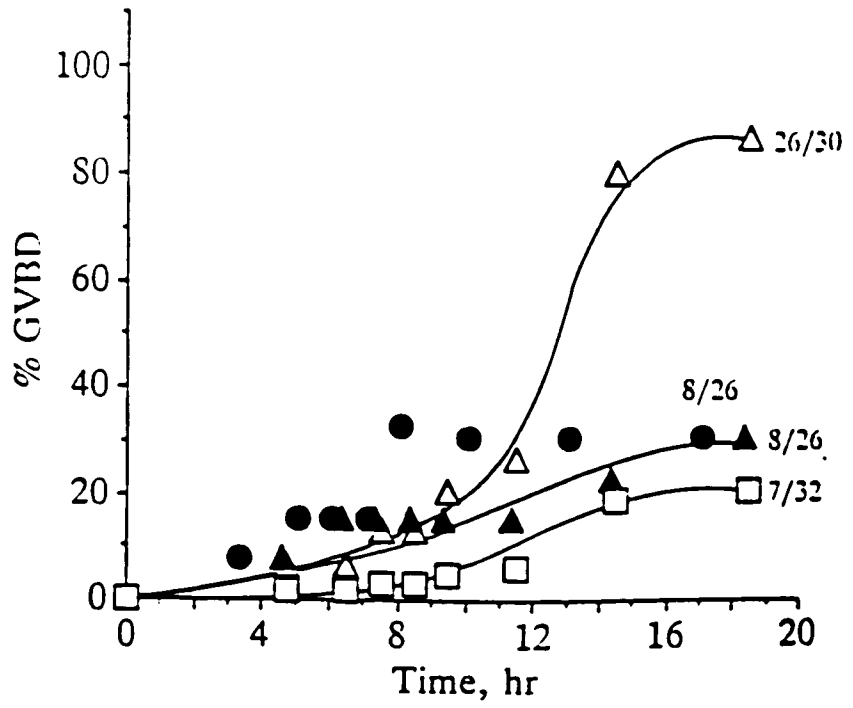
Confirmation of oocyte maturation was carried out by fixing oocytes in trichloroacetic acid and visualizing the dissolution of the germinal vesicle by slicing treated oocytes in half longitudinally (Figure 22). Sliced oocytes demonstrated intact germinal vesicles for vehicle injected oocytes as well as for α_o* plus PKC (19-36) peptide injected oocytes. Dissolution of the germinal vesicle was observed in oocytes with progesterone added and in α_o* injected oocytes.



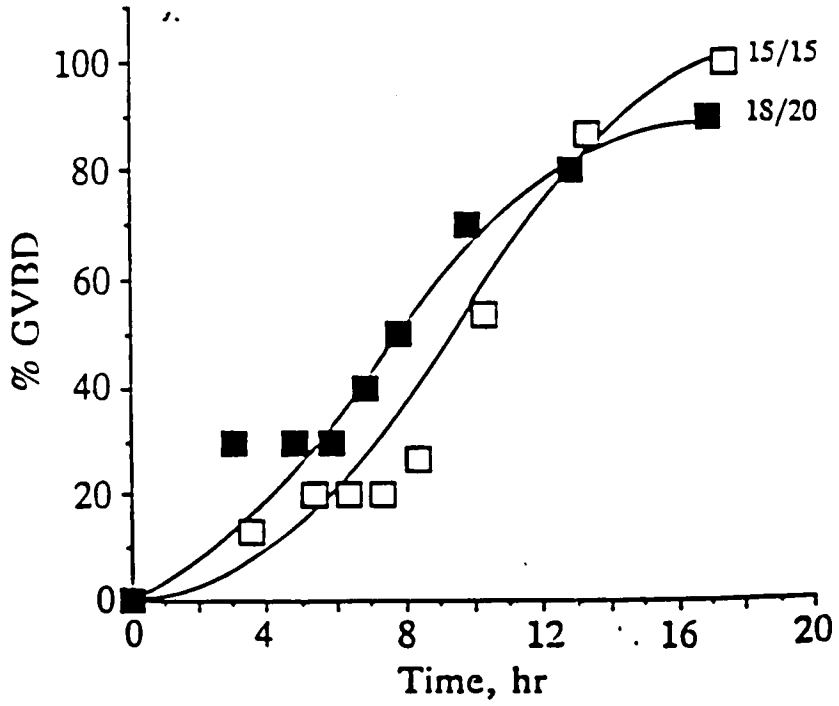
17. Schematic diagram of protein kinase C activation and pseudosubstrate inhibition/substrate assay.



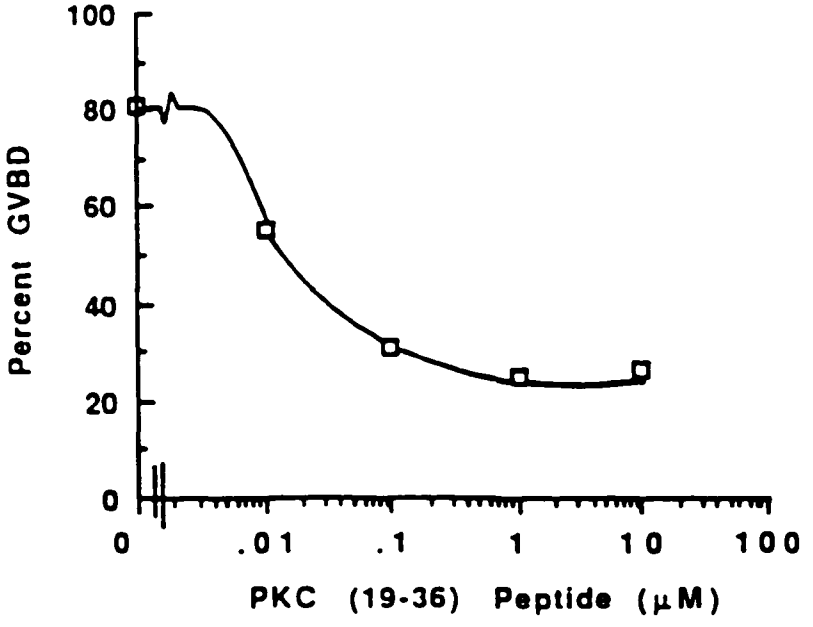
18. Comparison of protein kinase C (PKC) activity in the absence and presence of phorbol ester (PMA) in homogenates prepared from vehicle and α_o*-injected oocytes. α_o* was microinjected (3 nM final intracellular concentration) into ten oocytes fifteen minutes prior to oocyte homogenization. Either vehicle or phorbol-12-myristate-13-acetate (PMA) was added to the oocyte homogenates to determine maximum PKC activity. (N=3 +/- S.E.M.).



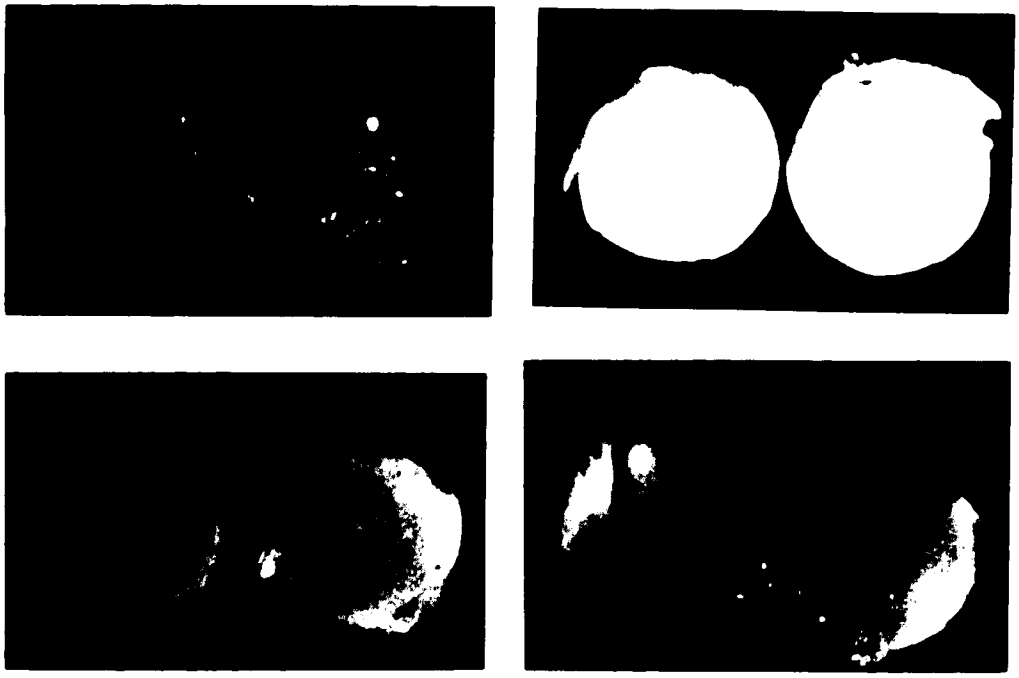
19. Time course of blockade of activated G_o-α subunit induced oocyte maturation by PKC(19-36) peptide. Effect of PKC (19-36) peptide microinjection (1 μM final intracellular concentration) on maturation induced by activated G_o-α subunit (3 nM final concentration) microinjection. PKC (19-36) peptide was coinjected with activated G_o-α subunit (▲). For comparison, activated G_o-α subunit alone (3 nM, Δ), PKC (19-36) peptide dissolved in vehicle (●), and vehicle alone were injected (◘). GVBD was recorded after 16 hours. Numbers at the end of the curve represent number of oocytes that display GVBD/total number of oocytes.



20. Effect of PKC(19-36) peptide on progesterone induced oocyte maturation. Time course of the effect of microinjection of PKC (19-36) peptide on progesterone induced oocyte maturation. PKC (19-36) peptide was microinjected at a final intracellular concentration of 1 μM immediately before the addition of progesterone to the oocyte bath (□) and compared to progesterone added to oocytes that were not previously injected with peptide (■).



21. Dose response profile of PKC(19-36) effect on activated G_o-α subunit induced oocyte maturation. α_o* subunit (3 nM final intracellular concentration) was co-injected with varying concentrations of PKC (19-36) peptide, diluted in vehicle. Germinal vesicle breakdown was recorded after 16 hours. At 3 nM α_o* subunit final concentration, a half maximal concentration of PKC (19-36) peptide for inhibition of GVBD was ≈ 10 nM.



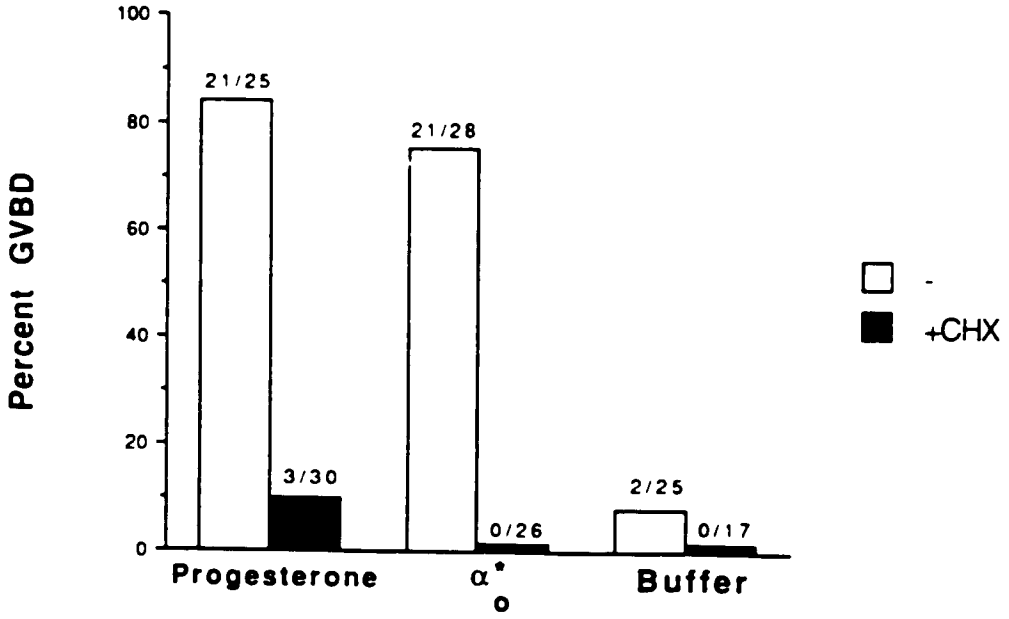
22. Microdissection of oocytes microinjected with vehicle (A), activated G_o-α subunit (B), incubated with progesterone (C) or activated G_o-α subunit plus PKC(19-36) peptide (D). Oocytes were fixed in 5 % trichloroacetic acid and sliced in half along the longitudinal axis with a scalpel under a dissecting microscope. Germinal vesicle breakdown was confirmed by the presence or absence of a vesicle in the top half of the oocyte half. Oocytes halves were photographed under a dissecting microscope using Kodak Ektar 100 film. Backgrounds were sprayed with green tone.

c-mos And α_0* Induced Maturation

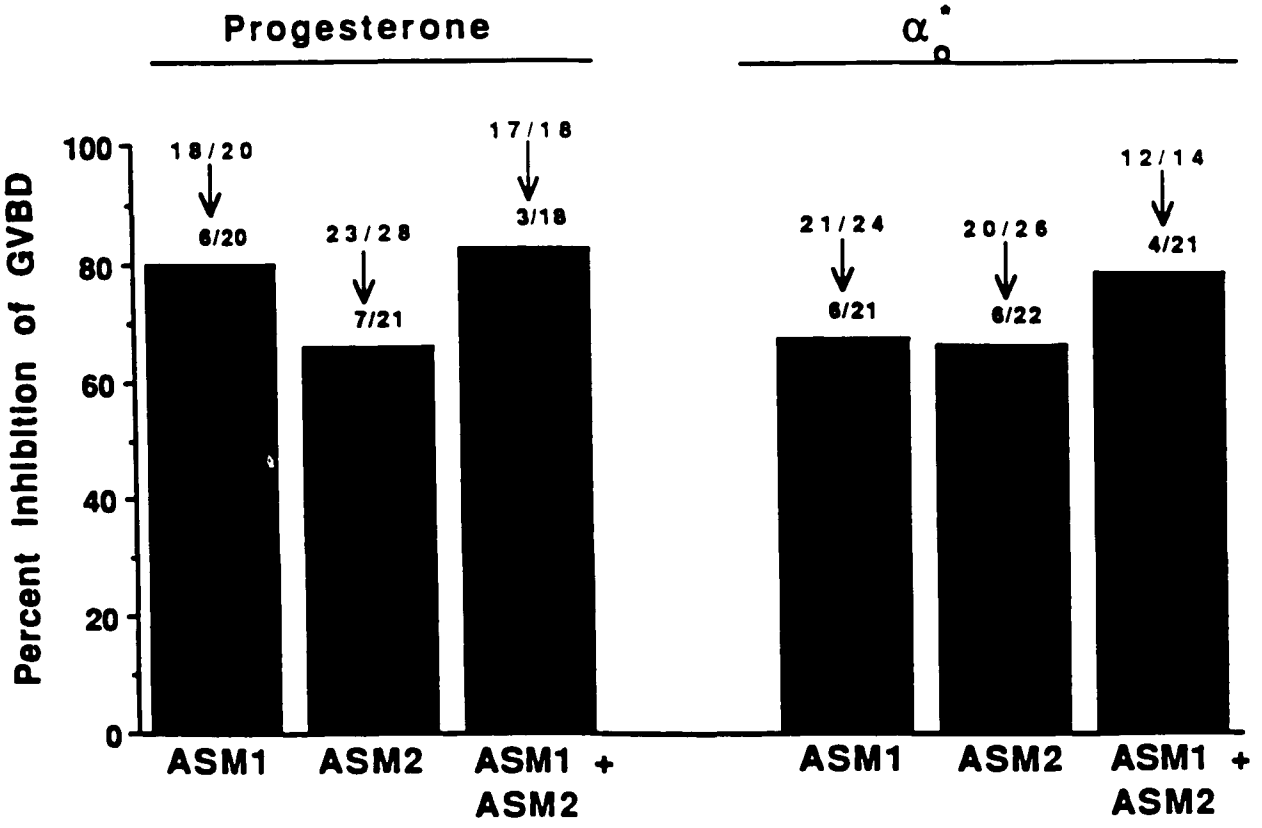
The progesterone effect is mediated at least in part through translation of pp39^{mos}, a ser-thr kinase protein product of the proto-oncogene *c-mos*^{xe}. Since protein kinase C is also a ser-thr kinase and required for the α_0* but not the progesterone effect, it was not a priori obvious whether the α_0* effect would require protein synthesis. Hence I tested if cycloheximide blocks the α_0* induced maturation. Treatment of oocytes with cycloheximide for 4 hours prior to the injection of α_0*, blocks the α_0* effect (Figure 23). In order to determine if the α_0* effect required translation of *c-mos*, two anti-sense oligonucleotides to the *c-mos* transcript were tested for their capability to block the α_0* induced maturation. It was found that both antisense oligonucleotides, when injected individually or together 4 hours prior to treatment, inhibited α_0* and progesterone induced maturation (Figure 24). Corresponding sense oligonucleotides did not have significant effects. Hence it appears that translation of the c-mos transcript is necessary for the α_0* effect, as it is for progesterone induced maturation

I next analyzed the role of protein kinase C in the α_0* effect on mos protein. Watanabe et al. (1989) have shown that the c-mos protein production is not present in measurable amounts in immature oocytes and maturation results in translation and phosphorylation of c-mos. At 4-6 hours after the start of the maturation signal, the amount of mos protein identified by ^{35}S and ^{32}P labeling was similar. Hence we utilized ^{32}P labeling of oocytes to determine the effect of protein kinase C activity on the

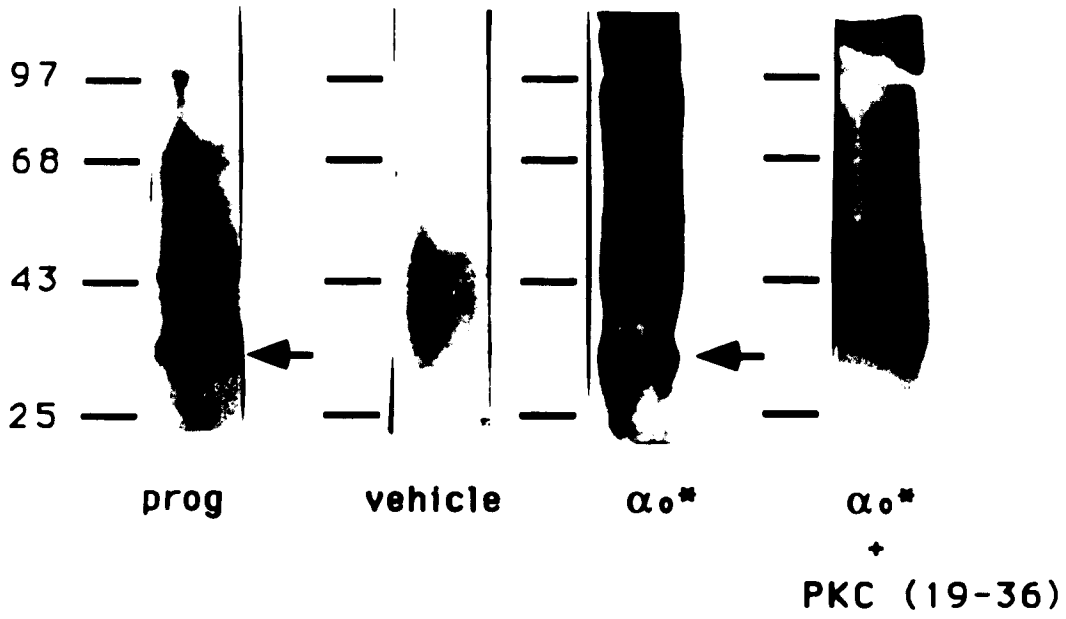
identification of mos by immunoprecipitation with a mos specific monoclonal antibody S5. It was found that no labeled protein was detected in buffer injected oocytes but could be detected in progesterone treated oocytes. Injection of α_0* resulted in the appearance of the 39 kDa band. When the PKC (19-36) peptide was injected, no 39 kDa immunoprecipitatable band was observed. (Figure 25) The experiments in Figures 16-18 indicate that mos synthesis is required for the α_0* effect. Since mos is synthesized and phosphorylated in the absence of protein kinase C activation in progesterone matured oocytes, it appears that the presence of ^{32}P labeled p39 mos in α_0* injected oocytes and its absence in α_0* + PKC (19-36) injected oocytes suggests that phosphorylation and possibly synthesis of mos by α_0* is regulated through protein kinase C.



23. Effect of cycloheximide on progesterone and α_o* induced maturation. Oocytes were incubated in 100 µg/ml cycloheximide 4 hours prior to progesterone addition (10 µM final concentration) or α_o* microinjection (3 nM final intracellular concentration). Buffer was the α_o* storage solution. Germinal vesicle breakdown was measured 16 hours after α_o* microinjection or progesterone addition.



24. Effect of injection of antisense oligonucleotides to the *mos* mRNA on progesterone and α_o^*-induced germinal vesicle breakdown. ASM1 is an oligonucleotide complementary to nucleotides +178 to +198 in the coding region of c-*mos* . ASM2 is complementary to nucleotides -21 to +6 and spans the ATG initiation codon. Oligonucleotides were microinjected 4 hours prior to progesterone addition (10 μM final concentration) or α_o^* microinjection (3 nM final intracellular concentration). Germinal vesicle breakdown was measured 16 hours after microinjection or progesterone addition. Percent of inhibition of germinal vesicle breakdown is calculated by determining the maximal level of oocyte maturation achieved with the sense oligonucleotide, normalizing this value as 100% and calculating the percentage inhibition of maturation elicited by the corresponding antisense oligonucleotide. All sense oligonucleotides gave greater than 80% germinal vesicle breakdown when microinjected into oocytes.



25. Immunoprecipitation analysis of pp39^{mos} in progesterone-treated, vehicle, α_o* and α_o* + PKC (19-36) peptide injected oocytes. Oocytes were incubated with ^{32}P for four hours. Radiolabeled oocytes were incubated with progesterone (10 μM final concentration), microinjection of vehicle, microinjection of α_o* (3 nM final intracellular concentration) or co-injection of α_o* (3 nM final intracellular concentration) and PKC (19-36) peptide (10 μM final intracellular concentration). Immunoprecipitation was done with a monoclonal antibody against pp39^{mos} protein.

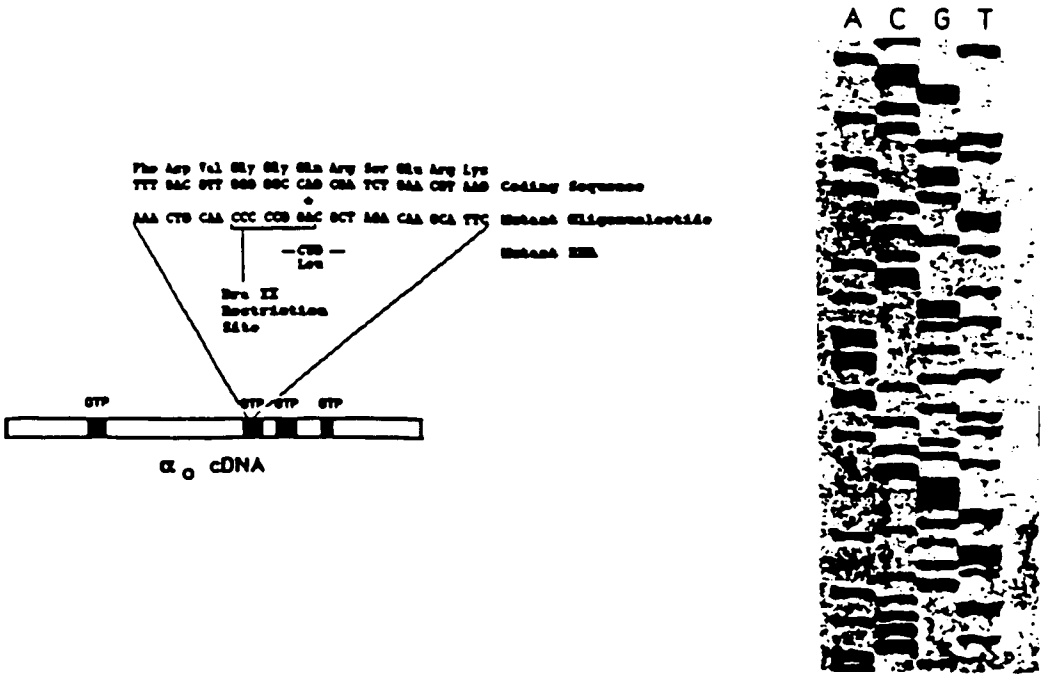
Construction and Expression of the Go-α mutant

Ras oncoprotein and G_o-α subunit share a region of homology in the guanine nucleotide binding domain, and it is in this region that a commonly found transforming mutation in ras occurs. The Q205L mutant of G_o-α, which is analagous to a mutation of the Q61 of ras, was constructed by oligonucleotide directed in-vitro mutagenesis and sequenced by the dideoxy oligonucleotide primed method (Figure 26). The oligonucleotide used was designed such that mutant cDNA contained a unique restriction site for the endonuclease DraII. This allowed for the restriction mapping of mutants by cutting with DraII and EcoRI restriction enzymes to yield a 613 base pair fragment.

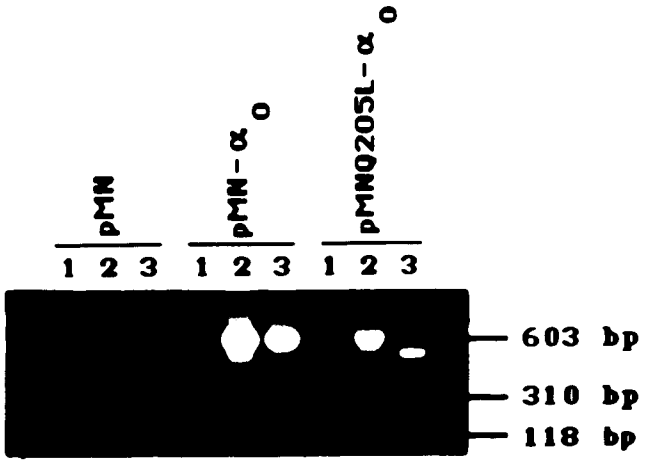
Subconfluent NIH-3T3 cells were transfected with pMN, pMN-α_o or pMN-Q205Lα_o by the calcium phosphate precipitation method. The cells were allowed to grow to confluency and then treated overnight (16h) with 10^{-7} M dexamethasone. The cells were harvested and RNA was prepared. The mRNA was reverse transcribed to make cDNA. G_o-α specific oligonucleotide probes (corresponding to positions 144-160 and 711-726 of the α_o cDNA) were used to amplify by polymerase chain reaction a fragment of the cDNA corresponding to the α_o message. No amplification of α_o cDNA was observed from cells transfected with pMN alone (Fig. 27, pMN(1,2,3)). This observation indicates that NIH-3T3 cells do not appear to contain any measurable amounts of G_o-α message. pMN-α_o and pMN-Q205Lα_o transfected cells showed a 600 base pair band of the predicted size (Fig. 27, lane 2 of pMN-α_o and

pMN-Q205Lα_o). The PCR amplified 600 base pair bands were observed only when the cells were treated with dexamethasone (compare lanes 1 and 2 for pMN-α_o and pMN-Q205Lα_o, Figure 27) This indicated that G_o-α messages were being transcribed from the vector in an inducible manner, as expected. When the α_o and Q205Lα_o PCR products were digested with DraII restriction enzyme, only the Q205Lα_o product yielded a 400 base pair fragment indicating the presence of the mutant transcript in these cells (Compare lanes 3 in pMN-α_o vs. pMN-Q205Lα_o).

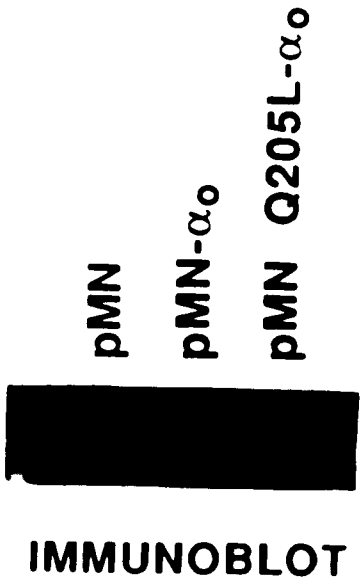
The presence of G_o-α protein was verified by immunoblotting with an α_o specific antipeptide antibody. Transfected cells were selected for neomycin resistance in the presence of 0.4 g/l G418 for ten days. The survivors were treated with dexamethasone and grown to confluency. The α_o protein was not detected when crude membranes (40,000 X g) prepared from these cells were used for immunoblotting. The α_o protein was also not detected in whole cell extracts. Hence, highly purified plasma membranes were prepared by sucrose density gradient ultracentrifugation. 100 μg of the purified plasma membranes were resolved by SDS gel electrophorhesis and then blotted with an α_o specific antisera. A 39 kDa band was observed in pMN-α_o and pMN-Q205Lα_o cell membranes but not in pMN transfected cell membranes (Figure 28).



26. DNA sequence of Q205L mutant of G_o-α subunit cDNA (Left panel). Wild type G_o-α subunit DNA was subcloned into the M13mp19 vector and mutated by site-directed, oligonucleotide primed mutagenesis. DraII restriction site corresponds to the region of the G_o-α subunit cDNA mutated by this procedure. Viral DNA was purified from a DraII sensitive mutant and sequenced by the Sanger dideoxy method using [^{32}P-α]ATP, electrophorhesed on an 8% urea/polyacrylamide gel and autoradiographed for two hours using Kodak X-Omat film. The mutant cDNA sequence is shown (right panel)



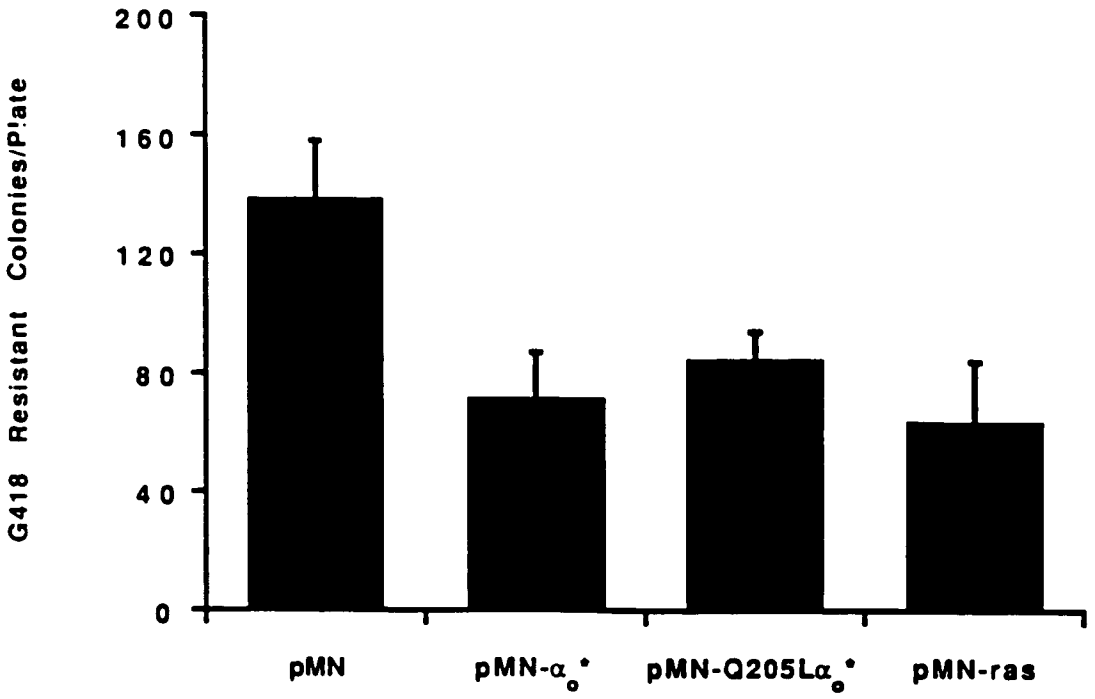
27. PCR amplified cDNA from transfected NIH-3T3 cells were resolved electrophoretically on an ethidium bromide stained 1% agarose gel. For each category of transfected cells, the conditions for the three lanes were as follows: (1) cDNA from transfected cells without dexamethasone treatment, (2) cDNA from transfected cells with 1 μM dexamethasone, (3) Dra II enzyme digest of cDNA from cells treated with 1 μM dexamethasone. Sizes are indicated in base pairs.



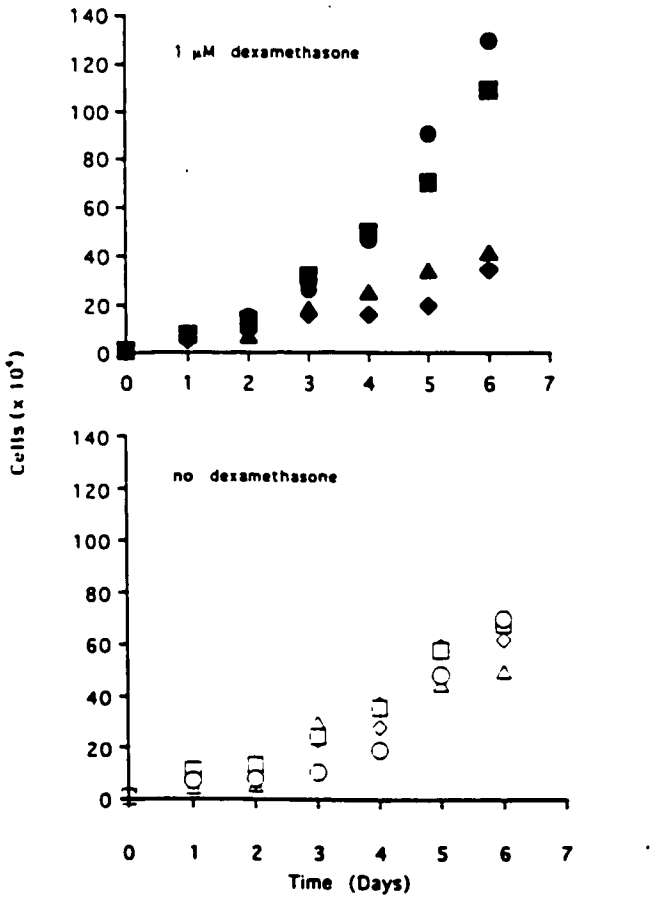
28. Immunoblotting of NIH-3T3 cell plasma membranes with anti-α_o antiserum. Purified plasma membranes from transfected NIH-3T3 cells treated with 1 μM dexamethasone were resolved by SDS-PAGE and then blotted with an α_o specific antiserum. Bands were visualized by the ECL system from Amersham. Lanes are as follows: membranes from cells transfected with (1) pMN vector, (2) pMN-α_o, (3) pMN-Q205Lα_o

Mitogenic Effects of Q205L-α_o

pMN contains a selectable marker under the control of SV40 promoter. Thus, constitutive expression of geneticin (G418) resistance can be used to select transfected cells in which antibiotic resistance is being expressed. A comparison was made of transfection efficiency based upon resistance to 400 μg/ml G418 (Figure 30), demonstrating that comparable levels of transfection efficiency was being attained with the various trasnsfected constructs. A characteristic which defines some types of transforming genes is their ability to enhance growth. Thus, I sought to determine whether Q205L-α_o had a growth enhancing effect. By comparing pMN-Q205L-α_o tranfected cells to pMN-α_o and pMN transfected cells, I was able to detect a decrease in doubling time for the pMN-Q205L-α_o transfected cells (Figure 31). Although only a small proportion of cells might actually contain the mutant gene, through the use of a computer simulation I was able to model a situation where an enhanced growth rate of those affected cells might alter the perceived doubling time of the whole cell population during the six day time course of the assay.



29. Transfection efficiency in NIH-3T3 cells. Cells were transfected with pMN, pMN-α_o or pMN-Q205Lα_o. Geneticin (G418) was added to a concentration of 0.4 g/l and, after three days, the number of surviving colonies were counted.



30. Dexamethasone dependence of mitogenesis in transfected NIH-3T3 cells. Cells were transfected with pMN (▲), pMN-α_o (◆), pMN-Q205Lα_o (■), or pMN-ras (●). The cells were then split, grown for the indicated number of days and counted. Upper panel, time course of cell number with addition of 1 μM dexamethasone on alternating days. Lower panel, open symbols, time course of cell number without addition of dexamethasone.

Transformation by Mutant G_o-α

The capability of the expressed pMN-α_o and pMN-Q205Lα_o to induce transformation of NIH-3T3 cells was assessed by three criteria; formation of foci in confluent cultures, anchorage independent growth of the transfected cells in soft agar and capability of the transfected cells to induce tumors in Nu/Nu mice. Transfection and feeding regimen are as follows: Subconfluent NIH-3T3 cells were transfected with pMN, pMN-α_o or pMN-Q205Lα_o. Fresh medium was added and treatment of the cells with dexamethasone was started. Cells were then allowed to grow for two days. At this time, each transfection plate was split into four plates and then grown to confluency, which takes 7-9 days. The plates were scored for the number of foci 14-16 days after the transfection. In spite of obtaining NIH-3T3 cells from several sources I was unable to obtain cells that showed no background foci. Typically 2-4 background foci per plate were observed. Transfection with pMN or pMN-α_o did not result in enhanced focus formation. Neither did the treatment of untransfected cells with dexamethasone. However, when cells were transfected with pMN-Q205Lα_o and treated with dexamethasone 3-6 times more foci were observed than in pMN-α_o transfected cells treated with dexamethasone (Figure 32). Non-transfected cells did not show significant focus formation under identical culture conditions (Figure 33) None of the transfected cells showed enhanced focus formation when they were not treated with dexamethasone (Table 2). I was unable to identify any unique morphology in pMN-Q205Lα_o induced foci. Typical regions of pMN, pMN-α_o and pMN-

Q205Lα_o plates are shown in Figure 34, parts A,B and C). The number of foci per plate was dependent on the concentration of DNA used during the transfection and was a saturable phenomenon (Figure 35). The concentration needed for half-maximal effect, determined in three seperate dose response experiments was approximately 1 μg DNA (vector plus insert). Transfection of fixed concentrations of plasmid DNA (20μg) in five individual experiments indicated that the effect of Q205Lα_o was highly significant (pMN-α_o vs. pMN-Q205Lα_o $p>0.004$). In the linear range, it was found that pMN-Q205Lα_o elicited 150-300 foci/μg G_o-α DNA under conditions where pMN-*H-ras* elicited 600-900 foci/μg *H-ras* DNA.

Transfected cells were assessed for anchorage independent growth by cloning in soft agar. NIH-3T3 cells were transfected with pMN, pMN-α_o or pMN-Q205Lα_o. The sub-confluent cells were grown to confluency and treated on alternate days with dexamethasone to induce transcription of the exogenous message. Fourteen to sixteen days later, cells were dispersed and seeded onto soft agar plates, at a concentration of 10^4 cells per plate. Ten to fifteen days after plating, the soft agar plates were observed for colony formation. Numerous colonies were observed in the plates containing pMN-Q205Lα_o transfected cells, in contrast to plates containing pMN or pMN-α_o transfected cells. A significantly lower number of colonies formed in non-dexamethasone treated cells that had been transfected with (Figure 36). Typical regions of plates containing cells transfected with pMN (A), pMN-α_o(B) or pMN-Q205Lα_o(C) are shown in

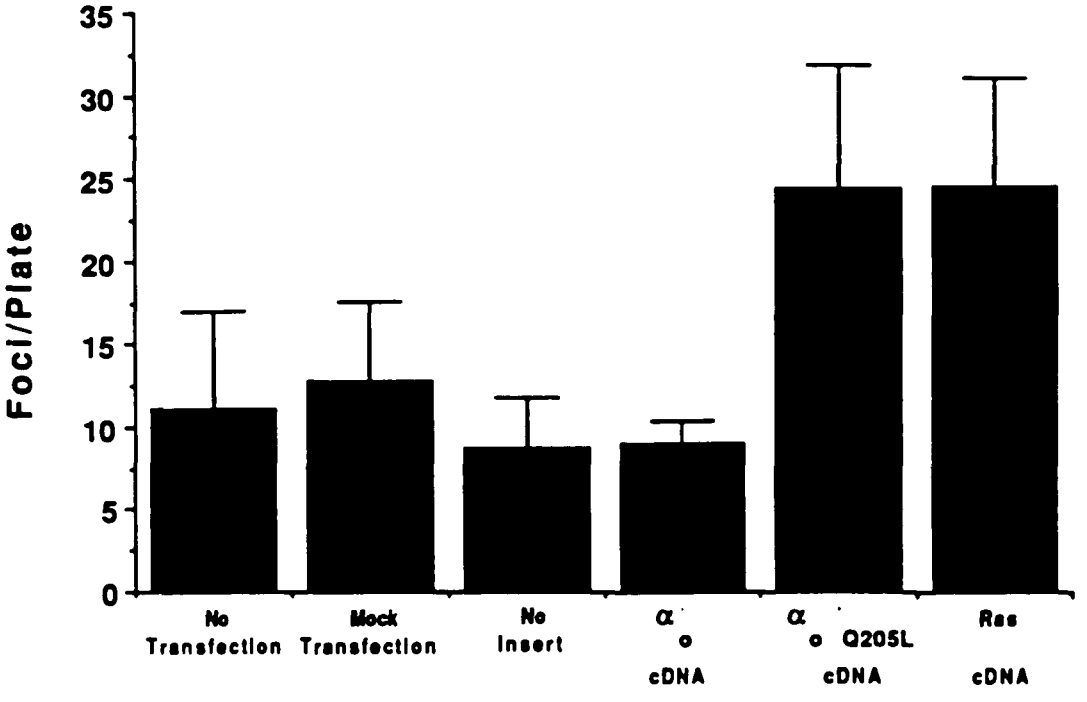
Figure 37. The plates were counted and a summary of three plating experiments each derived from independent transfections is shown. It can readily be seen that while pMN-Q205Lα_o forms 300-400 colonies per 10^4 cells, the pMN or pMN-α_o forms less than 30 colonies for the same amount of cells.

Since expression from pMN vector is an inducible event, I sought to address the question of how long the Q205L-α_o gene needed to be transcribed in order to deliver a transforming signal. This may not be important in the case of some oncogenes where mutations are irreversable. Yet in multistep progression towards transformation the temporal integration of inappropriate hormonal signals, plus the transient over-expression of proteins may be crucial to the transforming event. Thus I sought to determine for how long was induction of Q205L-α_o necessary in NIH-3T3 cells. For these studies, pMN-Q205L-α_o was transfected into NIH-3T3 cells and the cells were treated according to the normal alternating feeding/dexamethasone treatment schedule as described in Materials and Methods. Every other day for twelve days after transfection and subcloning of transfected cells, one plate of cells were trypsinized and plated in soft agar plates (10^4 cells/plate) as described above. Thus the length of time of induction necessary for transformation could be monitored over this twelve day time course. The formation of colonies in soft agar appeared to be dependent on the length of time the gene was induced by dexamethasone (Figure 38).

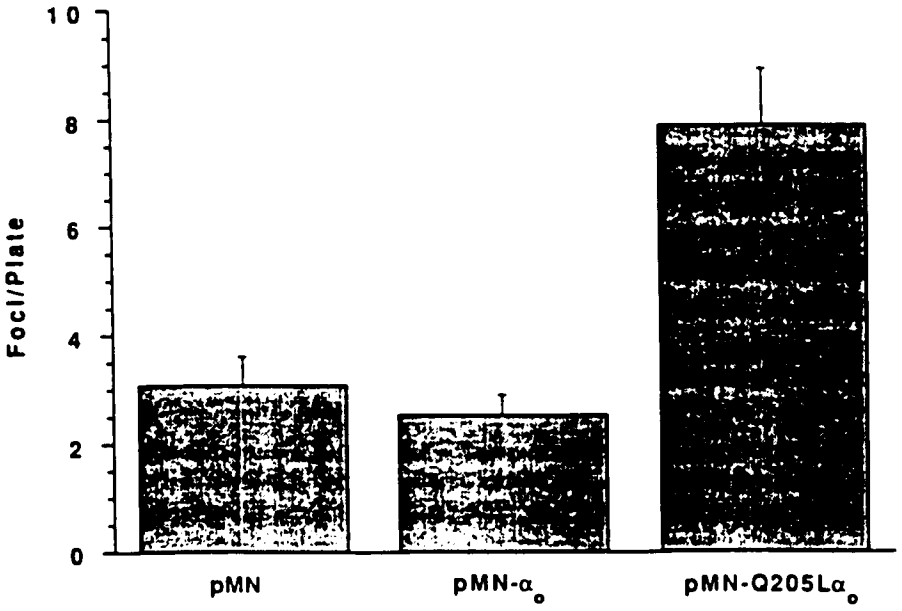
In order to determine whether cellular transformation by mutant G_o-α was cell-type specific, subconfluent Rat-1 fibroblast

cells were transfected with pMN, pMN-α_o, pMN-Q205Lα_o or pMN-v-H-ras by calcium phosphate precipitation. After 24 hours, fresh medium was added and treatment of the cells with dexamethasone was started. Cells were then allowed to grow for two days. At this time, each transfection plate was split into four plates and then grown to confluency, which takes 7-9 days. The plates were scored for the number of foci 21 days after the transfection. Rat 1 cells showed no background level of focus formation (Table 3). pMN-v-H-ras transfection led to significant focus formation, while pMN-205Lα_o showed no focus formation in this assay, indicating that the Rat-1 fibroblast cell line was not capable of transformation by the mutant G_o-α.

In order to determine whether the transforming effect of pMN-Q205Lα_o was due to the dexamethasone added to the culture medium, cDNA for α_o and Q205Lα_o were subcloned into the vector pRC/CMV. This vector allows for constitutive expression of the inserted gene under control of the cytomegalovirus promoter. Cells transfected with Q205Lα_o showed significantly enhanced capability to form foci and grow in soft agar (Table 3).



31. Focus Formation in confluent NIH-3T3 cells transfected with 20 μg DNA /75 mM plate of cells and treated with dexamethasone (1 μM) on alternating days. DNA was transfected by calcium phosphate precipitation. Two days after transfection, the cells were divided into three 75 mM plates and allowed to grow to confluency in 5% calf serum supplemented DME culture medium. Foci were counted 14 days after transfection by light microscopy. (N=8 +/- S.E.M.)



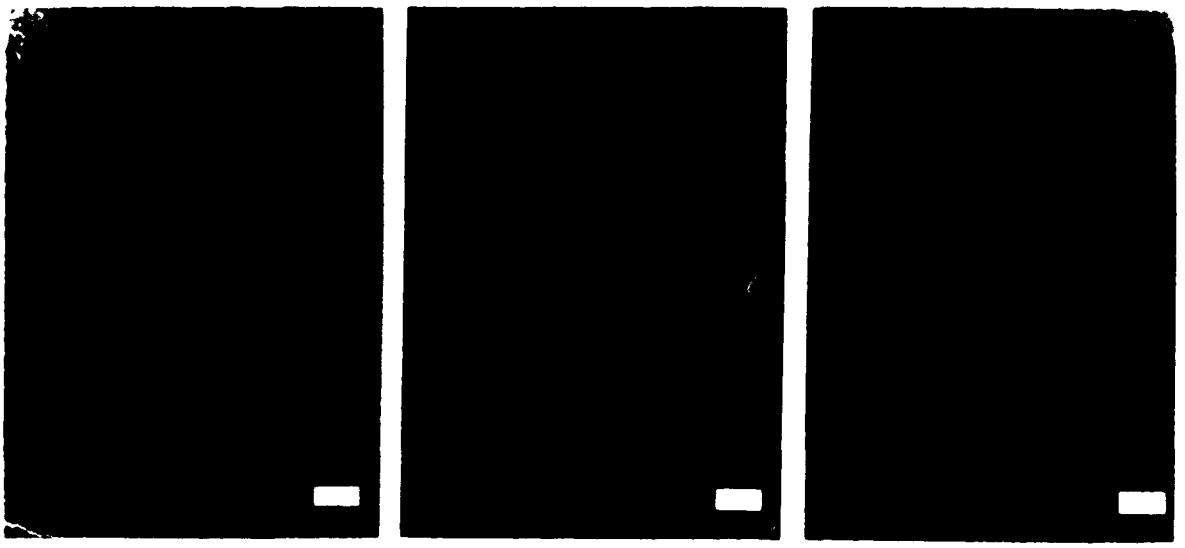
32. Focus Formation in confluent NIH-3T3 cells transfected with 20 µg DNA /75 mM plate of cells. Non transfection controls were cells grown according to the same transfection protocol as transfected cells (5% calf serum supplemented DME culture media). Mock transfected controls consisted of adding calcium phosphate with no DNA. (N=3 +/- S.D.)

Table 2

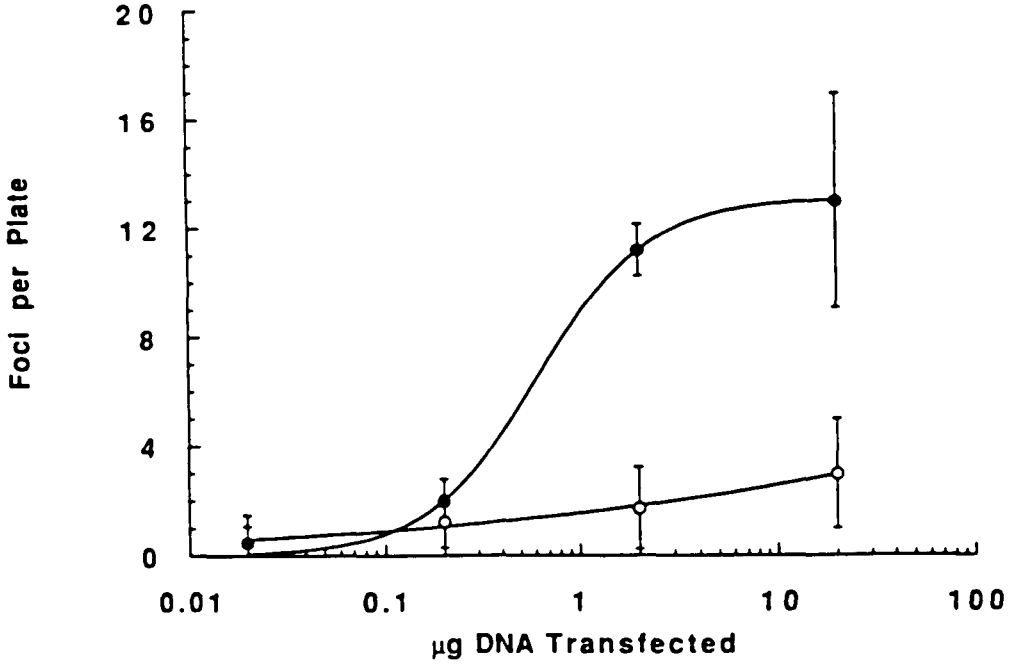
Focus formation in Transfected Fibroblast Cell Lines

Transfection Vector	Foci/ plate No Treatment	Dexamethasone	p Value
NIH-3T3 Cells			
pMN	2.3 ± 1.5	11.0 ± 2.6	0.007
pMN-α_o	4.7 ± 1.2	11.3 ± 2.1	0.009
pMN-Q205Lα_o	11.7 ± 3.5	33.7 ± 8.5	0.014
pMN-ras	11.0 ± 3.0	45.0 ± 5.6	0.001
RAT-1 cells			
pMN	ND	0	
pMN-α_o	ND	0	
pMN-Q205Lα_o	ND	0	
pMN-ras	ND	77.7 ± 14.5	

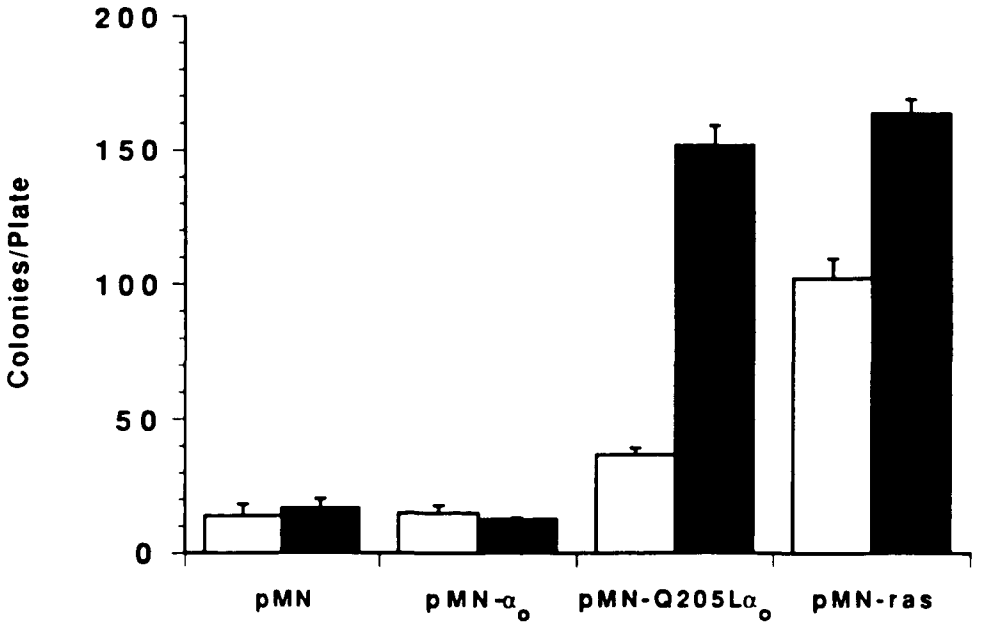
NIH-3T3 or RAT-1 fibroblasts were transfected with indicated vectors. After transfection, the cells were grown for 48 hours and then split into six 60 mm culture dishes. Dexamethasone (1 μM final) was added to three of these dishes on alternate days. Foci were counted 14-18 days after sub-culturing. Values represent mean ± S.D. of triplicate determinations from at least two independent transfections. p Values were determined by a one-tailed Student's t test and shows the effect of dexamethasone treatment. ND, not determined



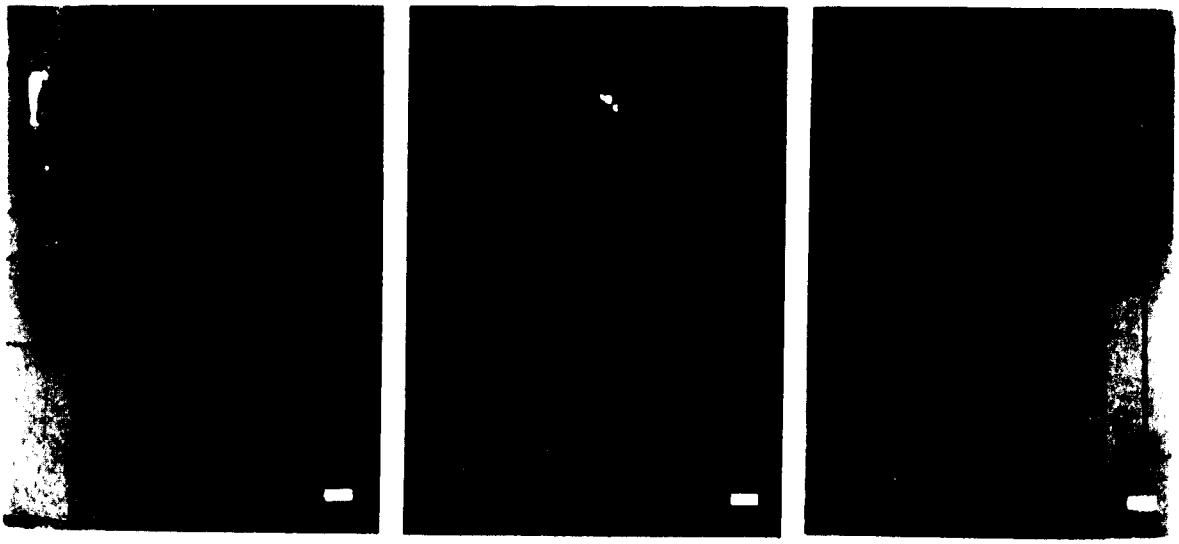
33. Photomicroscopy of regions in confluent culture, 16 days after transfection with pMN (A), pMN–α_o (B) or pMN-Q205Lα_o (C). The bar represents 300 μ.



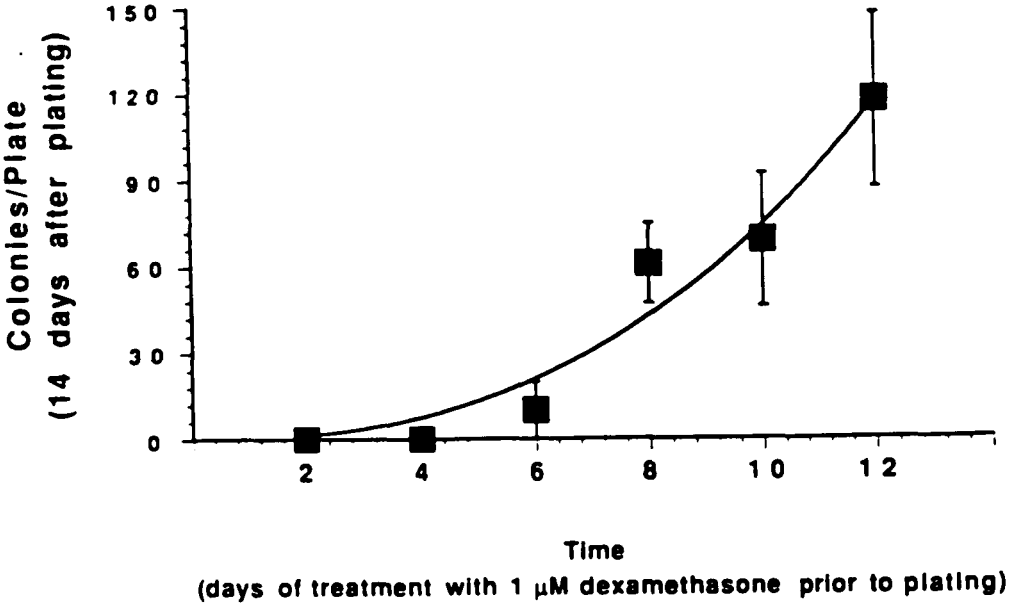
34. Dose response profile off pMN-α_o (O) and pMN-Q205Lα_o (●) cDNA transfected cells with respect to foci per plate. Carrier DNA (NIH-3T3 whole cell DNA was used to transfect each plate with a minimum of 20 µg. Two days after transfection, the cells were divided into three 75 mM plates and grown to confluency. Cells were treated every other day with 1 µM dexamethasone. Foci were counted 14 days after transfection. (N=3 +/- S.D.).



35. Anchorage independent growth of NIH-3T3 fibroblasts transfected with pMN, pMN-α_o or pMN-Q205Lα_o cDNA. Colony formation in soft agar medium with NIH-3T3 cells Open bars: colonies from cells without treatment with dexamethasone. Shaded bars: colonies from cells treated with 1 μM dexamethasone. Cells were transfected and cultured as monolayers for 14 days. Cells were then seeded into soft agar at a concentration of 10,000 cells/dish. Colonies were counted after an additional 21 days. The cells were not treated with dexamethasone during growth in soft agar in any of the conditions shown. (N=3 +/- S.D.).



36. Photomicroscopy of colonies formed in plates of NIH-3T3 cells transfected with (A)pMN, (B) pMN-α_o or (C) pMN-Q205Lα_o. Cells were transfected and cultured as monolayers for 14 days. Cells were then seeded into soft agar at a concentration of 10,000 cells/dish.



37. Effect of varying periods of dexamethasone treatment on anchorage independent growth. Time course of the number of days the cells were grown with alternate day dexamethasone treatment prior to plating in soft agar dishes. Cells were transfected and grown for the indicated number of days before being seeded into soft agar medium. During this time period, the cells were treated on every other day with 1 μM dexamethasone. Cells were then seeded into soft agar at a concentration of 10,000 cells/dish. The cells were not treated with dexamethasone during culture in the soft agar media. All colonies were counted 16 days after seeding. (N=3 +/- S.D.).

Table 3

Constitutive expression of Q205Lα_o in NIH-3T3 cells

Assay	Transfection Vector		
	pRC/CMV	pRC/CMV-Q205Lα_o	p Value
Foci/plate	1.3 ± 1.5	8.8 ± 3.0	0.009
Colonies/plate	0.33 ± 0.58	22.7 ± 6.10	0.02

Q205Lα_o cDNA was inserted by blunt end ligation into the ApaI site of the pRC/CMV vector multiple cloning region. Constitutive expression is maintained by the cytomegalovirus promoter. NIH-3T3 fibroblasts were transfected with indicated vectors. After transfection, the cells were grown for 48 hours and then split into six 60 mm culture dishes. Foci were counted 14-18 days after sub-culturing. The cells were then plated (10^4cells per plate) onto soft agar cloning dishes. Two weeks after plating, the numbers of formed colonies were counted. Foci and Colonies values represent mean ± S.D. of triplicate determinations from at least two independent transfections. p Values were determined by a one tailed Student's t test .

Tumorigenicity of Go-α mutant

We next tested if the pMN, pMN-α_o and pMN-Q205Lα_o transfected cells could induce tumors in Nu/Nu mice. It was found that pMN and pMN-α_o transfected cells induced tumors at very low frequency (0-16% of mice), however cells when transfected with pMN-Q205Lα_o induced tumors in more than 80% of mice injected. Three separate experiments are shown in Table 4. Since it appeared that all transfected NIH-3T3 cells induced tumors in mice over a long period of time, I sought to determine the latency of tumor formation in mice injected with the mutant, wild type or vector transfected cells (Table 5). These experiments displayed a time related response of tumor formation relative to tumor size. For these experiments, cells were transfected and grown for two weeks. Pools of these cells were then split and injected into individual mice. Latency of tumor formation appeared to occur over approximately twelve days after subcutaneous injection of 10^5 cells/animal. A typical tumor formed from cells transfected with pMN-Q205Lα_o is shown on an individual mouse in comparison to a mouse injected with cells transfected with pMN-α_o (Figure 40).

Table 4

Tumor Inducing Capability Of pMN-α_o and pMN-Q205Lα_o In Transfected Cells

Transfection Vector	Tumorogenicity mice developing tumors/mice injected		
	EXP I	EXP II	EXP III
pMN	1/10	0/6	1/6
pMN-α_o	2/10	1/5	1/5
pMN-Q205Lα_o	8/10	5/5	5/5

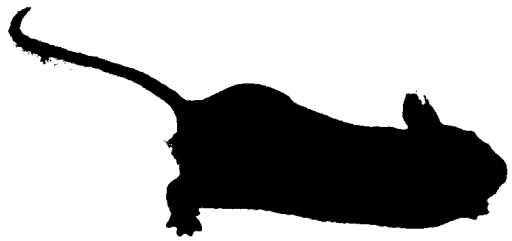
NIH-3T3 fibroblasts (10^5 per plate) were transfected with 20 μg of vector DNA (pMN) without or with the indicated insert and then grown to confluency for 14 days, with incubation on alternate days with 1 mM dexamethasone. The confluent cells were dissociated by trypsin-EDTA treatment and washed twice with serum free DMEM. Pools of 10^6 cells in 0.2 ml DME were injected per mouse subcutaneously. Tumor formation was scored 15 to 20 days later. Eventually (after 8 to 12 weeks), all animals began to develop tumors. Effect of pMN-Q205Lα_o was highly significant, by Student's one-tailed t test (pMN vs. pMN-Q205Lα_o : $p<0.005$; pMN-α_o vs. pMN-Q205Lα_o : $p<0.004$).

Table 5

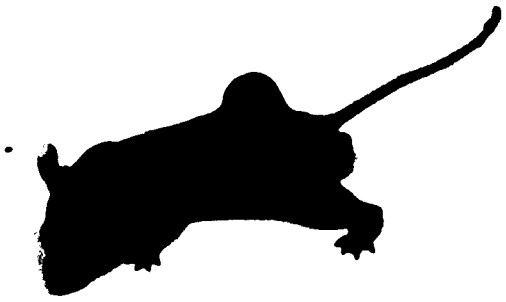
Tumor Latency In Nu/Nu Mice Injected With Transfected NIH-3T3 Cells

Transfection Vector	Tumor Diameter (mm) on Day: 4	8	12	16	26
pMN	0.0	0.0	0.0	0.0	1.0
	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0
pMN-α_o	0.0	4.0	3.0	3.5	8.0
	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	1.0
pMN-Q205Lα_o	0.0	0.0	0.0	0.0	0.0
	0.0	3.4	8.1	14.2	15.0
	0.0	0.0	2.5	5.9	6.5
	0.0	0.0	4.0	3.8	6.0

NIH-3T3 cells were transfected with 20 μg of vector DNA containing appropriate inserts and then selected with 400 μg/ml G418 and maintained in 200 μg/ml G418. Cells were grown to confluency for 14 days with incubation on alternate days with 1 μM dexamethasone. The confluent cells were dissociated by trypsin-EDTA treatment and washed twice with serum free DMEM. Pools of 10^6 cells in 0.2 ml DME were injected per mouse subcutaneously. Pools of cells were injected at a concentration of 10^5 cells subcutaneously into athymic mice and tumor formation was monitored in individual animals.



A



B

38. Photographic representation of mice injected subcutaneously with cells transfected with (A) pMN-α_o or (B) pMN-Q205Lα_o. NIH-3T3 fibroblasts (10^5 per plate) were transfected with 20 μg of cDNA. Cells were grown to confluency for 14 days with incubation on alternate days with 1 μM dexamethasone. The confluent cells were dissociated by trypsin-EDTA treatment and washed twice with serum free DME. Pools of10^6 cells in 0.2 ml DME were injected per mouse subcutaneously. Mice were photographed 28 days after injection of cells.

Discussion

The results presented in this thesis suggest that activation of G_o can promote growth, proliferation and transformation. These effects have been addressed independently, using different assays in order to demonstrate the overall effect of persistent activation of G_o. Mutations that block GTPase activity and thus persistently activate the G protein have been found in G_s and G_i isolated from cancerous tissues. It has been demonstrated that activation of G_s may be mitogenic on its own at least in certain cell types (Zachary et al. 1990). G_i-α subunits have also been shown to be both mitogenic and transforming in various cell types (Pace et al. 1991 , Gupta et al 1992).

The demonstration that the GTPγS activated G_o-α subunit can trigger the re-entry of the oocyctes into the cell cycle allowed for the unequivocal demonstration of growth promoting capability of a known G protein (Kroll et al 1991). The differential effects of the protein kinase C inhibitor peptide indicate G_o-α and progesterone act through distinct pathways to promote oocyte maturation. These observations suggest that multiple pathways may exist to trigger the re-entry of the oocyte into the cell cycle. Although this is not a new idea, as ras and insulin have been shown to induce GVBD utilizing mechanisms independent of parts of the progesterone pathway, the observation that the G_o-α subunit induces GVBD by means of protein kinase C activation is interesting. In addition, since activated G_o causes Ca^{2+} release from IP_3 sensitive stores (Moriarity et al 1990), it

is possible that, through the activation of the CAM sensitive phosphodiesterase, activated G_0 can lower cAMP levels. Thus, concurrent lowering of cAMP levels and the stimulation of protein kinase C may constitute one signal for re-entry into the cell cycle.

Even though there are different pathways to trigger oocyte maturation the final result is still the same, as assessed by germinal vesicle dissolution in the sliced sections of mature oocytes and the appearance of condensed chromosomes indicative of metaphase II. The convergence of the G_0-α, progesterone and ras maturation pathways at the synthesis of mos protein presents an intriguing site at which growth signals may come together. The limited distribution may make $pp39^{mos}$ a unique entity in oocytes. However, the activation of an analogous serine/threonine kinase downstream from protein kinase C activation is possible in somatic cells. There have been suggestions of kinase cascades in cellular growth control (Kamata and Fung 1990, Burkhand and Traugh 1983) as many growth associated proteins are both kinases and phosphoproteins. It has been reported that raf, a cytoplasmic ser-thr kinase is downstream of protein kinase C in NIH-3T3 cells and in T cells (Kolch et al 1991, Seigel et al 1990). Ser-thr kinases such as raf, pim and mos are oncogenic in somatic cells indicating that it is possible that similar pathways may exist. In addition, it has been shown that mos is activated by phorbol ester stimulation of protein kinase C in NIH-3T3 cells (Al-Bagdadi et al, 1990). Thus, activation of cell growth may be achieved by a phosphorylation cascade, similar to glycogenolysis in liver, in that kinases are responsible for phosphorylation of kinases, leading to a signal amplification and

ultimately to cell growth. This has not, as yet, been proven for cell growth.

Dexamethasone dependent pMAM-neo expression of α_o or Q205L-α_o appears to be a fairly well regulated process. The PCR amplification of reverse transcribed cDNA of the α_o or Q205L-α_o message shows that under the amplification conditions applied, no α_o or Q205L-α_o message could be detected in non-dexamethasone treated cells, although an amplifiable signal was detected after many (>40) PCR cycles. The ability of DraII restriction enzyme to discriminate the wild type and mutant forms of the amplified α_o message presents a useful technical means of detection and discrimination. Additionally, it appears that the mutation introduced into the α_o cDNA does not affect the ability of the gene to be expressed, nor does it affect the apparent molecular weight as assessed by SDS-PAGE and immunoblotting. This apparent lack of gross morphological change may be important in carcinogenesis because it suggests that such a mutation *in vivo* would not reduce G_o-α subunit synthesis. Expression of such mutant forms could be deleterious to the cell. Additionally, these results also suggest that cells expressing such a mutation can escape immunosurveillance due to its overall similarities to the native subunit.

Not all genes that promote cellular growth may be oncogenes. Clearly, there exist both promoters and initiators of transformation, as well as proteins which may act as both. Q205LG_o-α protein stimulates cell growth in culture and eventually promotes transformation. However the possibility cannot be excluded at this point that Q205LG_o-α protein acts in concert with other spontaneous

mutations either by stimulating the clonal expansion of pre-existing mutations in NIH-3T3 cells or inducing new mutations through enhanced growth rate and reduced fidelity of gene replication. Indeed, the mitogenic effect of Q205L-α_o in transfected cells may indicate that the oncogenic effect of this gene is due to expansion of other pre-existing, or newly acquired mutations. p21ras also appears to induce cellular growth, and it is important to note that ras, in primary embryonic fibroblasts, is not oncogenic on its own, but only in coordination with another oncogene (Land et al. 1983).

In order to ascertain that the effects of Q205L-α_o were not due to the concerted effect of some cellular process triggered by dexamethasone, it was necessary to determine whether a constitutively expressing vector containing Q205L-α_o could also induce cellular transformation. The pRC/CMV containing the Q205L-α_o vector induced transformation in NIH-3T3 cells. Thus, the transforming effect observed with pMN-Q205Lα_o appears to be specific for the transfection of the gene and not due to the activation of a cellular component by dexamethasone. Nevertheless, the observed minimum time necessary for intermittent dexamethasone treatment in order to obtain pMN-Q205Lα_o-induced soft agar colonies indicates that the protein must accumulate to a certain level before cellular transformation can occur. Alternatively, these results may be interpreted to suggest that a downstream effector of Q205Lα_o must be activated for a sufficiently long period of time before transformation can occur. It may also be that, while Q205L-α_o is expressed in sufficient levels even at earlier time periods, other events must be intergrated before cellular transformation can

occur. These other events, as described above, may be the expansion or aquisition of other mutations.

Because the NIH-3T3 fibroblast is an immortalized cell line, mutations have almost definitely been acquired during the immortalization process. Serial passage of these cell lines enhances the probability that additional mutations may occur. Additionally, prolonged contact inhibition probably selects for cells containing further genetic lesions for loss of contact inhibition, leading to "spontaneous" transformation. These factors also affect the athymic mouse assay, as most mice injected with NIH-3T3 cells ultimately develop tumors. Hence, it is necessary to observe the latency of tumor formation after injection of transfected cells. The earlier the formation of tumor, the fewer spontaneous events that are probably occuring, and thus, less temporal intergration of transforming events is necessary. By these criteria, cells transformed by Q205L-α_o are tumorigenic.

The failure of pMN-Q205Lα_o to transform RAT-1 fibroblasts may be due to the lower number of endogenous mutations found in these cell types. Both NIH-3T3 and RAT-1 cells are mesenchyme derived cells that undergo many gene expression changes during the course of terminal differentiation. However, RAT-1 cells appear to be less on the verge of transformation as indicated by the lack of spontaneous foci formation. RAT-1 cells transfected with pMN, pMN-α_o, or pMN-Q205Lα_o do not form foci under conditons were pMN-ras induces foci formation. This indicates that the effects of Q205L-α_o are cell type specific. The basis for this specificity is not clear. It may be that NIH-3T3 cells have acquired different, or more

numerous mutations during immortalization and subsequent cell passage. It is possible that RAT-1 cells may not have the necessary mutation for Q205Lα_o to act in synergy with, or to expand.

Mutations that lead to growth promotion and cancer have been found in many of the proteins that transduce extracellular signals to intracellular responses. Hormones, hormone receptors, kinases and transcription factors have all been identified as potential sites by which a mutation can induce neoplastic growth. Our current understanding indicates that there are at least three generalized mechanisms by which extracellular signals can be translated into an intracellular growth response. First, receptor tyrosine kinases phosphorylate intracellular effectors. An example of an oncogene product of this type is the erb-B receptor; second, G protein dependent receptors, which act by transmitting its signal via a G protein to an intracellular effector. An example of an oncogene product of this type is the mas receptor or the altered α_1-adrenergic receptor capable of inducing transformation ; and third, an intracellular hormone receptor that is translocated to the nucleus after binding a lipid soluble hormone. An example of an oncogene product of this type is the erb-A receptor. Although hormone activation of many different receptors have been shown to be mitogenic, until the studies presented in this thesis as well as the recent papers from Bourne's and Johnson's groups (Gupta et al. 1992, Pace et al. 1991) there has never been a demonstration that locking a G protein in a persistently activated state can induce growth.

The potential role of mutated G_O-α subunit as an oncogene may be important. It is apparent that oncogenicity arises from mutations in various components of cellular signal transduction mechanisms. As the mechanisms of cellular signal transduction are dissected, it is apparent that many pathways may be turned on by a single protein. The signal transduction mechanisms of the cell appear to contain exquisite regulatory controls to prevent over-amplification. It is possible that a mutation in a growth promoting G protein may be far enough upstream to affect several regulatory pathways. The G protein is the farthest upstream component of signalling pathways with the exception of the receptor. Receptors, however appear to be the primary targets of negative feedback regulation by several mechanisms including phosphorylation, sequestration or down-regulation. G proteins, however, do not appear to be extensively regulated by negative or positive feedback mechanisms. Hence, a mutated G protein that is persistently active can deliver a continous signal and thus induce aberrant growth by continuously activating multiple pathways.

The upstream location of G proteins in cell signalling, their relative insensitivity to feedback regulation, and apparent ability to undergo mutations during carcinogenesis makes G proteins a probable target for chemotherapeutic intervention. The design of pharmacological agents that inhibit G protein / effector coupling might prove useful in cancer therapy. Of course, the localization of the G protein in the plasma membrane makes drug design more difficult. In addition, the ubiquitous nature of G protein action in so many different hormonal signal transduction systems creates a

problem of specificity. Thus, a discrimination of which G proteins are utilized in a particular pathway will be important for future drug design. The continued attempt to map the three-dimensional nature of the G protein α-subunit, including it's effector enzyme activation domain, should ultimately lead to rational drug design, allowing for discrimination between G proteins as well as selective blockade of effector enzyme stimulation.

More than one growth factor is required for normal cellular proliferation, which is probably related to a requirement for interactions between signalling systems. The multistep progression model of cellular transformation suggests that each mutation or alteration that occurs as a step towards carcinogenesis is a potential target for chemotherapy. Intervention at any level may preclude the outcome that would normally occur without intervention. Thus, the characteristics of the G protein provide an excellent opportunity for chemotherapeutic manipulation. The inhibition of a signalling pathway that is activated by an oncogene product could lead to reversal of cell transformation. Since there are many pathways involved in growth-related signalling, it might be possible to inhibit a pathway activated by an oncogene, and leave other signalling pathways necesary for normal metabolism, intact.

These studies have also enhanced the understanding of the role of the phospholipase C/protein kinase C signalling system in cell cycle progression and transformation. Inhibitors of these pathways, like inhibitors of G proteins, would be expected to inhibit many cellular functions. There are many subtypes of effector enzymes such as phospholipase C, so specific inhibitors of subtypes may also

be of clincical use in blocking cellular growth. Presently, ilmofosine and hexadecylphosphocholine, which are ether lipid analogs and non-specific inhibitors of phospholipase C, are in clinical trials as anticancer agents (Powis 1991). These agents have been shown to inhibit cellular formation of inositol phosphates, and inhibit Ca^{2+} release.

Protein kinase C has already been implicated as the site of action of many currently utilized chemotherapeutic agents. The estrogen receptor antagonist, tamoxifen, is an inhibitor of protein kinase C, although it is unclear whether concentration needed for inhibition may be higher than therapeutic concentrations (Aitken and Lippman 1985). Additionally, the chemotherapeutic agent, doxorubicin, is an inhibitor of protein kinase C, yet the higher concentrations necessary for this action are much higher than those used in current cancer chemotherapy (Hannun et al. 1989). There are many different forms of protein kinase C and sub-type specific inhibitors may be useful. Additionally, it is paradoxical that tumor promoters, which activate PKC, stimulate growth in some cell types, yet induce terminal differentiation in others. Hence, it is unclear whether when seeking to design PKC specific drugs, specific activators or inhibitors should be sought.

Initially it was thought that insight into cellular transformation would require an understanding of normal cellular growth regulation. However, it is from the study of oncogenes and aberrant pathways that we are gaining an understanding of normal growth control. An understanding of cell cycle control and cell proliferation is fundamental to all aspects of medicine. It will provide not only

insights into cancer biology but also normal development, endocrinology, neurology as well as many other areas of biological interest. The elucidation and control of the mechanisms by which cellular growth are regulated will be one of the most important contributions to human knowledge. Persistent activation of cellular growth and transformation by G proteins is but one small piece of this complex puzzle.

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