ANALYSIS OF THE Gα4 SIGNAL TRANSDUCTION PATHWAY AND ITS ROLE IN THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

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NOMENCLATURE

Gα2	Gα2 gene
Gα4	Gα4 gene
Gα5	Gα5 gene
Gα2	Gα2 protein
Gα4	Gα4 protein
Gα5	Gα5 protein
gα4 null	Cells in which the wild type *Gα4* gene has been disrupted by insertional mutagenesis (*gα4* null mutant strain)
$G\alpha4^{HC}$	Cells which carry a high copy number of the *Gα4*expression vector (*Gα4* overexpression strain)
Gα2(Q208L)	Mutation in *Gα2* gene involving a substitution of glutamine208 to leucine
Gα4(Q200L)	Mutation in *Gα4* gene involving a substitution of glutamine200 to leucine
Gα5(Q199L)	Mutation in *Gα5* gene involving a Substitution of glutamine199 to leucine
cAMP	Cyclic adenosine 3', 5' monophosphate
cGMP	Cyclic guanosine 3', 5' monophosphate
WT	Wild type axenic stain KAx3

FA	Folic Acid
CotC/SP60	Prespore specific gene
ecmA/ecmB	Prestalk specific genes
lacZ	Gene that encodes for β-galactosidase activity
GFP	Green Fourescent Protein
Bs^r	Gene that encodes for blasticidin resistance
G-418^r	Gene that encodes for G-418 resistance
THY1	Gene that encodes for thymidine synthetase enzyme
PYR5-6	Gene essential for synthesis of uracil

CHAPTER 1

INTRODUCTION

Cells in a living organism have the ability to process and to respond to large amounts of information that is imparted to them via external signals such as hormones, neurotransmitters, growth factors, odorants and light. Although signals interact with intercellular components in many different ways, a large number of external signals interact with receptors on the cell surface through proteins which represent one element of a three-protein transmembrane signaling system whose components act in a sequential fashion. The class of proteins which, upon binding of the agonist to a receptor get activated due to their ability to bind guanine nucleotides are referred to as G-proteins. G-proteins are hetereotrimeric in nature being comprised of the α, β and γ subunits. These proteins in turn act as transducers and amplifiers of the signal and modulate the activity of cellular effectors within the cell (8, 74, 114). G-protein coupled receptors (GPCRs) form a large and functionally diverse protein superfamily (64, 156). They are heptahelical in nature and traverse the membrane seven times. GPCRs have contact sites for G-proteins and may or may not be very selective in their interaction. G-proteins are an integral part of several developmental processes such as mating in yeast, laying of eggs in the nematode *C. elegans*, chemotoxis in the slime mold *Dictyostelium*, immune responses, vision, hearing and olfaction in mammals (137).

G-proteins exert their effects on a wide array of effector systems, such as enzymes, ion channels and transporters, resulting in rapid changes in the concentration of intracellular signaling molecules such as cAMP, phosphatidylinositol, diacylglycerol, arachidonic acid, inositol phosphates and ion concentrations. Although, the basic elements of signal transduction events mediated by G-proteins include the receptor, G-protein and effectors, an increasing number of accessory proteins have been uncovered which modify the incoming signal. These accessory proteins include the GTPase activating proteins (GAPs), phosducin and the regulators of G-protein signaling (RGS) proteins (6, 79, 176). Coupled to this complexity are the presence of a large variety of Gα subunits and to a lesser extent, Gβ and Gγ subunits. This presents the possibility of different receptors being capable of interacting with several G-proteins at one time. The impact of this kind of divergent signaling at the cellular level would be enormous. Furthermore, the possibility exists for the Gα subunits to interact with several different Gβγ subunits upon dissociation (90, 113). Given the large number of possibilities of G-protein heterotrimer composition and the equally large number of of G-protein coupled receptors, heterotrimer composition can affect which G-proteins are activated by a receptor. This complex network of signaling components can thus have a major impact on the signaling mechanisms that affect developmental and physiological processes in a cell.

The complexity of signaling processes in higher systems has prompted the study of these processes in simpler living systems which often have analogous signals and components of the signaling pathways. One such example is the study of signal transduction events in the slime mold, *Dictyostelium discoideum*. This simple eukaryote

is a very convenient system for the study of signaling processes essential for chemotaxis, morphogenesis, gene expression and pattern formation. Many of the proteins involved in these processes have mammalian counterparts (38). The genetics and biochemistry of *Dictyostelium* provide powerful tools for the study of chemotactic processes and signal transduction (39). This simple system can therefore provide useful clues towards unravelling the complexities inherent in signaling processes of higher systems. So far, at least 8 Gα subunits, one Gβ and one Gγ have been discovered in *Dictyostelium*. Of these, three G-protein subunits have been observed to exhibit striking phenotypes in multicellular development (68, 70, 91). The Gα2 subunit is important for aggregation in response to cAMP, and the Gα4 subunit has been demonstrated to be important for folic acid responses and for proper spore development (68).

During the past several years, reports of Gα subunit mutations in mammalian tumors have prompted the idea that *Gα* genes can function as oncogenes (82, 95, 108). Some of these *Gα* mutations (i.e. Q227R of Gαs) involve replacement of a conserved glutamine residue within the G-3 region (as designated by Bourne et al (11)) of Gα subunits. Changes in this glutamine residue have generated considerable interest as analogous mutations (Q61L) in mammalian ras proteins were found to result in oncogenesis (4). In all cases involving Gα subunits and ras proteins with changes in this glutamine residue, reduced GTPase activity has been observed which presumably resulted in increased downstream signaling. We attempted to create mutations in the *Gα4* and *Gα5* genes of *Dictyostelium discoideum* that are analogous to those found in mammalian *Gα* oncogenes. This was done in order to study the effect of these mutations on vegetative and multicellular stages of development of the organism. In many cases,

the expression level of the G-protein genes that have a Q->L replacement in the conserved G-3 region is amplified by heterologous promoters or by insertion of multiple copies of the mutated gene (25, 69, 182). As artificially high expression levels can result in increased signaling, it is advantageous to study the effect of reduced GTPase activity on increased downstream signaling in a system like *Dictyostelium* where single copy insertions of the mutated gene can be achieved.

The mutations of interest in this study involved substitution of a glutamine with a leucine residue in the conserved G-3 region of the Gα subunit. Similar studies have been done before with the Gα5 subunit (70). Mutations *Gα5(Q199L)* and *Gα5(G197T*) did not seem to have any apparent effect on the rate of tip formation when a single copy of the mutant gene was used to replace the wild type gene. However, overexpression of the *Gα5(Q199L)* resulted in cell death (70).

The specific aim of this study is understanding the function of the Gα4 subunit in *Dictyostelium* development. This will be achieved by determining whether intercellular signals provided by wild type cells can rescue spore production in *Gα4* mutants. Further, it will be seen if transplantation of the missing anterior prestalk region in $G\alpha4^{HC}$ mutants will rescue spore development. This study could therefore, provide insights into G-protein mediated signaling pathways in development and cell type-specific differentiation. A second aim of this study is to analyze the effect of a Q200L mutation in the conserved G-3 region of the Gα4 subunit. This is expected to provide insights into situations where G-protein signaling is altered as in the case of cancer and other diseases.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

Cells are constantly being bombarded by signals from the external environment, and some of these signals are transduced to the cell's interior through G-proteins. G-proteins appear to regulate developmmental processes of eukaryotes such as cell division, chemotaxis, and differentiation (52, 80, 114, 137, 149).

Many developmental processes in eukaryotes involve G-proteins, and mutations in these G-proteins interfere with the normal functioning of the signal transduction pathways. Changes in G-protein function can result in outcomes as varied as oncogenesis or cell death. It is therefore important to understand the structure-function relationships in G-proteins that mediate these processes. These studies are simplified using a simple model system, *Dictyostelium discoideum*. This study aims at enlarging our understanding of the role of G-proteins in developmental processes.

Dictyostelium discoideum life cycle

The cellular slime mold *Dictyostelium discoideum* has been studied for many years as a simple model system for development and differentiation (103) . The life cycle of this cellular slime mold is shown in Figure 1. In the vegetative stage, single celled amoebae depend on bacteria for a food source which they locate by responding to folic

acid secreted by the bacteria. Upon starvation, the developmental stage of the organism ensues involving expression of genes distinct from those expressed in the single celled stage. Chemotactic responsiveness to folic acid is replaced with sensitivity to extracellular cAMP that allows the starving cells to aggregate and form a multicellular mound. This further develops into a migratory slug which travels in search of a suitable substratum upon which it can attach. The culmination of development occurs with the formation of the fruiting body structure. Early in development, particularly in the mound stage, cell differentiation occurs to give rise to two distinct types of cells (i.e. the prespore and the prestalk cells). The prestalk cells occupy the anterior portion of the mound while the prespore cells are present in the posterior and central protions (47, 65). Histochemical staining for β-galactosidase (β-gal) activity expressed from cell type specific promoters can be used to study the spatial distribution of these cells (103, 162). The prespore and prestalk cells subsequently form the spores and stalk, respectively, of the mature fruiting body. This process of differentiation and development can be manipulated to occur in a highly synchronous fashion in the laboratory, the entire process taking only 24 hours (154).

Molecular Genetics in *Dictyostelium*

Advances in molecular genetics have made the slime mold, *D. discoideum,* a convenient model system for the study of developmental life processes. This eukaryote is greatly amenable to biochemical and genetic analyses (103) . Being a haploid organism makes *Dictyostelium* a simple system to study genes, many of which are present in multiple copies in mammalian systems. Efficient transformation is possible using vectors

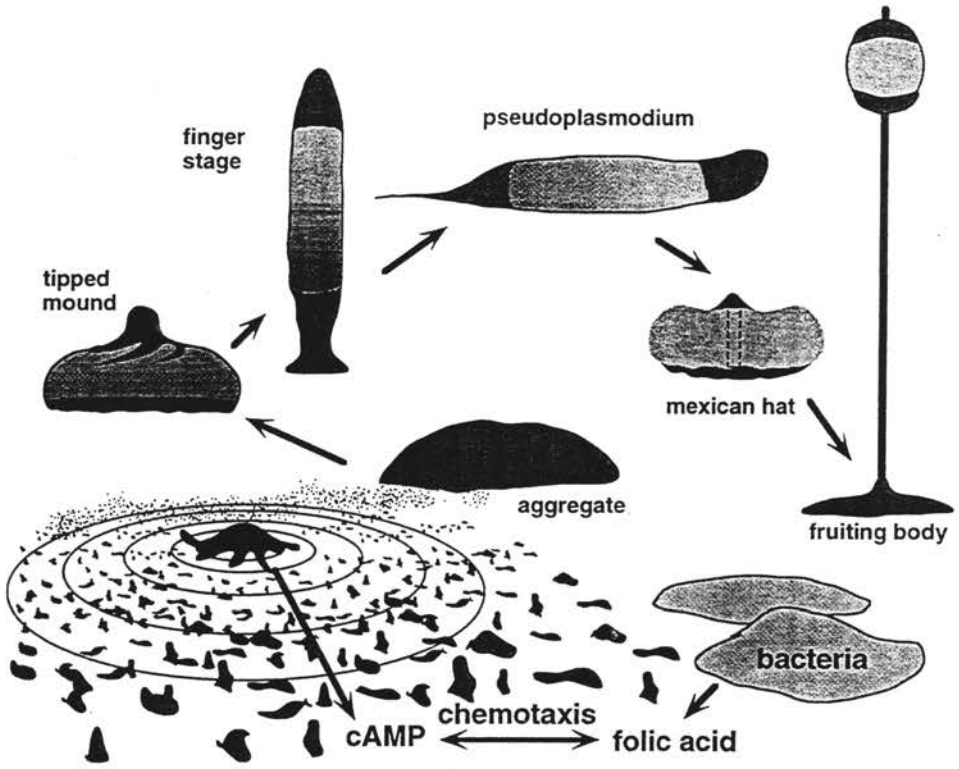


Figure 1: Developmental life cycle of *Dictyostelium discoideum*. In the vegetative stage, single celled amoebae feed on bacteria and upon starvation, aggregate in response to extracellular cAMP to form multicellular mounds. These mounds form a slug and ultimately a fruiting body at 24 hours post-starvation. The fruiting body carries the spores for dispersal which allow for propagation of the life cycle.

with a a selectable marker such as resistance to drugs like blasticidin (161) or G-418 (116). Gene disruption is one of the most common techniques employed to study gene function. This is achieved by homologous recombination which results in a loss of the phenotype conferred by the gene product. Such a null mutant can then be transformed with the original gene to determine if rescue of the phenotype occurs. For example, the analysis of *Gα2* gene was done by employing gene disruption which resulted in an aggregation deficient phenotype and the loss of cAMP receptor-mediated functions including activation of adenylyl cyclase, guanylyl cyclase and gene expression. These phenotypes were however, rescued by a vector expressing *Gα2* (91).

Until recently, *Dictyostelium* transformation vectors contained a marker gene for drug selection, usually *G-418*, that when successfully transformed into the cells and incorporated into host DNA allows growth of axenic cells in HL5 medium in the presence of the drug. The level of resistance to the drug is found to be directly related to the number of copies of the drug resistance genes inserted in the genome. It is common to find tandemly repeated copies of the drug resistance gene and vector sequence at a single site in the *Dictyostelium* genome (83).

When cells are transformed with a plasmid bearing a toxic gene along with a drug resistance marker (expressed at high levels) and then these transformants are selected using the drug, , it is quite possible that the tandem copies of the inserted genes may undergo recombination. Consequently, in such cases, single copy insertional events seem highly desirable. Genes involved in the biosynthesis of essential nutrients have been used extensively for this purpose. Cells which have the *thy1* gene disrupted require the addition of exogenous thymidine for growth in liquid media. Similarly, cells in which the

PYR5-6 gene has been inactivated require the addition of uracil for growth. Transformation of a single copy of either gene is sufficient for growth of cells on minimal medium lacking the nutrient (94). Another technique which is being used extensively to block gene expression uses the antisense approach. The introduction of vectors that constitutively produce high levels of antisense RNA to certain genes prevents translation of the mRNA of transcripts of thesegenes (102). This method can be used where non-essential genes such as the *dicoidin-I* (32) and the cellular *ras* gene (88) are involved.

G-protein mediated signal transduction: structure and function of components

Living cells are constantly being bombarded by chemical and physical signals on their surfaces. Some of these signals do not enter the cells but, instead, bind to receptors at the cell surface and initiate the flow of information to the cell's interior through transducers called G-proteins. G-protein coupled transmembrane signals require the sequential and reversible interaction of a machinery composed of three elements: a) a receptor, b) a transducer (i.e., a heterotrimeric G-protein) and c) an effector (i.e., an enzyme, ion channel or transporter). Figure 2 shows a typical example of a receptor coupled G-protein signal transduction pathway.

Receptors: The receptors for many hormones (such as gonadotropins, glucagon, etc.), light and odorants span the membrane seven times (43). In addition, receptors communicating through G-proteins include those for catechol amines, muscarinic cholinergic and $GABA_A$ eicosanoids and a number of peptide hormones and neuromodulators. When these receptors bind agonists, they promote the binding of GTP

to specific binding proteins called G-proteins. Despite the variety of agonists that stimulate the diverse second-messenger pathways activated by the family of G-protein coupled receptors, these receptors share considerable homology, reflecting their common mechanism of action. By analogy to the electron cryo-microscopy structure of the bacterial proton pump bacteriorhodopsin, it has been proposed that G-protein coupled receptors belong to a superfamily of integral membrane proteins (156). Cloning and sequence determination of genes for more than 100 members of this large family of receptor proteins which includes the light receptor, rhodopsin, show that these receptors are characterized by seven hydrophobic stretches of 20-25 amino acids, which are predicted to form transmembrane α-helices, connected by alternating extracellular and intracellular hydrophilic loops. The N-terminus of these heptahelical or serpentine receptors is located extracellularly; the C-terminus extends into the cytoplasm. Most of the primary sequence homology among this family of receptors is contained within the hydrophobic transmembrane domain, with the hydrophilic loop regions being more divergent. Important clues as to regions of receptors important for ligand binding have been obtained from a chimeric receptor approach. This strategy has been followed for the construction of chimeric α_2/β_2-adrenergic receptors (43). This approach led to the discovery that helix 7 region of these receptors was important for selective agonist binding. It also helped to localize the general regions of the β_1- and β_2-adrenergic receptors involved in selective agonist binding (43).

The structure-function relationships of G-protein coupled receptors have been the subject of extensive studies, the major approach being that of site-directed mutagenesis (141). In the emerging picture for the mechanism of receptor activation, the ligand would

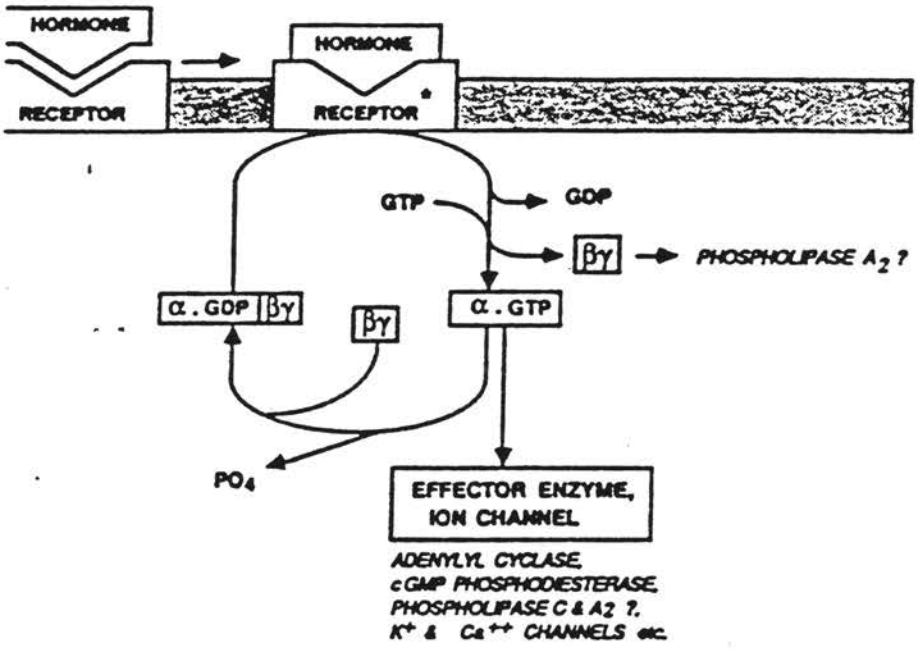


Figure 2: Schematic showing receptor activation of G-protein-regulated enzymes and ion Channels (80).

interact with specific residues of some of the transmembrane helices. In doing so, it would release a built-in constraint that maintains the unliganded receptor in its inactive state. The activated liganded receptor would then achieve a conformation allowing it to activate its cognate G-protein by way of interaction with the intracellular loops. Most of this picture comes from data accumulated on the activation of rhodopsin and adrenergic receptors. A subset of spontaneous mutations in transmembrane segments of rhodopsin was shown to activate it constitutively and cause retinitis pigmentosa or night blindness (23, 129). It is now clear that a single receptor can activate several different pathways in a given cell, although the predominant pathways may vary from one cell type to another. This observation is best exemplified by the α_2 receptor which can couple to four different G-proteins in the same cell (188). A different type of heterogeneity is exemplified by the β-adrenergic receptor which stimulates two second-messenger pathways (adenylyl cyclase and Ca^{+2} channels), both through the activation of G_s-α (8).

G-proteins: The low molecular weight GTP-binding protein family has more than 30 members and includes one of the most well studied prototypes -the ras protein. $p21^{Ras}$ (Ras) is primarily, but not exclusively, involved in signal transduction from the plasma membrane to the nucleus and regulating a wide variety of cellular processes (10). However, Ras is only one member of a diverse superfamily that includes regulators for virtually every event in the life of the cell: cytoskeletal rearrangement (Rho, Rac); nuclear import (Ran); cellular trafficing (Rab, ARF); translation (elongation factors such as EF-Tu and the signal recognition particle); mitogenesis (Ras, Rap); and receptor-mediated communication with the extracellular milieu (heterotrimeric G-proteins) (11).

The hetereotrimeric G-proteins, participate in the transduction of extracellular signals across the cell membrane. Binding of the extracellular signal to the receptor leads to activation of the G-protein which binds GTP and thereby allows it to regulate the activities of specific effector proteins within the cell. These effectors include transporters, ion channels, and enzymes, that synthesize cytoplasmic second messengers. G-proteins are thus involved in the regulation of varied processes such as growth, cellular metabolism, and cellular differentiation. G-proteins are responsible for first amplifying the signals from receptors and then directing them to the approporiate effectors. Thus, G-proteins are an important link in the complex information network from outside the cell to the inside. Signals from different receptors can be processed through one or more G-proteins to stimulate a single pathway inside the cell. Similarly, information received from a single receptor can also be directed to several different effectors through one or more G-proteins.

G-proteins are membrane associated heterotrimeric proteins composed of α, β, and γ subunits. The Gα subunit contains the guanine nucleotide binding site that in the inactivated state, is occupied by GDP. Analysis of signal transduction pathways in different systems has given rise to a simple model system to explain the process of signal transduction (43, 53, 80, 115, 136, 149). The model (see Figure 2 for G-protein activation cycle) involves activation by an external signal which leads to exchange of GDP for GTP in the Gα subunit which in turn leads to its dissociation from the Gβ and Gγ subunits.

While many external signals are yet to be identified, signals such as cAMP or folic acid have been implicated in the activation of G-protein pathways in the slime mold,

Dictyostelium discoideum (34, 124, 160). The Gα-GTP complex regulates the activity of specific enzymes and ion channels by binding to intracellular effector molecules that further amplify the signal. Low molecular weight second messengers such as cyclic nucleotides or inositol triphosphate in turn, generate dramatic intracellular changes including selective protein phosphorylation, gene transcription, cytoskeleton reorganization, secretion and membrane depolarization.

The Gα subunit is the primary transducer of the signal but in some cases such as the yeast mating response, the Gβγ subunits can assume this role (182). It was assumed for a long time that the Gα subunit bound to GTP activated effectors, while the Gβγ subunits acted as a negative regulator. Release of free Gβγ from an abundant G protein, such as G_i, was thought to deactivate other Gα subunits by forming inactive heterotrimers. Indeed, the Gβγ subunits can block activation of adenylyl cyclase by this mechanism (58). This paradigm changed with the discovery that the Gβγ subunit could activate the muscarinic K^+channel and the realization that Gα and Gβγ subunits positively regulate effectors (101). Subsequently, the Gβγ subunit was shown to be a positive regulator of a large number of effectors including adenylyl cyclase, phospholipase C_β (PLCb), phosphoinositide kinase (P13-kinase) and β-adrenergic receptor kinase (19). The Gβγ subunit may also act through ras to activate mitogen-activated protein (MAP) kinase pathways (31, 49). It is now known that many effectors are regulated both by Gα and Gβγ subunits, although the pattern of regulation is specific to the effector subtype (164). One subtype of adenylyl cyclase is activated by Gα and unaffected by Gβγ, a second subtype is activated by Gα and synergistically activated further by Gβγ, and a third subtype is activated by Gα but inhibited by Gβγ subunits. The Gα subunit has intrinsic

GTPase activity which is used to hydrolyze the GTP, allowing it to reassociate with the Gβγ dimer thus completing the activation cycle. At times, the downstream effector functions as a GTPase activating protein (GAP) to enhance the GTPase function of the Gα subunit. An example of a GAP is phospholipase C-β1 (PLC-β1), which accelerates GTPase activity of Gq/11, the G protein that stimulates PLC-β1 in response to M1 muscarinic cholinergic receptors. The intrinsic rate of GTP hydrolysis varies from one type of α subunit to another (17, 98). The rate of GTP hydrolysis appears to be a timing mechanism that controls the duration of both Gα and Gβγ subunit activation. Reactivation turns off both subunits and primes the system for another cycle. Thus, although the Gβγ subunit does not bind GTP, its active lifetime depends on the rate of GTP hysdrolysis by an α subunit. Overexpression of the Gα, Gβ or Gγ subunits of the G-protein results in increased activation of downstream effectors (25, 183). Similarly, loss of the subunits could result in a loss of signal transduction (93).

Gα subunit: The Gα subunits are a family of 39-52 kDa proteins which share 45-80% amino acid sequence similarity (132). They have been divided on the basis of their amino acid sequences into 4 classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ (177). Sequence homology among the Gα subunits and similarity of their biochemical properties suggest that their tertiary structures are similar and that they share definable functional domains (76, 100). The Gα chains of G-proteins can be specifically ADP-ribosylated by bacterial toxins. G_s and transducin are targets for cholera toxin, whereas G_i, G_o, and transducin are targets for pertussis toxin (57). Cholera toxin modifies an arginine residue located in one of the nucleotide binding regions of the Gα-subunit of G_s and G_t. A functional consequence is the abolishment of the endogenous GTPase activity resulting in continuous activation of

the Gα subunit. In contrast, pertussis toxin catalyses the transfer of an ADP moiety from NAD^+ to a cysteine residue of the α-subunit of the G_i/G_o and G_t. Since, the modified cysteine residue is located four residues upstream from the carboxy terminus, pertussin toxin-catalysed ADP-ribosylation prevents interaction of the receptor with the G-protein representing a functional receptor-G-protein uncoupling. Nevertheless, pertussin toxin-modified G-proteins still possess the capability to interact with effectors.

It seems likely that G-proteins evolved by duplication and divergence of a common ancestral gene. There are 23 mammalian Gα subunit genes which vary in range of expression as well as receptor-effector coupling. Their predicted amino acid sequences show at least 40% identity corresponding to conserved regions involved in guanine nucleotide binding (153). Before the crystal structure of Gα proteins was available, the understanding of the Gα chain's GDP/GTP binding structure relied upon the conservation of the GDP/GTP binding domain between G proteins, $p21^{Ras}$ and EF-Tu since, crytsal structures existed for these two proteins. The Gα subunit can be classified into five different regions that are critical for guanine nucleotide interactions. These are as follows:

i) The G-1 region or A region: The consensus sequence for this region is Gly-X-X-X-X-Gly-Lys. In Ef-Tu, this region is near the α-phosphate of GDP. The lysine is thought to neutralize the negative charge on the phosphate. Mutations in analogous regions of Ras reduce GTP binding and activity. Similar sequences are found in other purine nucleotide binding proteins.

ii) The G-2 region: This region appears to be important for Gα-subunit interactions with the effector. It is also observed to be important for GTPase activity.

iii) The G-3 or C region: The consensus sequence for this region is Asp-X-X-Gly. In the Ef-Tu structure, aspartic acid chelates a magnesium ion which is closest to the β-phosphate of GDP while the glycine forms a hydrogen bond with GDP γ-phosphate. The glycine thus forms a 'pivot' for conformational change induced by GTP. A mutation of Asp-X-Ala-Gly to Asp-X-Thr-Gly in ras results in autophosphorylation of the threonine if GTP is used as a substrate. Mutation of other residues near this sequence in Ras results in reduced GTPase activity (100).

iv) The G-4 or G region: This region has a consensus sequence Asn-Lys-X-Asp and is appropriately named as it determines nucleotide binding specificity. The aspartate forms hydrogen bond with guanine ring and the amides of asparagine and lysine stabilize the GDP/GTP binding site by hydrogen bonds to residues in the G-1 region. A mutation of aspartic acid to asparagine in Ef-Tu alters the nucleotide specificity of the protein to favor xanthine diphosphate over guanosine diphosphate (100). Mutations in this region of Ras reduce, alter, or abolish nucleotide binding (50).

v) The G-5 or I region: This region may be involved in nucleotide binding but has not been very well defined. It is located after the G-4 region and contains a cluster of invariant amino acids.

In addition to these five regions, an E region has been described as per the nomenclature of Halliday (72). This region is characterized as a very hydrophobic region between the C and G regions. It oftens contains an alanine that is located 26 amino acids distal to aspartic acid in the C-region consensus sequence. In Ras, mutation of the analogous alanine to threonine results in 30-fold reduction in GTP affinity (50). The consensus sequences in domains G-1 through G-4 are signatures for a large number of GDP/GTP

binding proteins (11).

A new era in understanding the structural basis for Gα subunit function was born with the elucidation of the crystal structure of GTP- and GDP-liganded transducin and $G\alpha_{11}$ (26, 118). The crystal structures of these proteins suggest that all Gα subunits share a common overall structure comprised of two major domains: Gα-guanine nucleotide domain and a Gα-helical domain that may serve to keep the guanine nucleotide tightly bound as well as to enhance the GTPase activity of the Gα subunit (26, 110). Three regions within the Gα subunits that have highly conserved sequences fold to form the nucleotide binding site. Each Gα subunit has a distinct receptor binding domain that is structurally and functionally conserved but is sufficiently distinct to be selective for appropriate receptors. Structures of the GDP and GTP bound Gα subunits highlight two residues critical for normal GTPase activity: glutamine 227 (equivalent to 61 of ras) and arginine 201 (the site of the cholera toxin catalysed ADP-ribosylation) in G_s-α. The first 25 amino acids of the Gα subunit appear to be essential for βγ binding (55), but their position is unknown as they are mobile and do not show in the crystal (26). The carboxy terminal region is thought to be involved in receptor binding or with receptor-regulated nucleotide exchange. This region is the site of the unc mutation in α_s, which blocks the ability of G_s to respond to receptors (159), and which in the $G\alpha_{is}$ and $G\alpha_o$ is ADP-ribosylated by pertussis toxin (180). The C-terminus has an important role in defining the specificity of G-protein receptor interactions, at least for some G-proteins. Conklin et al (27) coupled $G\alpha_q$ with receptors that normally interact with $G\alpha_{1-2}$ by replacement of three amino acid residues at the C- terminus but are found to interact with different receptors (89). The C-terminus is thus not the only determinant of specificity.

Equivalent mutations of the GTP-binding site in different Gα subunits seem to cause very similar phenotypes as the GTP-binding site is their most conserved part.

Gβ subunit: The Gβ subunit is predicted to contain two types of structures: an N-terminal region thought to form an amphipathic α helix such as might form coiled coils (106), followed by seven repeating units of approximately 43 amino acids each (149). The repeating units in β are examples of a class repeating sequences (WD repeats) found in a family of proteins engaged not only in signal transduction, but also in control of cell division, transcription, processing of pre-mRNA, cytoskeletal assembly and vesicle fusion. The Gβγ subunit has been known to interact directly with at least seven kinds of proteins, none of which have any obviously common βγ binding motif. The number of different interacting proteins suggests that no single repeating unit specifies a partner and that each partner interacts with more than one repeating unit. The Gβ and Gγ subunits bind very tightly to each other and can only be separated by denaturants.

Gγ subunit: The Gγ subunit is predicted to be largely α-helical (106). The Gα subunit seems to able to interact with both Gγ as well as Gβ (128). Binding of GDP-liganded Gα subunit to Gβγ blocks the ability of Gβγ to stimulate effectors (101), either because Gα–GDP induces a conformational change in Gβγ, or because it covers the site of Gβγ-effector interaction.

Effectors regulated by G-proteins: Effectors regulated by G-proteins are diverse as they include adenylyl cyclases, phosphodiesterases, phospholipases, potassium channels and phosphoinositide 3 kinase (7, 19, 166, 191) (Figure 2). Both Gα and Gβγ subunits have been demonstrated to activate phosphodiesterases, adenylyl cyclases and phospholipase

C. Two of the relatively well defined G-protein-effector interactions include the G_t-phosphodiesterase and G_s-adenylyl cyclase.

Phosphodiesterase: The phosphodiesterase isolated from bovine retinal rod outer segment is a heterotrimeric protein composed of an α subunit of 88 kDa and a β subunit of 84 kDa (73). Two identical γ subunits of 11-13 kDa are firmly attached to the $\alpha\beta$ complex (73). The $\alpha\beta$ complex is the catalytic domain which can be blocked by the γ subunits. Accordingly, in order to activate phosphodiesterase, the γ subunits must be deactivated or removed. Direct regulation of the phosphodiesterase by rhodopsin and transducin (G_t) has been demonstrated in reconstituted phospholipid vesicles containing the purified proteins (157).

Phospholipase C: Inositol phospholipid specific phospholipases C (PLCs) catalyze the hydrolysis of the three common inositol containing phospholipids, generating second messengers, diacylglycerol and IP3 (117, 133). Protein isolation and molecular cloning studies have revealed the existence of multiple PLC-isoenzymes in mammalian tissues (134). The various PLC isoforms appear to be activated by different receptors and different mechanisms. The catalytic activities of all three types of PLCs (α,β,γ) are dependent on Ca^{+2} (73).

Adenylyl cyclase: Adenylyl cyclase exists as multiple molecular species. The cyclase has been purified from heart (126) and brain (30) using affinity chromatography techniques. The enzyme appears to be a single polypeptide with a molecular weight of approximately 150,000. Most adenylyl cyclases are associated with the plasma membrane, although certain bacterial enzymes and perhaps, one form in mammalian sperm, are cytosolic (163). The regulation of adenylyl cyclases is complex with some being activated by the

Gα subunits while others are inhibited by them. A case in point is the activation of type I adenylyl cyclase by GTPγS-bound G_s- α, although this activation is inhibited by Gβγ-subunits. On the other hand, type II and type IV adenylyl cyclases are only weakly activated by G_s- α alone, but activation is enhanced by Gβγ-subunits (56).

Ion channels: Several examples of Gβγ-subunits activated ion channels have emerged in recent times. A single G-protein may have several membrane targets (15) including more than one type of ionic channel (145), and G-proteins may wire these targets into membrane circuits capable of producing complex electrical and metabolic responses. Examples of ion channels modulated or regulated by G-proteins include muscarinic atrial K^+ channels, photoreceptor non-selective cation channels, voltage gated Ca^{+2} channels and Na^+ channels (15). The identification of single channel K^+ currents of atria that were activated by adenylyl cyclase was made in isolated heart tissue where the activation mechanism was seen to be dependent on the G-protein, G_k (14, 139). Similarly, K^+ channels were also demonstrated to be regulated by G_k through the action of adenylyl cyclase (192) in rat pituitary cell membranes. G-proteins also regulate Ca^{+2} channels from rabbit skeletal T-tubules (15) and from guinea pig or bovine cardiac sarcolemma (15).

Other effectors of G-proteins include membrane transport proteins such as in the case of Mg^{+2} transport which is inhibited by a G-protein dependent, cAMP independent mechanism (136).

Role of subunit diversity in signaling mediated by G-proteins:

So far, 23 different mammalian Gα subunit genes have been cloned. Also, at least

5 mammalian Gβ subunits and >10 Gγ subunits have been identified. Some degree of specificity has been observed by which Gβ subunits form dimers with Gγ subunits and Gα subunits form heterotrimers with Gβγ dimers. Despite the limitation of specificity, a large number of combinatorial possibilities exist by which heterotrimers can be formed. This resultant diversity could account for the specificity of receptor-effector coupling. In the simplest possible case, a single receptor could couple to a G-protein which in turn activates an effector (such as an enzyme or an ion channel). This type of signaling is shown in Figure 3A. However, most cells will probably exhibit more complexity in their signaling pathways. Several G-protein coupled receptors exist in a cell which can therefore, associate with a variety of G-proteins to regulate intercellular signaling processes. It is quite possible that several receptors can converge on a single effector system (Figure 3B). An example of this type of signaling is the activation of adenylyl cyclase to generate cAMP (75). Such convergent receptor signaling was observed in the analysis of cyc⁻S49 cells that lack α_s but showed loss of adenylyl cyclase activity to two other hormones (135). Further, disruption of a $G\alpha_i$ gene in the mouse cells has been demonstrated to result in parallel decreases in receptor mediated inhibiton of adenylyl cyclase in response to three different agonists (138). It is also now clear that receptors can activate more than one G –protein subtype, leading to divergent signaling pathways in cells (Figure 3C). This was effectively demonstrated in the case of the β-adrenergic receptor which could activate G_t as well as G_i subunits (75). Yet another example of divergent G-protein pathways activated by the same receptor subtype is that of activation of the G_i and G_s subunits by the α_2-adrenergic receptor (75). In transfection experiments, the coupling of the human TSH receptor to G_i, G_z, G_s and G_q was demonstated (96).

Another mechanism by which cells may have bifurcating pathways is through use of dual signaling whereby upon activation of the agonist bound receptor, the Gα-subunit conveys a signal to effectors, but the Gβ-subunit can also convey part of the information (19). Several cloned receptors such as the M_2 and M_4 muscarinic receptors have been seen to mediate inhibition of adenylyl cyclase and activation of phospholipase C (63). In this type of dual coupling, adenylyl cyclase inhibition occurs at low agonist concentrations and is independent of receptor density. Activation of phospholipase C on the other hand, requires considerably higher concentrations (20-50 fold) and is directly correlated to receptor abundance. Activated Gα subunits are thought to mediate inhibition of adenylyl cyclase, whereas Gβγ subunits released by activated G_i are thought to activate phospholipase C. Thus, considerable diversity seems to exist in signaling pathways in a living sytem. G-protein mediated signal transduction can be looked upon as a highly complex network that allows some cross talk due to the presence of diverging and converging transduction steps, but also provides dominant pathways which guide the main flow of information.

G proteins in *Dictyostelium*:

The slime mold, *Dictyostelium discoideum* provides a convenient model for studying signal transduction processes. G-proteins in *Dictyostelium* have been the focus of intensive research. Thus, at least eight genes encoding Gα subunits and one gene encoding a Gβ subunit have been identified (13, 71, 91, 127, 190). There appears to very little identity between the different Gα subunits, outside of the region found to be homologous in other eukaryotes. The Gα subunits exhibit different temporal expression

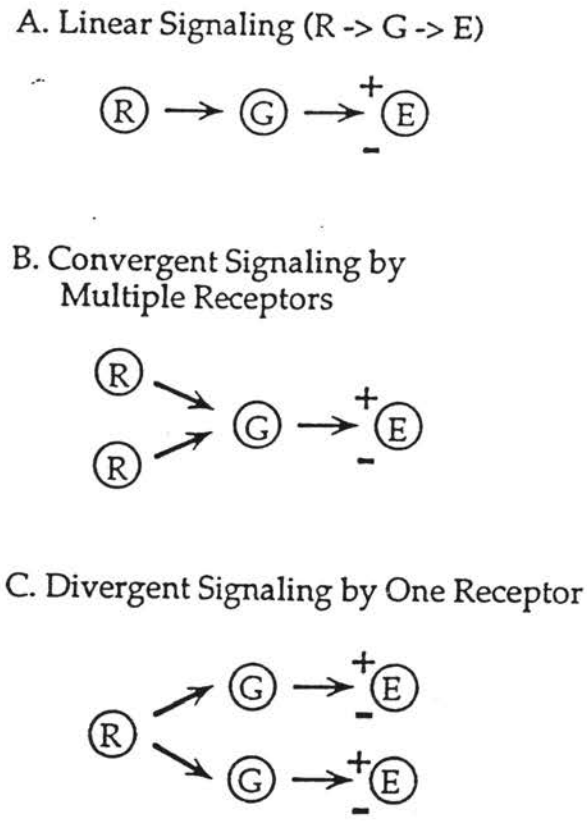


Figure 3: Signaling mechanisms based upon receptor/G-protein interactions. A. Linear Signaling. B. Convergent Signaling by Multiple Receptors. C. Divergent Signaling by One Receptor (75).

patterns and are therefore unlikely to share identical functions in development. Four of the eight Gα subunits (Gα2, Gα3, Gα4 and Gα5), play important roles in development, as suggested by analysis of null mutants created by gene disruptions (13, 71, 91). Figure 4 shows temporal expression patterns of the *Gα* genes as well as the cAMP-binding receptors in *Dictyostelium*. Of the eight subunits known, the Gα6 is expressed exclusively in the growth or vegetative stage of development; Gα1, Gα2, Gα3, Gα7 and Gα8 are expressed during early development; and the Gα4 and Gα5 are expressed primarily in the multicellular stages. The eight Gα subunits share about 35-50% amino acid sequence identity with each other and with their mammalian counterparts. Although they do not fall into distinct classes or subfamilies, they do contain all of the highly conserved features of the guanine nucleotide binding domains found in mammalian Gα-subunits. The Gα8 subunit is different from the rest in that it has a longer and more divergent C-terminal region which may be the site of receptor interaction (189). The functions of seven of the eight Gα subunits have been elucidated by gene disruptions. Only the Gα2, Gα3, Gα4 and Gα5 show striking phenotypes due to gene disruption; deletion of others has not displayed major growth or morphological aberrations. *gα1* null mutants show slight abnormal morphogenesis amd conditional defects in phospholipase C (PLC) regulation (125).

The *Gα2* gene is normally expressed at low levels during vegetative growth and at high levels during the aggregative stage of development. *gα2* null mutants are aggregation deficient and are unable to respond to extracellular cAMP, which mediates aggregation of cells to form a multicellular mound during early part of development (160). These mutants show no cAMP-induced stimulation of adenylyl cyclase, guanylyl

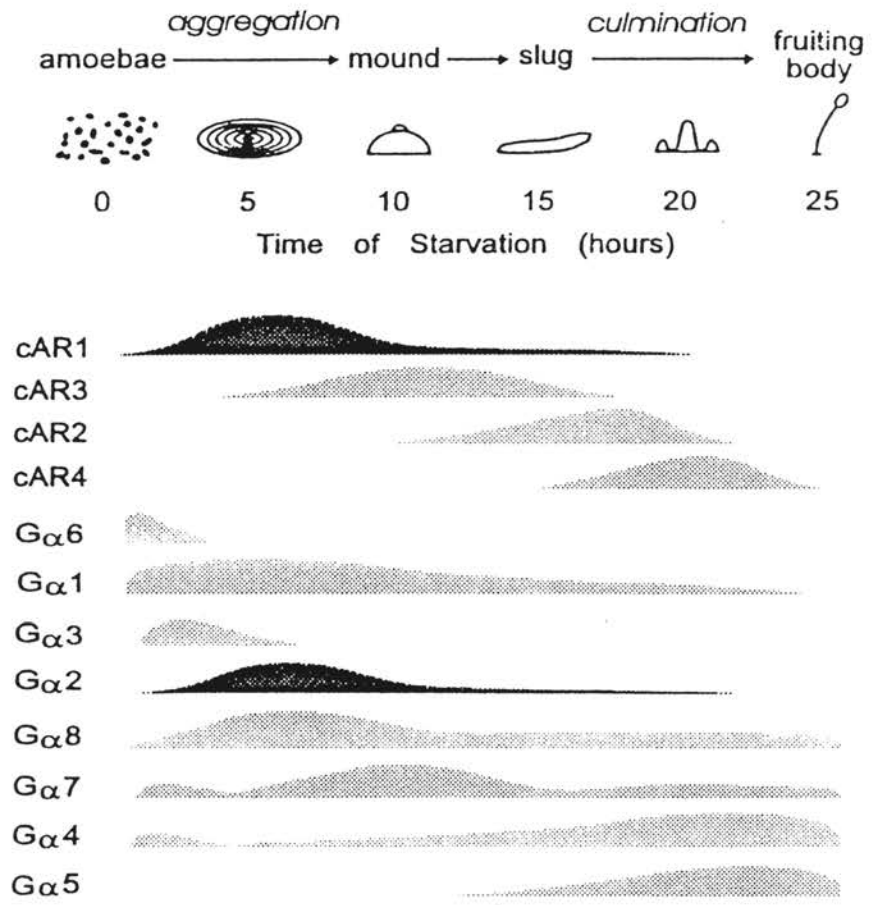


Figure 4: Time course of expression of major RNA transcripts in *Dictyostelium*. Top panel illustrates the developmental stages appearing after the onset of starvation. The expression patterns of cAMP receptors and Gα subunits are shown below. Components essential for early development are heavily shaded (38).

cyclase, PLC or actin cytoskeleton. The *gα2* null mutants have also been tested to determine whether receptors for other agonists use Gα2. Folic acid-induced responses appear to be normal in these cells (87, 91), while platelet activating factor (PAF) seems to mediate some of its effects through the Gα2 subunit. The intracellular concentration of cAMP increases following the addition of PAF (125). The *Gα4* and *Gα5* genes are primarily expressed during multicellular stages of development and to a lesser extent, during vegetative growth (68). The *Gα4* and *Gα5* genes encode the two most closely related subunits (51% amino acid sequence identity) of the eight identified in *Dictyostelium*. However, these two closely related subunits play different roles in development, as suggested by the phenotypes of the respective null mutants (71). The *gα5* null mutant does not show any morphological abnormalities but does show a delay in tip morphogenesis (70). The focus of this study is the *Gα4* gene that is expressed primarily during multicellular development (71). The developmental phenotypes of *gα 4* null and overexpression mutants suggest that Gα4 function is required for proper spore production (68) (see Figure 5). Null mutants of *Gα4* show both growth and developmental phenotypes (68). On bacteria, *gα4* null cells exhibit slower growth rates than wild-type cells. Upon starvation, *gα4* null mutant cells aggregate to form multicellular mounds but terminate development as mounds with extended tips. These phenotypes are consistent with the expression pattern of the Gα4 subunit: low in vegetative phase, absent in the aggregation stage and at high levels after the mound stage of development. Morphologically, the *gα4* null cells are blocked in development and exhibit a finger morphology as their terminal phenotype. *gα4* null cells appear to suffer from a deficiency in the production of prespore cells. The $G\alpha4^{HC}$ cells form large

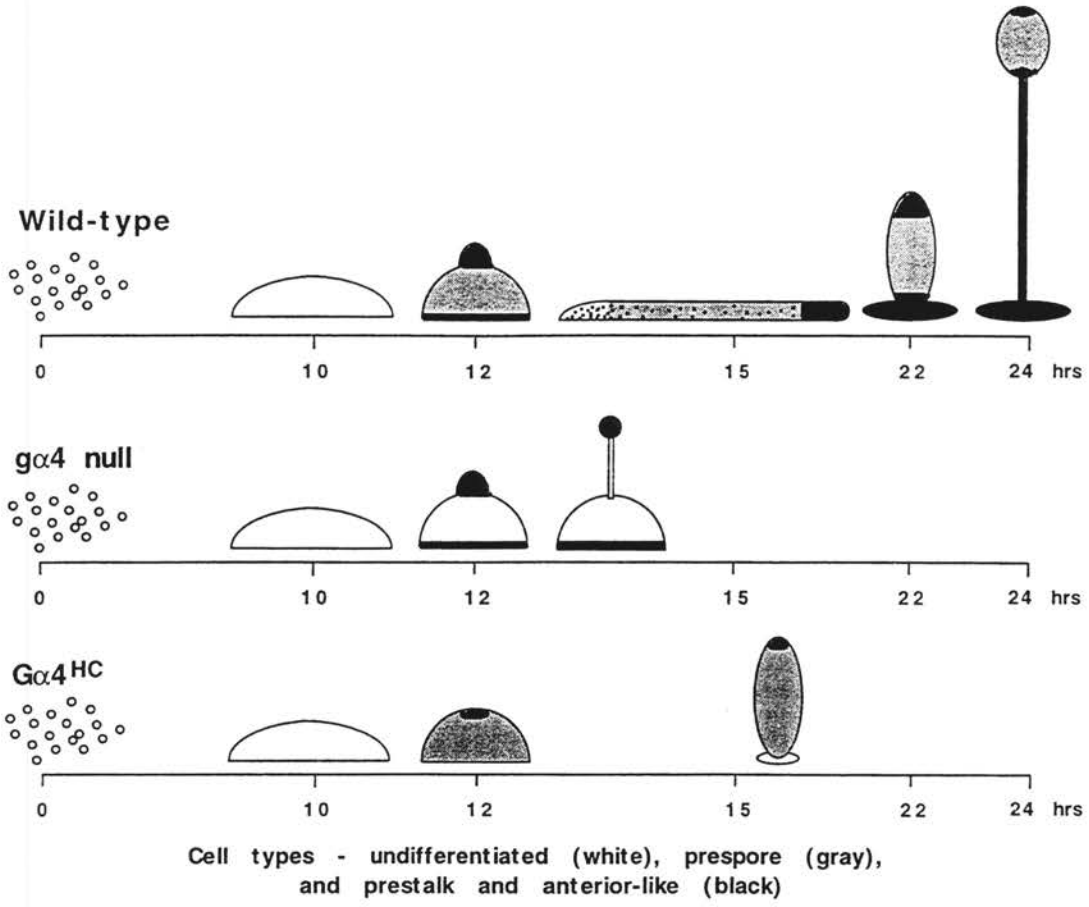


Figure 5: Developmental phenotypes associated with the *Gα4* mutants and wild type cells over time.

mounds and are also blocked in development. In contrast to the *gα4* null mutants, cells that contain high copies of the *Gα4* gene, $G\alpha4^{HC}$, are deficient in prestalk cell production as suggested by their inability to form proper tips on aggregates. $G\alpha4^{HC}$ cells are also deficient in spore production but, this deficiency can be overcome by developing them in presence of wild-type cells (71).

Biochemical analysis of wild-type and *gα4* null cells has revealed that Gα4 couples to specific subtypes of folic acid receptors expressed in vegetative and differentiated cells (69). Although, *gα4* null cells do not exhibit folic acid mediated responses, they retain normal cAMP-mediated adenylyl and guanylyl cyclase activation as well as chemotaxis (68).

The identification of multiple G-proteins in *Dictyostelium* has raised the possibility of there being functional redundancy. It is not entirely clear whether several G-protein pathways converge on a single effector system or whether these pathways function independently of one another. Although, chemoattractants such as folic acid and cAMP bind to different cell surface receptors (171) and intracellular G-proteins, it is believed that at some point the signals merge into one pathway. The cGMP responses to folic acid and cAMP are not additive, thereby suggesting that these two pathways converge at or before activation of guanylyl cyclase (170) (Figure 6). This points towards the possibility that inhibiton or activation of one G-protein pathway may affect other G-protein pathways.

Signal molecules regulating growth and gene expression in *Dictyostelium*.

Throughout the *Dictyostelium* life cycle, several secreted factors regulate the expression

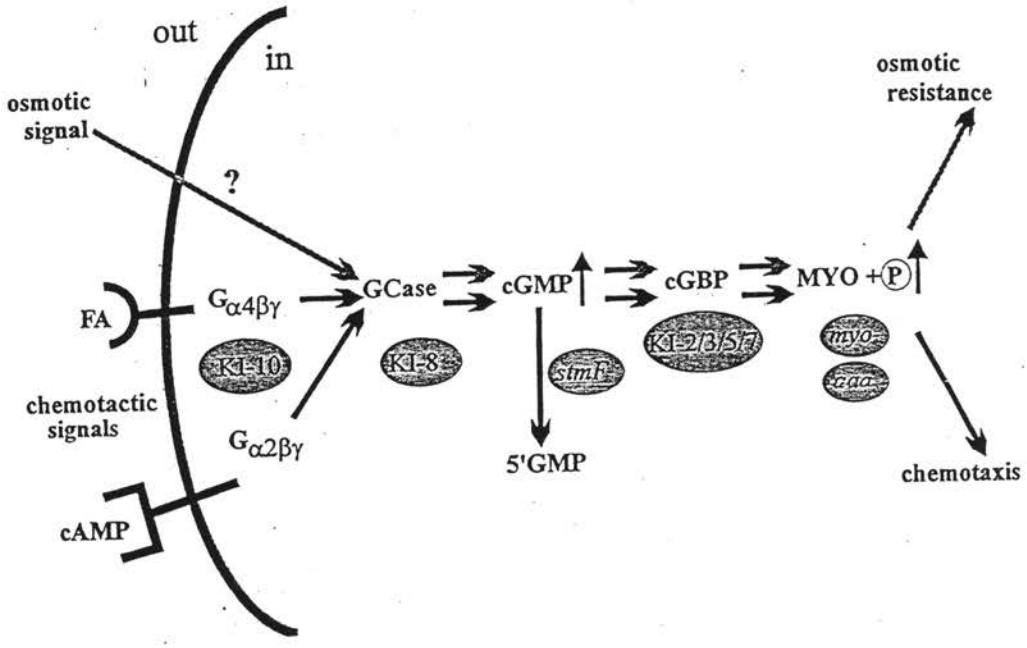


Figure 6: Model of signal transduction of chemoattractants and osmotic stress via cGMP in *Dictyostelium*. Folic acid (FA) and cAMP bind to different surface receptors that interact with different G-Proteins. These signals meet before guanylyl cyclase(Gcase) producing the second messenger cGMP, that interacts with a cGMP-binding protein(cGBP) or is degraded by a cGMP-specific phosphodiesterase. Besides, guanylyl cyclase, chemoattractants activate several other second messenger pathways that are probably not involved in chemotaxis (172).

of genes essential to growth, development and differentiation. The best studied signal molecules in *Dictyostelium* are cAMP and folic acid. Both of these signaling molecules involve the use of extracellular enzymes to degrade the signal, i.e. cAMP phosphodiesterase and folate deaminase, respectively (54, 124). Also, cAMP and folic acid interact with cell surface receptors that are coupled to G-proteins (35, 173). During development, cAMP pulses secreted by the starving *Dictyostelium* cells allow them to aggregate. cAMP also serves to regulate the expression of genes involved in differentiation. Extracellular cAMP is important in the early as well as late stages of development and its function during aggregation has been extensively characterized. It is spontaneously secreted at six minute intervals by small groups of cells and in turn, initiates propagated waves of cAMP that guide chemotactically sensitive cells to the centre of aggregation. The propagation of waves of cAMP results from the relay of the chemotactic signal, as cAMP induces the transient synthesis and secretion of additional cAMP. The system is resensitized by degradation of the signal by an external phosphodiesterase. This relay system allows cells to communicate over a distance of several centimeters. The components that are essential for the process include cAMP receptors (cARs), phosphodiesterase, adenylyl cyclase and other molecules that regulate their functions. The surface receptors that bind cAMP are encoded by four genes, cAR1, cAR2, cAR3 and cAR4 which are expressed transiently at different stages of development (Figure 4). Mutants with cAR1 deleted are defective in propagation of and response of cAMP signals. As cAR3 is expressed at a later stage in development, deletion of this receptor is required in addition to the cAR1 in order to abolish all responses to exogenous cAMP (78). Ectopic expression of cAR2 or cAR3 in car1-/car3-

cells rescues many of the functions of cAR1. Thus, each of these receptors appear to be coupled to the same signal transduction pathways. It is likely that the cAMP receptors differ in affinities so as to function appropriately in the environment in which they are expressed. When the cells are solitary, they express the higher affinity receptors but switch to expressing lower affinity receptors when they aggregate. So, cells appear to maintain similar mechanisms of signal transduction and regulation but respond to changes in their cellular environment by switching receptor subtypes. Persistent activation however, leads to desensitization of the response. Of the four cARs, cAR2 has been demonstrated to be coupled to the Gα2 signal transduction pathway in *Dictyostelium discoideum* (142).

Folic acid secreted by bacteria allows for the *Dictyostelium* amoebae to locate their food supply. In addition, folic acid also regulates gene expression (9). The coupling of folate receptors to G-proteins has been infererred by experiments where there is a decrease in receptor affinity towards folic acid in the presence of guanine nucleotides (35, 36). Therefore, in addition to early development, the Gα4 subunit is absolutely required for chemotaxis to folic acid, a mechanism by which *Dictyostelium* cells seek out nearby bacterial food sources (124). Folic acid binds cell surface receptors and activates chemotactic movement as well as a rapid accumulation of cAMP (~12 sec to reach maximum level), rapid association of actin to the cytoskeleton and a slower accumulation of cAMP (~8 sec to reach maximum level) (34, 35, 111). When feeding on a bacterial lawn, *Dictyostelium* cells will create a clearing or plaque which is dependent on the ability of cells to respond to folic acid. $G\alpha4^{HC}$ cells are supersensitive to folic acid and produce plaques at a faster rate than wild type cells on a bacterial lawn (68). Gα4

function is required for prespore cell development, as well as all known responses to folic acid in *Dictyostelium*, suggesting a possible role for folic acid as a developmental morphogen that activates the Gα4-mediated signal transduction pathway.

Monapterin, a pterin compound similar to folic acid, has been found to trigger a chemotactic response in the multicellular stage of development but is unable to elicit cellular responses before aggregation (168). The developmental specificity of the response to monapterin suggests the presence of another class of pterin receptors during multicellular development that are distinct from those found in cells prior to aggregation. All of these deveopmental responses to pterin compounds are dependent on Gα4 function which points towards involvement of the Gα4 subunit in signaling pathways driven by pterin compounds during multicellular development (69). Folic acid is also known to be the signaling molecule mediating cellular aggregation in the development of *D. minutum*, a species related to *D.discoideum* (36). The cellular composition of the multicellular aggregates in *D.minutum* appears to be different in that it is composed entirely of prespore cells. This is in contrast to the situation in *D. discoideum* where roughly 80% of the multicellular aggregate is composed of prespore cells with the prestalk cells accounting for the remaining population. If folic acid is involved in prespore cell differentiation, it would be expected to bias cell ratios in favor of a prespore population in *D. minutum*.

Differentiation growth factor (DGF) is secreted by vegetative cells and is believed to regulate mitogenesis (181).

Conditioned media factor (CMF) is secreted by starving cells and serves as an inducer of prestalk and prespore cell differentiation (21).

Differentiation inducing factor (DIF-1) has been shown to induce the expression of prestalk-specific genes and to inhibit prespore development (84).

Ammonia which is released as a byproduct of protein catabolism, inhibits the culmination stage during development (62).

Prestarvation factor (PSF) is secreted throughout vegetative growth and enables *Dictyostelium* amoebae to monitor their own density in relation to the bacterial food supply. PSF also induces the expression of a number of genes involved in early development (21). Several genes have been observed to be regulated by PSF in *Dictyostelium*. Among these are genes important for cell streaming (discoidin-I), cAMP signaling (cAMP receptor, cAR1 and the cyclic nucleotide phosphodiesterase), cell-cell adhesion (gp24 or contact sites B) and lysosome function (α -mannosidase) (130, 131).

The expression of *discoidin-I* is induced by PSF during vegetative growth at the late exponential phase (22, 131). During multicellular development, discoidin expression is induced by CMF which is secreted by starving cells (61, 112). *Discoidin-I* expression also appears to be downregulated by cAMP during aggregation (174, 185). In contrast, *discoidin-I* expression is upregulated in axenic mutants when grown in axenic medium (85, 174). The promoter regions upstream of the *discoidin-Ir* gene which were sufficient to regulate expression were found to include the 'discoidin induction element' (dIE) that is responsible for developmental activation of the gene (174). It was also observed that exogenous folic acid can repress transcription via the dIE element (9). Thus, folic acid can be used to repress discoidin gene expression. It has been reported that wild type cells produce high levels of discoidin at low cell densities in axenic medium and there is only a moderate (two to three-fold) increase in discoidin mRNA accumulation with the onset of

development. Analysis of the effectiveness of the discoidin promoter has been done by using pAV-CAT-Disc fusions and testing for transcriptional activity using CAT assays. These studies have reported that expression of discoidin genes during development is two-fold higher in the absence of folic acid as compared to when cells are treated with folic acid (9). Therefore, downregulation of discoidin expression mediated by folic acid does not appear to be very significant at this stage.

The *discoidin-Ir* gene promoter as a repressible expression system

The functions of essential proteins cannot be determined by the usual genetic techniques of gene disruption or blockage of gene expression using an antisense transcript. The use of an inducible system which allows for controlled expression of the gene at will, enables one to overcome the problem of lethality associated with that particular essential gene. The *Dictyostelium* transformation vector, pVEII consists of the discoidin promoter upstream of a multiple cloning site (9). This allows for the cloning of the gene of interest downstream of the discoidin promoter which can then be controlled using exogenous folic acid. Originally, the discoidin promoter construct was used for the controlled expression of an antisense transcript for calmodulin, an essential protein. The gene expression was controlled at low density or by addition of exogenous folic acid. At high cell density, the absence of normal calmodulin levels caused the cells to be unable to complete cytokinesis. This allowed the elucidation of another role for calmodulin (99).

The folate regulated discoidin expression system has also been used to overexpress a truncated *cyclin B* transcript. It was found that overexpression of this gene led to mitotic arrest and a modification of the developmental patterns of cell type

differentiation (105). Recently, this inducible system has also been used for the overexpression of an activated *rasG* gene during growth. Transformants that expressed the activated *rasG* gene failed to aggregate upon starvation and therefore could be defective in cAMP production (88). Thus, the discoidin expression system under the control of folic acid has been shown to be a powerful tool for the analysis of functions of essential genes.

Defects in G-protein and G-protein receptors as a cause of disease

In the past three decades, the tremendous amount of research in the field of G-protein coupled signaling has contributed to a wealth of information in this area. G-proteins convey information through diverse extracellular first messengers by coupling a distinct class of receptors, called G-protein-coupled receptors (GPCRs) to diverse effectors. The extensive study of all components involved in G-protein signaling has permitted the identification of specific defects in the transduction of these signals which result in a wide variety of human diseases. The first evidence that G-protein dysfunction could cause human disease involoved the actions of the cholera and pertusssis toxins . Since then, many mutations have been identified in both G-proteins and GPCRs in numerous disorders. It is impotant to note that defective signal transduction could be a result of qualitative as well as quantitative alterations in GPCRs and/or G-proteins, including changes in expression level, post-translational modifications and mutations. The major determinants of phenotypic expression of mutations in G-proteins and GPCRs include i) the range of expression of the mutated gene as a mutation in a ubiquitously expressed gene will cause more generalized manifestations than those caused by a

mutation in a gene which is restricted in expression; ii) whether the mutation is germline or somatic in nature as in general, germline mutations of genes with restricted range of expression and somatic mutations of ubiquitously expressed genes will cause more focal manifestations; iii) whether the mutations cause gain versus loss of function.

Loss of function mutations include those that block normal mRNA and/or protein synthesis as well as mutations that permit protein synthesis but block normal membrane targeting (step 1 of Figure 7). Many GPCR mutations have been observed to cause loss of function by impairing agonist binding to receptor or receptor activation by agonist (step 2 of Figure 7) or by preventing receptor coupling to and activation of G-protein (step 3 of Figure 7). For G-proteins, loss of function mutations include those that block receptor coupling or activation by GTP (step 3 of Figure 7) or those that impair effector interaction (step 4 of Figure 7). Gain of function mutations cause inappropriate or constitutive activation. A constitutively activated GPCR will lead to activation of the pathway in the absence of agonist binding. Likewise, a constitutively activated G-protein will result in signaling to the downstream effectors in the absence of upstream signals conveyed by agonist bound receptor. Such mutations may either result in the acclerated release of GDP, leading to receptor independent activation (step 3 of Figure 7) or prevent the hydrolysis of GTP which terminates GTP activation (step 5 of Figure 7). Examples of loss of function mutations in GPCRs leading to defective signaling include those of the TSH, LH and GHRH receptors (152). Gain of function mutations of the GPCRs include those in the LH receptor causing familial male precocious puberty (152). Loss of function mutations in G-proteins are involved in a large number of disorders. Pseudohypothyroidism (PHP) is a disorder caused by resistance to parathyroid hormone.

This results in a subnormal urinary cAMP response to parathyroid hormone suggesting a defect in signal transduction proximal to cAMP formation. The phenotypic manifestations of this disorder include obesity, short stature, mental retardation and certain bony anomalies which are collectively termed Albright herditary osteodystrophy (152). Gain of function mutations have also been described in G-proteins. Examples of these include mutations of the G_s- α gene which have been implicated in GH-secreting pituitary somatotroph tumors and hyperfunctioning thyroid adenomas (107). These mutations have involved two residues (arginine-201 and glutamine-227) known to be critical for GTPase activity, which result in constitutive activation of G_s which in turn lead to inappropriate cAMP stimulation. Activating mutations at arginine-201 have also been found in the McCune-Albright syndrome (179).

Abnormal functions of G proteins:

Certain alterations of proteins involved in mitogenic signaling have been observed to cause profound alterations in cell growth, including malignant transformation (1). An example is the activation of mutated *ras* genes, whose altered forms have been implicated in a large number of naturally occuring tumors (4). The ras proteins belong to a large number of signal transducing molecules that exchange GDP for GTP due to their interaction with specific activated receptors. Mutationally activated *ras* genes encode proteins which are impaired in their ability to hydrolyze GTP, thus remaining in their active GTP-bound form (4). Similar GTPase impairing mutations have been found in G_s and $G_{12}\alpha$ subunits (95, 108, 169), the former involved in activation and the latter in the inhibition of adenylyl cyclase. Furthermore, a mutated Gα subunit of the G_q protein was

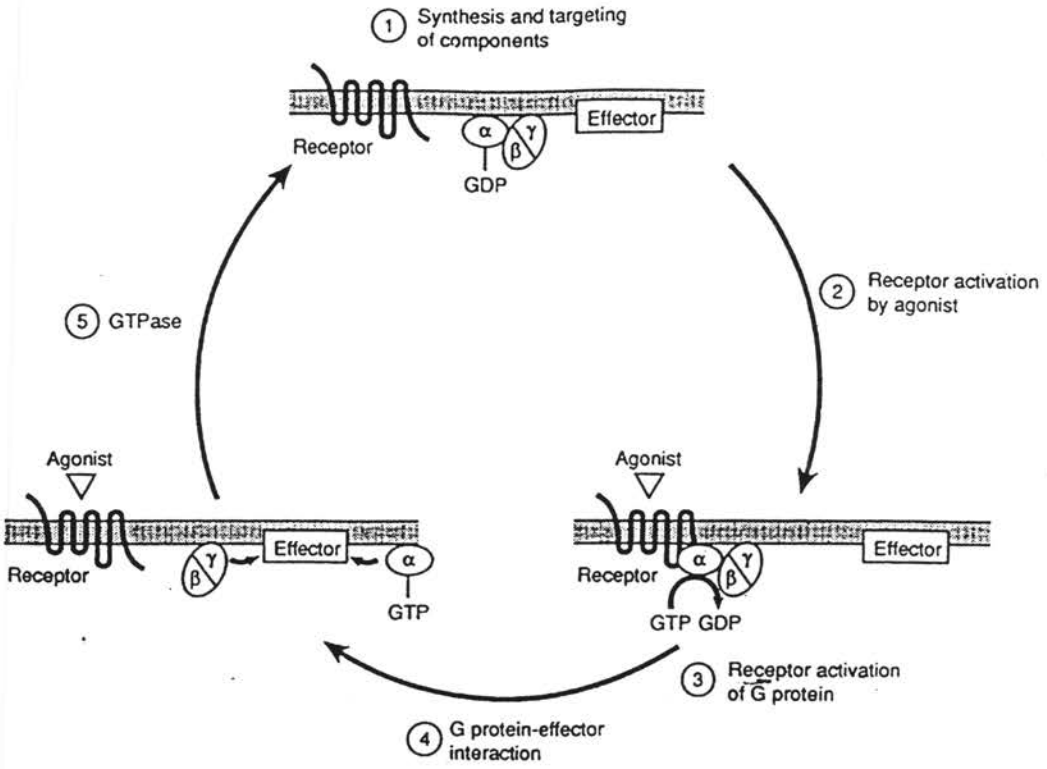


Figure 7: The G-Protein GTPase cycle. Potential sites for disease-causing abnormalities are numbered. In each panel, the shaded region denotes the plasma membrane, with extracellular above and intracellular below (152).

found to induce malignant transformation in NIH3T3 cells (82). Certain residues have been shown to be critical for the Gα subunit GTPase activity. Arginine and glutamine residues (201 and 227; respectively in the long form of G_s-α) are conserved in all Gα subunits (Table 1). The glutamine residue is identical to the Q61 of *ras*, known to be an oncogenic 'hotspot'. In the case of $G\alpha_s$, the mutation leads to constitutive activation of the adenylyl cyclase pathway which in turn leads to accumulation of high levels of cAMP in human pituitary tumors. Similar mutations have been found frequently in other types of thyroid and pituitary tumors and in adenocortical and parathyroid adenomas (158, 186, 193). Activating mutations at arginine 201 of G_s- α have also been found in the McCune-Albright syndrome. This disorder is caused by a somatic mutation of the G_s- α which leads to increased cAMP formation in many tissues. Since cAMP can stimulate proliferation as well as differentiated function of certain cell types such as endocrine cells and melanocytes, an activating mutation in $G\alpha_s$ could explain many of the features associated with this syndrome. Constitutively active $G\alpha_{i2}$ mutations also have been reported to cause cell transformation in certain systems such as in Rat1 fibroblasts (123). In addition, the expression of mutant $G\alpha_q$ has been seen to induce transformation of NIH3T3 fibroblasts (82) while constitutively active $G\alpha_z$ has been reported to transform Swiss 3T3 fibroblasts (187). Another example of a constitutive active G-protein is that of the one involving a missense mutation, G38D, in the rod transduction Gα-subunit which causes a disorder termed Congenital Stationary Night blindness (CSNB). Patients with this disorder have normal daytime vision, mediated by cone photoreceptors, but are blind

when light is sufficiently dim that rod photoreceptors alone mediate vision. A large body of evidence thus supports the presence of constitutively active mutations in G-proteins which result in alteration of their GTPase activity.

Study of activating mutations in G-proteins of *Dictyostelium*.

The study of putative constitutively active *Gα1* mutations involved amino acid substitutions in the highly conserved Gα-subunit domains known to reduce the intrinsic GTPase function in mammalian ras and $G\alpha_s$ (11). The *Gα1* gene with the mutation Q206L when expressed in wild-type cells and *gα1* null cells exhibited a phenotype similar to that of the *Gα1* overexpressors and formed slugs that culminated into fruiting bodies with uneven stalks and elongated basal discs (41). The expression of the G45V mutation on the other hand, resulted in an aberrant aggregation phenotype. The severity of the phenotype was seen to depend on the level of expression of the mutant Gα1 protein. Although mutations of conserved residues in $G\alpha_s$ have been observed to be dominant active due to constitutive activation of adenylyl cyclase, certain other mutations within the same highly conserved GTP binding/GTPase domains have been seen to be hypomorhic as the mutations result in reduced in vivo activation of adenylyl cyclase (97). In order to study the role of Gα2 subunit in aggregation, a molecular genetic approach was employed which involved mutations homologous to those known to reduce the intrinsic GTPase activity in other Gα subunits. Two of the mutations studied, *Gα2(G40V)* and *Gα2(Q208L)* were found to result in dominant negative phenotypes. Wild-type cells transformed with the *Gα2(G40V)* or *Gα2(Q208L)* exhibited substantial delay in development and lacked cAMP-activation of guanylyl cyclase and

phosphotidylinositol-phospholipase C(PI-PLC). Cells expressing the *Gα2(Q208L)* gene were delayed in aggregate formation until about 20h with only a small proportion (20-40%) forming mounds. These aggregates formed fruiting bodies which were very small after a period of about 36h (121). Thus, the *Gα2(Q208L)* gene appears to cause delays in both aggregation and culmination, and therefore seems to affect both early and late development. The expression of these mutant alleles in *gα2* null mutants was not observed to result in restoration of the ability of these cells to aggregate or exhibit normal adenylyl cyclase responses. The *gα2* null mutant cells expressing the *Gα2(G40V)* were seen to aggregate when plated at 5°C for 16h and then incubated at 20°C. However, in the case of the *gα2* null cells expressing the *Gα2(Q208L)*, only about 50% of the cells formed aggregates which did not proceed further in development (121). Analogous mutations were made in the *Gα5* gene in order to examine the role of the Gα5 subunit in kinetics of tip morphogenesis (Table 1). When wild-type cells were transformed with the *Gα5(Q199L)* gene, transformants selected for high copies of the mutant allele appreared to die out, suggesting that the gene was lethal in high doses (70). Cells selected for low copy numbers of the mutant plasmid did not display any alterations in phenotype. Transformants bearing single copies of this mutant gene with no wild-type *Gα5* allele did not show any alterations in phenotype either, suggesting that the *Gα5(Q198L)* allele could complement the loss of the wild-type *Gα5* gene. Transformants bearing single copies of this mutant allele were also observed not to show any aberrant phenotypes (70). In addition, another mutant allele, *Gα5(G196T)*, was also seen to result in cell death when expressed in high copies in wild-type cells. This mutation is analogous to the mutations seen to act as a dominant negative allele in the mammalian $G\alpha_s$ gene (122) as well as the

Dictyostelium Gα2 gene (121). However, none of the viable transformants displayed any aberrant phenotypes. Also, transformants with a single copy of the *Gα5(G196T)* allele and no wild-type allele did not appear to exhibit any aberrations in developmental phenotypes, suggesting that the *Gα5(G196T)* gene may not function as a dominant negative allele.

In an attempt to understand the Gα4 signal transduction pathway mediated by folic acid, we replaced the highly conserved glutamine 200 residue with a leucine by employing site-directed mutagenesis. Expression of this mutant *Gα4* gene in high copies in wild-type *Dictyostelium* cells was seen to result in cell death (Table 1). However, expression of this mutant *Gα4* gene in low copies was seen to allow transformants to come up which exhibited an aggregation deficient phenotype (agg^-). Since cAMP mediates aggregation through cAR1 which is coupled to the Gα2 signal transduction pathway, the ability of the *Gα4(Q200L)* gene to produce an agg^- phenotype suggests that mutation in one G-protein pathway such as Gα2, can affect another G-protein pathway, such as Gα4. Furthermore, when *Gα5(Q199L)* and *Gα2(Q208L)* genes were expressed in wild-type cells of *Dictyostelium*, they were seen to exhibit aggregation deficient phenotype as well. This suggests that there might be crosstalk between the different G-protein pathways. Previous studies have demonstrated that the two most related G-proteins in *Dictyostelium*, Gα4 and Gα5, promote complementary developmental processes that function antagonistically (Natarajan et al, unpublished data). The rapid cell death of the *Gα4(Q200L)* and *Gα5(Q199L)* mutants when selected at high copy number of the mutant *Gα* genes might be due to programmed cell death, a phenomenon also referred to as apoptosis. Apoptosis is a normal process in development and

Table 1: G-3 regions of Gα subunits and ras proteins

Gα subunit	*	Mutations
Mammalian Gαq:	DVGGQRSERRKW	Q>L transforms NIH 3T3 cells
Mammalian Gαs:	DVGGQRDERRKW	Q>L proliferation of Swiss 3T3 cells Q>R associated with pituitary tumors
Mammalian Gαi2:	DVGGQRSERKKW	Q>L proliferation of NIH 3T3 cells
Mammalian ras:	DTAGQEEYSAMR	Q>L oncogenic
Dictyostelium Gα2:	DVGGQRSERKKW	Q>L inhibits cAMP and folic acid chemotaxis
Dictyostelium Gα4:	DVGGQRSQRRKW	Q>L inhibits cAMP and folic acid chemotaxis, lethal when over-expressed.
Dictyostelium Gα5:	DVGGQRSERRKW	Q>L inhibits cAMP and folic acid chemotaxis, lethal when over-expressed.

morphogenesis. As an example, in the embryonic tissues of *C. elegans*, 131 of a total of 1090 cells die due to programmed cell death (46). Programmed cell death also removes unwanted or abnormal cells, maintains specific cell types, protects against infections and regulates maturation of the immune and nervous systems in the adult organism (48, 66). However, improperly regulated apoptosis can contribute to several pathological conditions including cancer. Apoptosis occurs when cells have sufficient time to organize and participate in their own demise (29). Cells undergoing apoptosis exhibit shrinkage, membrane blebbing, chromatin condensation and extensive nuclear fragmentation (24). Many cells can be activated to undergo apoptosis following the interaction of selected ligands with cell surface receptors. Examples of these include the Fas/Apo-1 (apoptosis inducing protein 1) and tumor necrosis factor receptor 1 (TNFR1) (150, 167). The timing and regulation of apoptosis appears to vary among different eukaryotic organisms and even tissue types but common regulatory genes appear to be important in many systems. Some of the regulatory genes are protooncogenes such as the *c-myc* gene or tumor suppressor genes such as *p-53*. The phenomenon of programmed cell death has been studied in *Dictyostelium* as well. *Dictyostelium discoideum*, which multiplies unicellularly, and upon starvation undergoes formation of a multicellular structure composed of prestalk and prespore cells. The prespore cells form the viable spore cells which can be propagated indefinitely. Differentiation to form the prestalk cells seems to require the sequential action of of cAMP and DIF (151). The stalk cells are vacuolated and are considered non-viable based on the their inability to regrow in culture medium (28). This is an instance of programmed cell death in *Dictyostelium*. The important features of programmed cell death in *Dictostelium* include massive

vacuolization in the stalk cells. This is similar to the vacuolization of cells involved in programmed cell death of other organisms such as *C. elegans* (28). Cytoplasmic condensation is another prominent feature of *Dictyostelium* apoptosis. In apoptosis, early nuclear lesions lead to the appearance of large DNA fragments (300 and/or 50 Kbp) revealed by pulsed field gel electrophoresis (119). However, no clear-cut ladder pattern of DNA fragmentation has been observed in *Dictostelium* which could be explained by the fact that nucleosomal organization of *Dictostelium* chromatin is irregular (28). One method that has been reported widely to test viability of cells in other systems is the use of dyes such trypan blue or propidium iodide (33). The simultaneous use of two dyes such as propidium iodide and Hoechst 33342 (HO 342) (33) provides a means to discriminate between necrotic versus apoptotic cells. In this method, cells are first stained with propidium iodide which is excluded by cells that have preserved their membrane integrity (i.e apoptotic and live cells) but, is taken up by cells that have lost their membrane integrity (necrotic cells). Cells are then stained with the dye Hoechst 33342 in the presence of ethanol. Apoptotic cells are distinguished from the necrotic cells by the presence of fragmented genomes. This method also allows apoptotic cells to be distinguished from the necrotic cells by the absence of propidium iodide. This method offers an attractive assay to differentiate between live, apoptotic and necrotic cells. Apoptotic cells can be distinguished from necrotic cells through the combined use of these two different stains. This method has been applied to human lymphocyte cultures treated with X-rays, mitomycin C or bleomycin (33). In the case of *Dictyostelium*, the use of ethanol allows to overcomes the problem associated with poor staining of cells since these cells are highly resistant to xenobiotics which includes the DNA-specific dye,

HO 342. *Dictyostelium* cells have been reported to exhibit active efflux of this DNA–specific stain mediated by extracellular vesicles (165). Early irreverible impairment of re-growth is an additional feature associated with apoptotic cells in *Dictyostelium*. However, this is seen to be followed by detectable morphological alterations characterized by membrane lesions only after a significant delay (28).

Common signal transduction pathways may be involved in the processes of oncogensis and apoptosis as both the processes involve changes in cell growth and differentiation. Since mutant G-proteins have been associated with oncogenesis, it is quite possible that they may also be involved in apoptosis. Whereas many of the known mammalian subunits function in fully differentiated cells, the Gα4 and Gα5 subunits in *Dictyostelium* appear to function primarily in undifferentiated cells. Thus, the mutant Gα4 and Gα5 subunits may be causing apoptosis by stimulation of developmental signals which are incompatible with vegetative growth.

The role of the *Gα4* gene in development of *Dictyostelium* has not been completely elucidated and the focus of the research outlined in this study is the Gα4 subunit and its role in *Dictyostelium* development. This will be achieved by examining the effect of the *Gα 4(Q200L)* mutation on development in *Dictyostelium*, and by determining whether intercellular signals provided by wild type cells can rescue spore production in *Gα4* mutants.

CHAPTER 3

CELL TYPE SPECIFIC LOCALIZATION AND DIFFERENTIATION MEDIATED BY G-PROTEIN FUNCTION: ROLE FOR Gα4.

Introduction

Development in most organisms is initiated by external signals which can control cell fate by directing cell differentiation and morphogenesis. It is thus very important to be able to identify these signals which dictate cell fate. The identification and dissection of molecular mechanisms which operate at the level of reception and relay of the signals can contribute towards understanding the processes that govern cell fate determination. During the life cycle of *Dictyostelium*, several signals control steps involved in differentiation. Cell sorting plays a significant role in localizing cells to the prespore and prestalk regions that go on to form the spore and stalk cells respectively, in the mature fruiting body (16, 45, 77, 155, 184). *Dictyostelium* cells aggregate in response to extracellular cAMP which is relayed between cells. This multicellular aggregate then goes on to form a slug which culminates with the formation of the fruiting body. Early in the aggregation process, cell sorting is evident as the prespore and prestalk cells become segregated to the central and anterior regions, respectively. This process appears to be influenced by vegetative parameters such as growth conditions and cell cycle progression (3, 59, 60, 120, 175, 178). The movement of the cells to the anterior prestalk region is

mediated by chemotaxis to cAMP as cells moving to this region exhibit chemotactic resonsiveness to cAMP (45, 144, 155). Further, cells that do not have a functional carB (cAR2) cAMP receptor gene form aggregates lacking tips (142). During the course of multicellular development, prespore cells are localized to the central region of aggregates where they form a defined border with the anterior prestalk cell region (37, 65). Although the movement of cells to the anterior prestalk region is well documented, very little is known about the process involving localization of cells in the central prespore region.

In *Dictyostelium*, several signals important for chemotaxis are mediated through receptors and transduced by heterotrimeric G-proteins to downstream effectors within the cell (18, 40). Analysis of G-protein genes in Dictyostelium has revealed that cAMP is important for the aggregation process mediated by the Gα2 signal transduction pathway(86, 121). The Gα subunit encoded by the *Gα4* gene appears to be essential for chemotactic responses to folic acid, but not cAMP (68). Although the *Gα4* gene is not required for responses to cAMP, other components of the Gα4 pathway such as the Gα subunit, adenylyl and guanylyl cyclases are important for responses to both cAMP and folic acid (170, 190). Expression of the *Gα4* gene is induced in cellular aggregates and the spatial pattern of expression is typical of genes expressed in undifferentiated cells rather than genes expressed in prestalk or prespore cells (68, 69, 71). *Gα4* null cells arrest as multicellular mounds with protruding tip structures composed primarily of prestalk cells (68). This terminal morphology is severely deficient in spore production which is indicative of a role in spore development for Gα4. Cells overexpressing the *Gα4* gene, referred to as the $G\alpha4^{HC}$ cells, arrest in development as aggregates lacking a defined

tip structure or a prestalk region. This suggests that excessive Gα4 function can block prestalk cell development. The *Gα4*^{HC} cells also are seen to accumulate higher levels of cGMP in response to folic acid as compared to wild-type cells which is consistent with a role for Gα4 function in cellular responses to folic acid (69). The requirement of the Gα4 subunit for folic acid chemotaxis and spore developement suggests that the Gα4-mediated signal transduction pathway might play an important role in directing movement of prespore cells in the multicellular aggregate during development.

The goals of this project include determining if the *Gα4* mutants can be rescued with respect to their spatial disrtibution, developmental gene expression and spore production by wild type cells in a chimera and whether Gα4 function stimulated by folic acid is required for prespore/prestalk cell development.

Materials and Methods

Strains and Culture conditions

All strains used in this study were haploid, axenic and isogenic to the wild type strain KAx3 except at the loci noted. The strain JH8 was constructed from the wild type strain KAx3 by site specific disruption of the *PYR5-6* gene (68). Transformants were selected in HL-5 medium containing uracil and 5-FOA to select for cells that are auxotrophic for uracil and deficient at the *PYR5-6* locus. This JH8 strain was subsequently used to create another strain, JH10, which was obtained by disruption of the *THY1* locus by insertion of the *PYR5-6* gene. This strain, JH10, is therefore auxotrophic for thymidine. The *Gα4*^{HC} strain was created by co-transforming a *Gα4* expression plasmid pJH56 (68) and plasmid pJH60 into JH10 cells and selecting for thymidine

prototrophs. Plasmid pJH60 has a 3.2 Kb fragment of the wildtype *THY1* gene inserted into the *Bam*HI site of the phagemid pT3T718U (Pharmacia) that allows for complementation of thymidine auxotrophy in JH10 cells. The aberrant developmental morphology associated with the $G\alpha4^{HC}$ strain was used as a screening technique for selection of transformants with a high copy number of the *Gα4* gene.

Verification of increased *Gα4* gene dosage and expression was obtained by genomic DNA blot (140) and RNA blot using procedures described previously (109, 140). The *gα4* null strain was created by disruption of the *Gα4* gene in the JH8 strain using a *Gα*4 fragment previously disrupted with the *PYR5-6* gene as described previously (69). *Gα4* gene disruption was verified by genomic DNA blot. In addition, *gα*4 null morphology during development was used as a means of verification.

Clonal isolates were obtained by plating cells on lawns of *Klebsiella areogenes* as described (22). Briefly, bacteria were plated on SM^{+3} (104) nutrient agar plates before inoculating droplets of *Dictyostelium* cultures. Solitary *Dictyostelium* amoebae fed on the bacteria, resulting in a plaque or clearing in the bacterial lawn. With increase in the size of the plaque, the cells in the center underwent multicellular development with the onset of starvation. Developmental morphologies of the aggregates closest to the center of the plaque were then noted (52). For shaking cultures, cells were grown in axenic HL-5 medium at 180 rpm at 22°C as described (20). Cultures of JH10 (*thy*$^-$) and JH8 (*PYR5-6*$^-$) required the addition of 100 μg/ml thymidine and 20 μg/ml uracil respectively. Cell density was determined by counting the amoebae in a haemocytometer.

RNA blot analysis

Total cellular RNA was isolated as described previously (116). Cells were developed either for 5 hours or 14 hours (as aprropriate) and then lysed with diethylpropyl carbonate (DEPC) and 20% sodium dodecyl sulphate (SDS). RNA was purified in a series of steps employing phenol/chloroform (1:1). RNA yields were determined by measuring absorbance at 260 nm. Samples were electrophoresed in a 1.2% agarose gel with 37% formaldehyde and subsequently blotted onto nylon membrane (MSI Inc.) by overnight capillary transfer in 10X SSC as described (109). Standard amounts of RNA (2-6 μg) were run on each lane of the gel. Blots were fixed by U.V. crosslinking. The DNA restriction fragements used for making probes were a part of the *Gα4* ORF, i.e., a 1.5 Kb *EcoRI/BamHI* fragment from plasmid 317 (68). The *ecmA* and *cotC* probes were gifts from Dr. Williams and Dr. Gaskins, respectively.

DNA probes were labeled with [α-^{32}P]-dATP using the protocol described earlier (51). Hybridization was performed in hybridization solution (20X SSC, 0.1M sodium phosphate buffer, pH 7.0; 0.4M EDTA, 100X Denhardts solution and 20%SDS) at 65°C in a Techne hybridizing oven. The blots were washed once with 2X SSC, 0.1% SDS at 37°C for about 30 minutes and then with 0.1X SSC, 0.1% SDS twice for 15 minutes each. All blots were exposed to X-ray film. Densitometry was used to quantitate bands on the autoradiograms. For RNA blots, the concentration of each sample was normalized using the corresponding ribosomal RNA band.

Southern blot analysis

Genomic DNA from *Dictyostelium discoideum* was purified as described

previously (116). Samples were digested with individual restriction endonucleases that either did not cut or cut once within the *Gα4* gene. Samples were then electrophoresed in a 0.8% agarose gel and blotted to nylon membrane (MSI Inc.) by overnight capillary transfer.

Random primer DNA probes employing radiolabeled ^{32}P were generated as described earlier (51). A 1.5 Kb *Eco*RI/*Bam*HI fragment comprising a part of the *Gα4* ORF from plasmid pJH317 (68) was used for preparation of the *Gα4* DNA probe. Hybridization of the labeled probe to the membrane was carried out as described eariler for RNA blot analysis. After hybridization, the blot was washed twice for 20 minutes in 2X SSC, 0.1% SDS at 37°C, followed by one wash for 15 minutes at 1X SSC, 0.1% SDS also at 37°C.

Analysis of morphology

Cells were grown axenically in shaking cultures of HL-5 medium to mid-log phase (1-2x10^6 cells/ml). For analysis of morphology, cells grown axenically were washed in 12 mM Na/K phosphate buffer (pH 6.1), hereafter referred to as "phosphate buffer". Cells were resuspended in phosphate buffer at 2x10^6 cells/ml and spread on non-nutrient agar plates for developement. Under these conditions, wild-type cells were seen to form fruiting bodies in 24-26 hours.

Analysis of cell sorting and gene expression in chimeric organisms

Cells were grown to mid-log phase, washed in phosphate buffer and resuspended at 2x10^7 cells/ml in the phosphate buffer in all cases except where specified otherwise.

Cells of different strains were mixed in predetermined ratios before development on non-nutrient plates containing 1.5% agar in phosphate buffer. Cells were allowed to develop on nitrocellulose membrane filters (Millipore) which were placed over non-nutrient agar plates.

Histochemical staining of β-galactosidase activity was performed as described (65). Cells were fixed for 5 minutes in 0.5% glutaraldehyde in Z-buffer. Cells were then washed in Z-buffer and stained with 1 mg/ml Bluo-Gal (Sigma) in the presence of potassium ferrocyanide and pottasium ferricyanide at 37°C (42, 109). The Bluo-Gal substrate provided greater contrast between staining and non-staining cells as compared to the substrate X-Gal. For quantitative β-galactosidase assays, cell suspensions of $1x10^8$ cells/ml were spotted onto Whatmann No. 50 paper filters that were placed on non-nutrient plates for development. A standard enzyme assay was used for the measurement of β-galactosidase activity of *Dictyostelium* cell extracts (42). Protein concentration of cell extracts was determined using Bradford's dye binding assay (12). Cell labeling with the fluorescent dye, 5-chloromethylflourescein diacetate (CMFDA or Cell Tracker Green), was performed as previously described (147). Cells were grown in HL-5, washed two times in phosphate buffer, and resuspended in 1mM CMFDA (C-2925; Molecular Probes) in phosphate buffer at 10^7 cells/ml. The cells were gently shaken for 15 minutes, washed, and resuspended in HL-5 for 1hr at $2x10^6$/ml. They were subsequently rewashed and resuspended at 10^7/ml in phosphate buffer. Labeled cells were mixed with unlabeled cells in a 1:9 ratio and plated on non-nutrient plates for development. The plates were incubated in the dark at 23°C until analyzed. That labeling had no effect on the kinetics or morphology of development was ascertained by

developing non-labeled cells in chimeras in an identical manner except for the absence of CMFDA. Cells were then analyzed by flourescence microscopy using a Nikon Optiphot-2 microscope.

In tip transfer experiments, cells from the anterior tip regions of labeled slugs were transferred onto mutant cell aggregates in the presence of low light levels and then these chimeric organisms were returned to the dark for further development.

Chemotaxis assays

Cells were grown to mid-log phase and washed in phosphate buffer. Low cell-density gradient assays were performed by placing 1 µl droplets of cell suspensions at $1x10^6$/ml on non-nutrient agar plates at a distance of 2 mm from droplets of chemoattractant (1 µl of either folic acid or cAMP). For gradient chemotaxis assays of cells from multicellular aggregates, cell suspensions at a density of $1x10^8$ cells/ml were plated on non-nutrient plates and allowed to develop for 10 hrs until the mound stage of development. Cell aggregates were then dispersed in phosphate buffer by repeated pipetting before being used for gradient chemotaxis assays. The number of cells that migrated beyond the perimeter of the original droplet 3 hrs after spotting was counted and taken as reflecting their chemotactic responsiveness. In order to account for random or non-chemotactic movement, the number of cells migrating outside the perimeter of the droplet in absence of the chemoattractant was counted and subtracted from the original count.

High cell density gradient assays were carried out with a prestarvation period of 6 hrs on nonnutrient agar plates, after which 1 µl droplets of cell suspension were re-plated

on non-nutrient plates with the chemoattractant (1 µl folic acid) spotted about 2 mm away from the cell suspension droplet. The pattern of chemotaxis was recorded after 3 hrs.

Electroporation of plasmids into *Dictyostelium*

Electroporation was carried out as described previously (44). Briefly, cells were grown to a density of $2x10^6$/ml in HL5 medium and harvested after mixing with electroporation buffer (12 mM Na/K PO_4 buffer, 50 mM Sucrose) and centrifugation at 1000g for 2 min. Cells were then resuspended in electroporation buffer and electroporated with DNA at 1.2 KV. Cells were mixed with healing solution (50 mM $CaCl_2$, 20 mM $MgCl_2$) in a petri dish before being provided with HL5 medium. Drug selection was done after 24 hr using either G-418 or blasticidin.

Results

Developmental gene expression in clonal aggregates of $G\alpha4^{HC}$ and $g\alpha4$ cells

In order to use reporter genes for analysis of the $G\alpha4^{HC}$ mutant, a $G\alpha4^{HC}$ mutant was created in the lab which was sensitive to the drug G-418 so that this drug could be used for selection of transformants which had been electroporated with the reporter genes conferring resistance to the drug. The morphology of the strain was verified as being blocked in tip morphogenesis and the insertion of the *Gα4* gene in multiple copies was verified using Southern blot (data not shown). Although a $G\alpha4^{HC}$ strain was available as reported previously in literature (69), it was important to obtain this new $G\alpha4^{HC}$ strain which is sensitive to the drug G-418 as several reporter genes of interest had been cloned

into vectors which were already G-418 resistant and hence, selection would not have been possible of $G\alpha4^{HC}$ cells transformed with these reporter constructs. Furthermore, Northern analysis showed that the expression of the *Gα4* gene in this strain was similar to the pattern seen in wild-type cells and increased with the onset of multicellular development (Figure 8). In contrast, expression of the *Gα4* gene in *gα4* null cells was different from wild-type cells as a smaller transcript (obtained due to disrutpion of the *Gα4* gene) was seen to be weakly expressed. Expression of the prestalk specific genes *ecmA* and *ecmB* was dramatically reduced in the $G\alpha4^{HC}$ cells when compared to the wild-type as well as *gα4* null cells. Expression of the prespore specific gene *cotC* was also seen to be severely reduced in the $G\alpha4^{HC}$ cells compared to the *gα4* null cells and wild-type cells. It is important to note that the *cotC* gene expression seen in the *gα4* null strain was different from results published earlier (69) which claimed that the *cotC* gene expression was dramatically lower than seen in the case of wild-type cells. However, other *gα4* null cells created in the lab confirmed that the level of *cotC* gene expression is the same in the *gα4* null strain as in the wild-type strain. The previous reports of low levels of *CotC* expression may have been a result of inappropriate developmental conditions which might have affected normal levels of *CotC* gene expression.

It was also observed through experiments performed by others in the lab that the level of *Gα4* gene expression dictated the severity of the developmental phenotype exhibited by the $G\alpha4^{HC}$ strain as losses in gene copy number resulted in reversion to the wild-type phenotype.

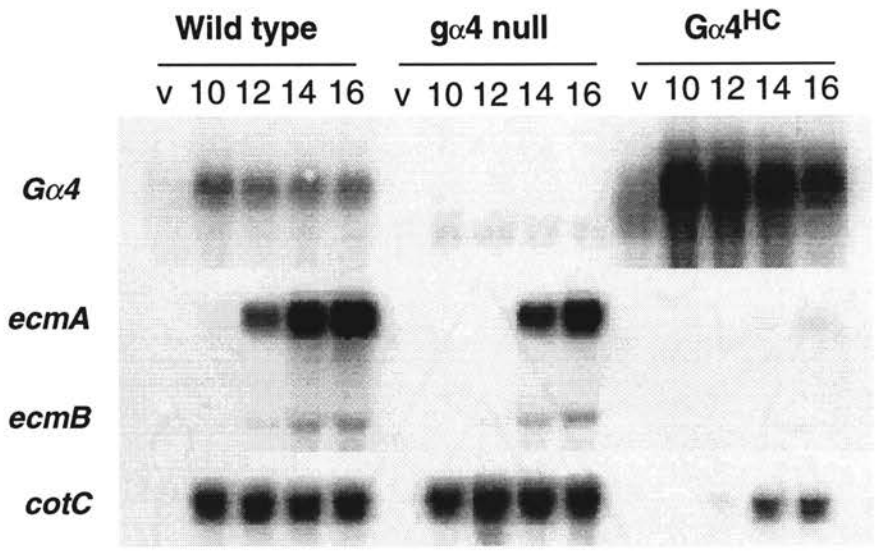


Figure 8: Developmental expression of the *Gα4*, *ecmA*, *ecmB* , and *cotC* genes in wild type, *gα4* null and *Gα4^{HC}* cells. Total RNA was isolated from cells at times indicated during development (10, 12, 14, and 16 hours after starvation) and size-fractionated on horizontal formaldehyde gels. All lanes contained 4 μg of total RNA and loading consistency was verified by ethidium bromide staining of ribosomal RNA bands. The RNA was then blotted to a nylon membranes and hybridized with a *Gα4* , *ecmA* probe (which also detects *ecmB* transcripts by cross hybridization), or *cotC* probe. Lanes marked (V) represent RNA isolated from vegetatively growing cells.

Spatial distribution of the *Gα4* mutants in chimeric organisms formed with wild-type cells

In order to understand the role of *Gα4* gene in cell localization, the spatial distribution of *gα4* null cells and $G\alpha4^{HC}$ cells was examined in chimeric organisms which were composed mainly of a mixture of wild-type cells and the *Gα4* mutants. The mutants were transformed with a reporter construct consisting of *actF:: lacZ* fusion. The actinF promoter is expressed ubiquitously in *Dictyostelium* and was therefore used. This was done so as to enable us to visualize the *Gα4* mutants in the chimeric organisms. When wild-type cells carrying the lacZ reporter constructs mixed with other wild-type cells were stained for β-galactosidase activity, it was seen that the blue staining cells were distributed uniformly throughout the aggregate, although the β-galactosidase activity seemed to be higher in prestalk cells when the staining period was limited. When a low ratio of mutant cells to wild-type cells (1:3) was used and staining was carried out for a short period of time, it was found that the *gα4* null cells were found primarily in the extreme posterior region and are severely underrepresented in the prestalk (or anterior) and prespore (or central) regions of the chimeric slugs (Figure 9). This distribution of the *gα4* null cells is similar to that observed for anterior-like cells which could serve as precursors to anterior prestalk cells and prespore cells. The *gα4* null cells were observed to eventually occupy the upper and lower cups of the chimeric fruiting bodies, which is also similar to the pattern seen for anterior-like cells at that stage of development.

The $G\alpha4^{HC}$ cells, in contrast, were seen to be localized in the central prespore region of the chimeric slug with a large majority of the cells in the region separating the anterior prestalk region from the central region. It is of importance to note that $G\alpha4^{HC}$

cells were not observed in the anterior prestalk region even after long periods of histochemical staining. The $G\alpha4^{HC}$ cells remained localized in the central prespore region in mature fruiting bodies. $G\alpha4^{HC}$ cells expressing the prestalk-specific ecmA::lacZ reporter gene were also found throughout the central and posterior regions but not in the anterior prestalk region where this gene is primarily expressed in wild-type cells. Other studies showed the same spatial pattern for $G\alpha4^{HC}$ cells expressing the prestalk-specific gene *ecmB::lacZ*. These prestalk gene expressing $G\alpha4^{HC}$ cells might be analogous to wild-type anterior-like cells that express prestalk genes but do not reside in the anterior region (43, 177).

In order to overcome limitations of expression parameters of reporter genes, the spatial distribution of the *Gα4* mutants was also examined using the flourescent dye, CMFDA (5-chloromethylfluorescein diacetate). Wild-type cells labeled with CMFDA mixed in a ratio of 1:9 with unlabeled wild-type cells were seen to maintain an even distribution in mixed aggregates throughout development. This indicates that the labeling conditions employed did not adversely affect cell localization. In contrast, CMFDA-labeled *gα4* null cells were evenly distributed in the chimeric organisms only till the mound stage of development and later, these cells were found to accumulate near the posterior end as the mounds differentiated into slugs (Figure 10). This is consistent with the observations made with the lacZ reporter experiment. CMFDA-labeled $G\alpha4^{HC}$ cells were also seen to be evenly distributed upto the mound stage of chimeric mounds but, these cells were subsequently excluded from the anterior region as the mounds differentiated into slugs. The $G\alpha4^{HC}$ cells remained in the prespore region throughout development which is consistent with the observations made in the lacZ reporter

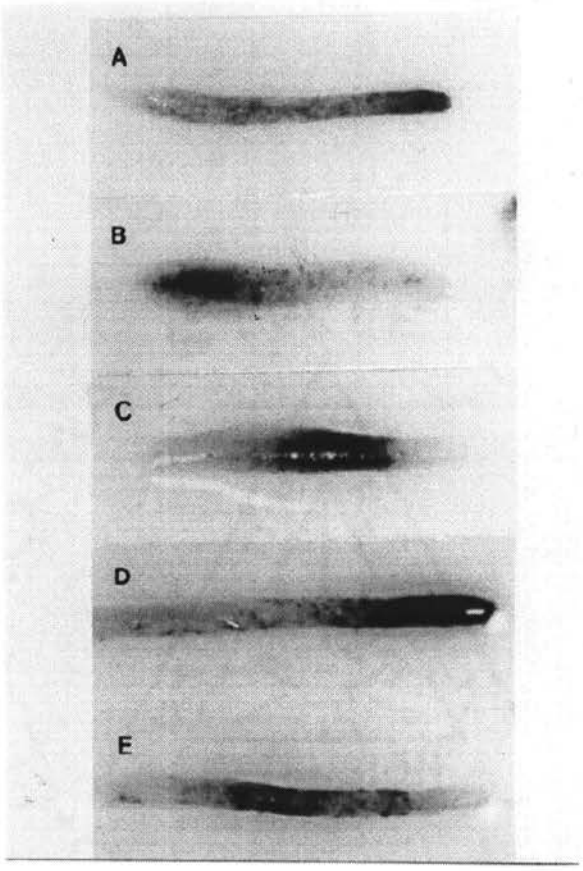


Figure 9: Histochemical staining of wild type, *gα4* null, or $G\alpha4^{HC}$ cells carrying the *actF::lacZ* or *ecmA::lacZ* reporter gene that have been developed with wild type cells containing no reporter gene. Cells were grown as described in Materials and Methods and mixed in a 1:3 ratio with wild type cells lacking the reporter gene. Cell mixtures were developed to the slug stage (18 hrs) on nitrocellulose filters overlaying nonnutrient plates and then histochemically stained for β-galactosidase activity as described in the methods. Panel A - wild type cells (*actF::lacZ*), panel B - *gα4* null cells (*actF::lacZ*), panel C - $G\alpha4^{HC}$ (*actF::lacZ*) panel D - wild type cells (*ecmA::lacZ*), and panel E $G\alpha4^{HC}$ (*ecmA::lacZ*). Organisms in panel A and B have been intentionally stained for a shorter period to emphasize the uneven pattern of reporter gene expression in wild type cells and the uneven distribution of *gα4* null cells, respectively.

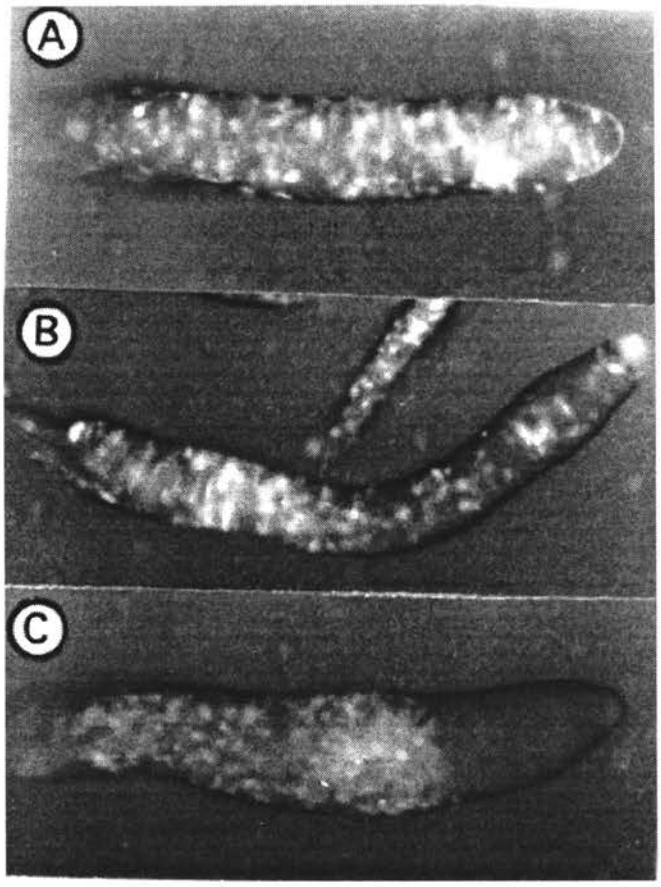


Figure 10: Localization of mutant and wild type cells labeled with CMFDA in slugs containing a 1:9 ratio of labeled cells to unlabeled wild type cells. Cells were labeled with CMFDA and then mixed with unlabeled wild type cells as described in Materials and Methods. Cell mixtures were plated on nonnutrient agar plates and the labeled cells were detected throughout development using fluorescence microscopy. Slugs with labeled wild type (A), *gα4* null (B), or $G\alpha4^{HC}$ (C) cells at 18 hrs of development. All slugs are oriented with posterior ends to the left and anterior ends to the right.

experiments. Before the use of CMFDA for examining spatial distribution of the *Gα4* mutants, we attempted to make use of the wild type version of the Green Flourescent Protein (GFP) for the same purpose. However, $G\alpha4^{HC}$ and *gα4* null mutants transformed with the reporter construct carrying either the wild type version of GFP or the mutated version of GFP did not show flourescence above the background flourescence of *Dictyostelium* cells, even though these transformants were selected with high drug (>10μg/ml G-418) concentrations.

Chemotactic properties of the *Gα4* mutants

In order to examine the role of Gα4 function in cell movement, the chemotactic responsiveness of the *Gα4* mutants was measured after aggregation. This was achieved by measuring relative number of *gα4* null and $G\alpha4^{HC}$ cells that can chemotax to gradientsof folic acid or cAMP. It was seen that $G\alpha4^{HC}$ cells displayed greater responses to folic acid at 10h of development and traveled greater distances as compared to wild-type cells which is consistent with the notion that the higher response to folic acid results from increased *Gα4* expression in the $G\alpha4^{HC}$ mutants (Table 2). This observation is similar to the response to folic acid in $G\alpha4^{HC}$ mutants prior to aggregation. In contrast to the $G\alpha4^{HC}$ cells, the *gα4* null cells from aggregates were not chemotactically sensitive to gradients of folic acid which is consistent with their response to folic acid prior to aggregation.

The $G\alpha4^{HC}$ mutants were also observed to exhibit greater responses to cAMP as compared to the wild-type cells. However, the response to cAMP does not seem to require Gα4 function as the *gα4* null mutant also was seen to be chemotactically

A	Relative number of cells chemotactic to folic acid			
Strain (hrs of development)	1 μM	10 μM	100 μM	1 mM
wild type (0 hrs)	0	9 ± 5	52 ± 28	261 ± 21
Gα4 ^{HC} (0 hrs)	16 ± 6	35 ± 13	182 ± 16	394 ± 38
gα4 null (0 hrs)	0	0	0	0
wild type (10 hrs)	2 ± 2	28 ± 8	74 ± 6	209 ± 27
Gα4 ^{HC} (10 hrs)	18 ± 6	142 ± 12	269 ± 46	449 ± 97
gα4 null (10 hrs)	0	0	0	0

B	Relative number of cells chemotactic to cAMP			
Strain (hrs of development)	1 μM	10 μM	100 μM	1 m M
wild type (10 hrs)	0	4 ± 4	6 ± 0	3 ± 1
Gα4 ^{HC} (10 hrs)	101 ± 3	478 ± 17	943 ± 100	278 ± 66
gα4 null (10 hrs)	4 ± 1	15 ± 6	88 ± 10	6 ± 2

Table 2: Chemotactic responsiveness of wild type, *Gα4*^{HC}, and *gα4* null cells to gradients of folic acid and cAMP during development. Chemotactic responsiveness of these strains was assayed during development at the times indicated by counting the number of cells that had migrated toward gradients of folic acid or cAMP after 3 hours (see Materials and Methods for details). The results of the chemotaxis assay were obtained from a typical experiment performed in duplicate. Error intervals represent the deviations in cell counts.

responsive to cAMP.

Developmental gene expression in *Gα4* mutants in the presence of wild-type cells

Spore production in *gα4* null and $G\alpha4^{HC}$ cells can be increased by developing them with wild-type cells in chimeric organisms. However, it has not been determined whether this increase is due to changes induced by wild-type cells in developmental gene expression or other factors such as morphogenesis or spore dispersal (68). In order to examine the developmental gene expression of *gα4* null and $G\alpha4^{HC}$ cells in the presence of wild-type cells, the expression of developmentally regulated lacZ was measured in the mutant cells during development with wild-type cells or mutant cells of the same type lacking the reporter genes. The reporter genes in *gα4* null cells were seen to be the same irrespective of whether these cells were developed with wild-type or other *gα4* null cells (Table 3). This pointed towards the fact that the wild type cells did not provide any signals which would alter developmental gene expression in the *gα4* null mutants. However, the $G\alpha4^{HC}$ cells exhibited a significant eight fold increase in the expression of the prespore-specific *cotC::lacZ* gene when developed with wild-type cells. This observation is consistent with the ability of wild-type cells to rescue spore development in $G\alpha4^{HC}$ cells (68). No major changes in levels of gene expression were seen in the case of the other reporter genes (*ecmA::lacZ*, *ecmB::lacZ*, *rasD::lacZ*, *ptpA::lacZ*, *cprB::lacZ* and *Gα4::lacZ*) when the mutants were developed with wild-type cells. This might be expected from the inability of the $G\alpha4^{HC}$ cells to localize in the prestalk region. Also, other studies in the lab indicate that two of the reporter genes, *ecmA::lacZ* and *ecmB::lacZ*, are highly expressed in the anterior prestalk cells of wild-type aggregates,

A	β -galactosidase activity		
Reporter gene	*gα4/gα4* aggregates	*gα4* /wildtype aggregates	Ratio of activity *gα4* /wildtype *gα4* / *gα4*
cotC::lacZ	39.2 ± 2.9	55.3 ± 2.7	1.41
ecmA::lacZ	2.15 ± 0.03	2.12 ± 0.08	1.17
ecmB::lacZ	82.3 ± 11.2	67.1 ± 10.9	0.81
rasD::lacZ	86.3 ± 3.4	95.5 ± 10.4	1.1
ptpA::lacZ	41.4 ± 4.1	45.7 ± 13.9	1.1
cprB::lacZ	97.4 ± 0.5	95.2 ± 4.0	0.98
Gα4::lacZ	64.2 ± 4.4	60.3 ± 4.1	0.94

B	β-galactosidase activity		
Reporter gene	$G\alpha4^{HC}/G\alpha4^{HC}$ aggregates	$G\alpha4^{HC}$/wildtype aggregates	Ratio of activity $G\alpha4^{HC}$ /wildtype $G\alpha4^{HC}/ G\alpha4^{HC}$
cotC::lacZ	3.08 ± 0.58	23.6 ± 0.56	7.66
ecmA::lacZ	0.37 ± 0.03	0.48 ± 0.07	1.3
ecmB::lacZ	7.78 ± 0.43	10.7 ± 1.8	1.38
rasD::lacZ	62.2 ± 9.1	109 ± 11	1.76
ptpA::lacZ	280 ± 17	288 ± 26	1.03
cprB::lacZ	251 ± 2	418 ± 44	1.67
Gα4::lacZ	29.3 ± 3.4	30.2 ± 2.1	1.03

Table 2: Developmental gene expression of *gα4* null and $G\alpha4^{HC}$ cells in chimeric aggregates. Cells were developed on filters overlaying nonnutrient plates for 19-21 hrs (slug stage for mutant/wild type chimeras) and then assayed for β-galactosidase activity as described in the methods. The β-galactosidase activity of *gα4* null cells carrying *lacZ* reporter genes was measured after cells were developed with either wild type (*gα4*/wild type aggregates) or *gα4* null (*gα4/gα4* aggregates) cells lacking the reporter gene (A). The β-galactosidase activity of $G\alpha4^{HC}$ cells carrying *lacZ* reporter genes was measured after these cells were developed with either wild type ($G\alpha4^{HC}$/wild type aggregates) or $G\alpha4^{HC}$ ($G\alpha4^{HC}/G\alpha4^{HC}$ aggregates) cells lacking the reporter gene (B). All aggregates contained a 1:2 ratio of cells carrying the reporter gene to cells lacking the reporter gene and both sets of aggregates (mutant/mutant and mutant/wild type) were created concurrently using equal amounts of the same culture of cells with the reporter gene. Values of β-galactosidase activity are the means and standard deviations of three independent experiments. The β-galactosidase enzymatic activity is expressed in units of pmol substrate/(min.μg protein) using ONPG as the substrate. Basal levels of reporter gene expression in the *gα4/gα4* and $G\alpha4^{HC}/G\alpha4^{HC}$ aggregates result from a combination of promoter strength and copy number of reporter gene. Cell-type specificity of reporter gene promoters is as follows: *cotC* (*SP60*) - prespore cells; *ecmA* and *ecmB* - prestalk and anterior-like cells; *rasD*, *ptpA* (*PTP1*), and *cprB* (*CP2*)-prestalk and nonprespore/nonprestalk cells; and *Gα4* - nonprestalk/nonprespore cells.

but the *Gα4*^{HC} cells expressing these genes are absent from the anterior prestalk region in chimeric organisms.

The ability of wild-type cells to increase the prespore specific gene expression in *Gα4*^{HC} cells suggests that wild-type cells are capable of rescuing spore development in *Gα4*^{HC} mutants by providing an intercellular signal. Since the *Gα4*^{HC} cells lack a defined prestalk region, we decided to check if transplantation of the missing anterior region onto *Gα4*^{HC} aggregates would rescue these mutants in their ability to form spores. Anterior tips of wild-type aggregates were transplanted on to mounds of cells resulting in chimeric organisms consisting of *Gα4*^{HC} /wild-type cells. These chimeric organisms were observed to form normal fruting bodies with large spore masses. The wild-type cells used for transplantation experiments had been previously transformed with an *act::lacZ* gene which allowed for discriminating them from the *Gα4*^{HC} cells. The transplanted wild-type cells remained segregated to the anterior prestalk region and later formed the stalk of the mature fruiting body as evidenced by the blue staining from β-galactosidase activity of the wild-type cells (Figure 11). This experiment was also performed with wild-type cells pre-labeled with CMFDA. The transplanted wild-typecells remained segregated to the anterior region of the developing chimeric organisms and later formed the stalk in the mature fruting body (Figure 12). This complete lack of mixing between the mutant and wild-type cells suggests that the rescuing signal might be a diffusible factor secreted by the prestalk cells. The production of this factor does not appear to be Gα4 dependent as *gα4* null cells have been reported to be capable of rescuing spore development in *Gα4*^{HC} cells (68). It has been reported previously that cAMP is capable of increasing the prespore gene expression in wild-type

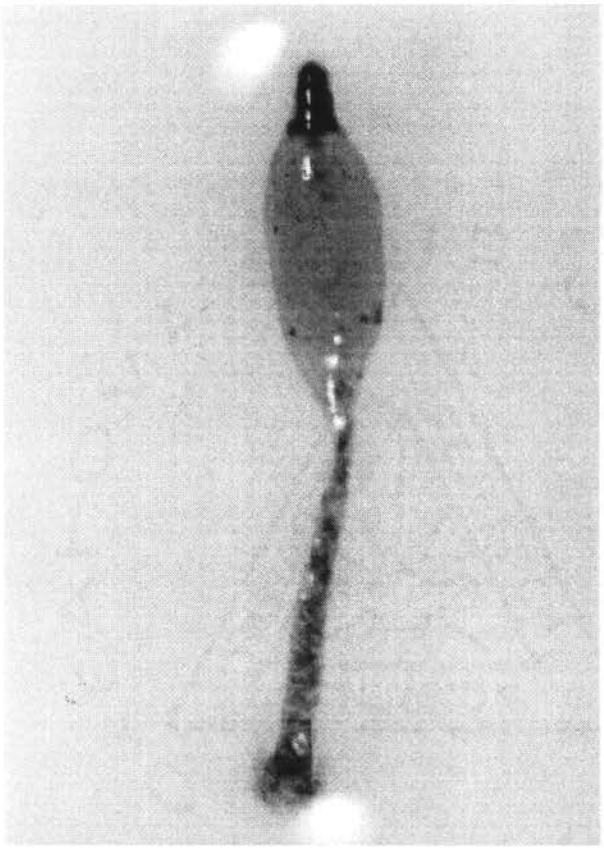


Figure 11: Histochemical staining of a chimeric organism composed of $G\alpha4^{HC}$ cells and wild type anterior cells. Wild type cells carrying the *actF::lacZ* reporter gene and $G\alpha4^{HC}$ cells without a reporter gene were grown and prepared for development as described in the Materials and Methods section. Wild type cells were developed directly on nonnutrient plates and $G\alpha4^{HC}$ cells were developed on nitrocellulose filters overlaying nonnutrient plates. After 16 hours of development, anterior cells (approximately 20% of total aggregate) from wild type slugs were transplanted on to $G\alpha4^{HC}$ aggregates (without mixing) and the resulting chimeric organisms were allowed to develop 8 hours longer before staining. Chimeras were histochemically stained for β-galactosidase activity as described in Materials and Methods.

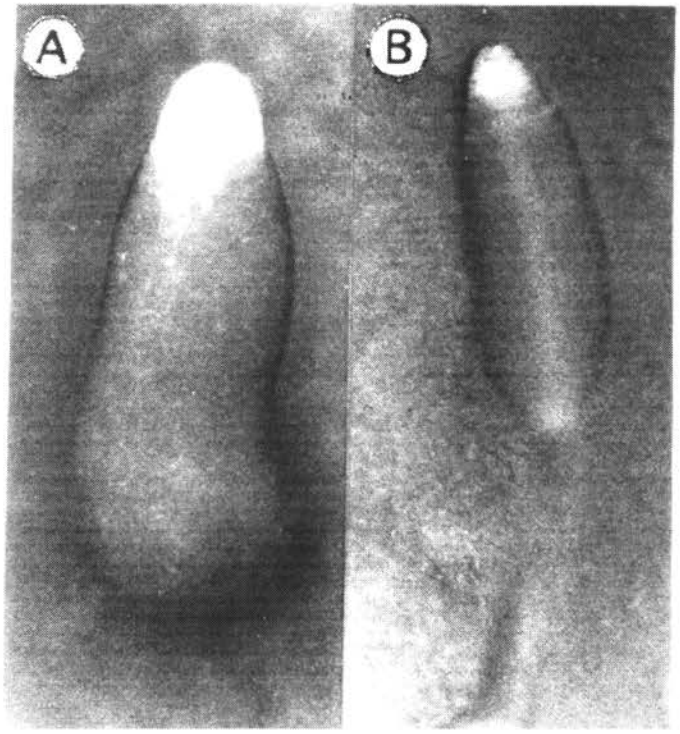


Figure 12: Distribution of CMFDA-labeled anterior cells from wild type slugs after transfer to mounds of *Gα4*^{HC} cells. Wild type cells were labeled as described in Materials and Methods and then developed synchronously but separately from *Gα4*^{HC} cells on nonnutrient agar plates. After 16 hours of development, anterior cells were transferred to *Gα4*^{HC} mounds as described in Materials and Methods but under very low illumination. The spatial distribution of the labeled wild type cells in the chimeras during subsequent development was examined by fluorescence microscopy. Chimeric slug 4 hours post tip transfer (A) and early culminant 8 hours after tip transfer (B).

cells.

In related studies performed by other people in our laboratory, the possibility of this signal being cAMP was ruled out as no significant increase in prespore gene expression was noticed when aggregates of $G\alpha4^{HC}$ cells were treated with exogenous cAMP.

Discussion

In developing $G\alpha4^{HC}$ cells, cell-type specific gene expression is absent which is consistent with the termination of morphogenesis at the mound stage of development. The lack of prespore specific gene expression in these mutants is probably due to the absence of an intercellular signal that is normally produced by anterior prestalk cells. This is consistent with the observation that prespore gene expression is rescued in $G\alpha4^{HC}$ mutants when developed with wild-type cells in a chimeric organism. Other studies also point towards signaling from prestalk cells which are important for spore differentiation (2, 148). However, prestalk gene expression does not appear to be restored by this form of intercellular signaling. This indicates that the Gα4 subunit may be inhibiting prestalk cell differentiation in a cell autonomous fashion. The term cell autonomous is used here to refer to the fact that loss of Gα4 function in the *gα4* null mutants cannot be complemented by the presence of wild type cells in the chimera. However, factors other than Gα4 function may be important for regulating prestalk cell differentiation as loss of Gα4function in *gα4* null cells does not restore normal levels of prestalk gene expression. This is consistent with reports of both cAMP and differentiation inducing factor (DIF) being important for prestalk gene expression and differentiation (5). Studies of spatial

distribution of *Gα4*^{HC} cells in chimeric organisms composed of wild-type cells indicates that since *Gα4*^{HC} mutants are excluded from the anterior prestalk region, it is possible that signaling mechanisms mediated by the Gα4 subunit prevents movement of cells into this region in a cell-autonomous fashion and/or promotes movement of cells into other regions of the multicellular aggregate. Although *Gα4*^{HC} mutants exhibit responsiveness to cAMP which is a chemoattractant for prestalk cells, it is likely that this responsiveness may not be sufficient in frequency or amplitude to mediate localization of these cells to the anterior prestalk region. In fact, other studies have demonstrated that multiple cAMP receptors differing in their affinities for binding cAMP are expressed differentially during various stages of multicellular development which could point towards different forms of cAMP signaling (81, 92, 142, 143, 194).

In other studies carried out in our laboratory, *Gα4*^{HC} cells expressing the prestalk specific reporter gene *ecmA::lacZ* were seen to be excluded from the anterior prestalk region, indicating that the ability of cells to migrate to this region cannot be restored by expression of prestalk cell-specific genes. It appears that expression of the prestalk specific genes *ecmA* and *ecmB* is not sufficient to confer the ability on the cells to localize in the anterior region of the aggregate. Furthermore, it is possible that overexpression of the *Gα4* gene might block some step in prestalk cell differentiation that may be required for localization of cells to the anterior region. In fact, the overexpression of the *Gα4* gene might promote cell localization to the central prespore region by chemotactic movement of cells, as *Gα4*^{HC} cells appear to retain chemotactic responsiveness to both folic acid and cAMP even after cellular aggregation. The terminal morphology of the *gα4* null cells which is characterized by the inability of most prespore

cells to follow the extending prestalk region supports this theory (69). It is likely that Gα4 function is necessary for localization of cells in the prespore region as *gα4* null cells are not seen to be evenly distributed in the multicellular organism. If this localization is in the form of a chemotactic movement mediated by Gα4, it is likely that this function occurs early in the sorting process as *Gα4* expression does not appear to be very prominent in the central prespore region (65, 66). Since $G\alpha4^{HC}$ cells exhibit amplified responses to pterin compounds such as folic acid and monapterin as compared to wild-type cells, and as these responses are absent in *gα4* null cells, Gα4 function seems essential for these pterin responses. These pterin responses have previously been reported to stimulate association of actin with the cytoskeleton (162). Pterin chemotaxis has been shown to be an important part of development in a related slime mold species, *Dictyostelium minutum* where folic acid rather than cAMP mediates aggregation of cells to form multicellular aggregates (139, 140). It is possible that in both *D. discoideum* and *D. minutum*, folic acid promotes prespore development.

In related work performed by others in our laboratory, it was observed that treatment of wild-type cells with exogenous folic acid substantially delayed tip morphogenesis which could mean that the phenotype of $G\alpha4^{HC}$ cells was due to increased signaling of the Gα4 signal transduction pathway. Further, the expression of the prestalk specific genes *ecmA* and *ecmB* was delayed in wild-type cells treated with folic acid, which is similar to the expression patterns observed in $G\alpha4^{HC}$ cells. The expression of the prespore specific gene *cotC* was also delayed in folic acid treated wild-type cells. This expression pattern is consistent with with the delay in developmental morphogenesis. The strongest evidence for this delay in tip morphogenesis being

mediated by the Gα4 signal transduction pathway comes from the observation that the *gα4* null cells do not appear to be affected in phenotype or gene expression by treatement with exogenous folic acid. It is possible that the phentotype of the $G\alpha4^{HC}$ mutants may be due to the overexpressed Gα4 subunit interfering with other pathways such as the Gα5 signal transduction pathway. Since, the Gα5 subunit is not essential for tip formation, it is likely that the $G\alpha4^{HC}$ cells are not defective only in the Gα5 signal transduction pathway. It is however, clear that the developmental phentoypes of $G\alpha4^{HC}$ cells are specific for the Gα4 signal transduction pathway as similar phenotypes are not observed when other structurally related Gα subunits are overexpressed (70, 92).

This body of work demonstrates that signal transduction mediated by the Gα4 subunit in a cell autonomous fashion is important for the promotion of prespore andinhibition of prestalk development which points towards the possibility of pterin compounds such as folic acid being regulators of cell fate choice during development. The theory that folic acid signaling may be used to target cells with high energy reserves (i.e prespore cells) to form mature spores is strengthened by the observation in this study that folic acid mediated delays in development are most effective when cells have been recently provided with nutrients. Additional support for this idea come from other studies which have reported that cells grown in the absence of glucose respond to cAMP earlier and these cells sort to the anterior prestalk regions of chimeric organisms (120, 175). Folic acid may therefore be regulating multicellular development in *Dictyostelium discoideum* through the Gα4 signal transduction pathway.

CHAPTER 4

ANALYSIS OF ACTIVATED Gα MUTANTS AND THEIR ROLE IN DEVELOPMENT OF *DICTYOSTELIUM*

Introduction

Heterotrimeric G-proteins transduce signals from cell surface receptors to downstream signaling pathways, allowing eukaryotic cells to respond to a wide variety of external stimuli. Of the three subunits (α, β, and γ) that form these G-proteins, the α-subunit plays a key role in regulating the active and inactive states of this signal transduction component. Stimulated receptors activate G-proteins by facilitating the loss of bound GDP from the Gα subunit, and then the Gα subunit binds GTP and dissociates from the Gβγ dimer. The active state of the G-protein is terminated by the hydrolysis of GTP to GDP, a process that is carried out by the intrinsic GTPase activity of the Gα subunit and enhanced by other Gα interacting components. Genetic and biochemical analyses have identified conserved regions of Gα subunits as being important for this GTPase activity and the inactivation of Gα subunit function. Many aberrant signaling phenotypes have been associated with specific mutations within these conserved regions, particularly with substitutions of leucine for the glutamine residue in the G-3 region of the Gα subunit. This mutation in some mammalian *Gα* genes can result in altered cell growth control and tumor formation. In some thyroid and pituitary tumors, elevated

levels of cAMP suggest that the mutant $G\alpha_s$ subunit is constitutively activating its downstream effector, adenylyl cyclase (158, 186, 193). Constitutively active $G\alpha_{i2}$ or $G\alpha_z$ have both been reported to cause cell transformation in fibroblasts and the analogous mutation in Gα can produce tumor formation (82, 123, 187). These phenotypes somewhat resemble those observed for analogous mutations in ras proteins that also interfere with GTPase activity.

While G-protein function has been examined in a wide variety of organisms, the role of these proteins in developmental processes has been intensively studied in *Dictyostelium discoideum*, a soil ameobae with a relatively simple developmental life cycle. *Dictyostelium* grows vegetatively as a solitary amoebae but upon nutrient deprivation these cells aggregate (~ 10^5 cells/aggregate) to form a multicellular aggregate that can undergo a series of morphological stages before development culminates with a fruiting body structure, consisting of a mass of spores on top of a stalk. Genetic analyses of G-protein function have indicated that several G protein mediated signal transduction pathways are important for carrying out the multicellular developmental cycle. Of the many Gα subunits identified in *Dictyostelium*, at least 4 appear to be involved with some aspect of multicellular development and the one known Gβ subunit appears to be important for chemotactic movement of individual cells and multiple cellular development (68, 70, 71, 91, 190).

While *Dictyostelium* development is relatively simple compared to higher eukaryotes, the roles of the individual *Dictyostelium* Gα subunits appears to be quite distinct with respect to developmental morphology and cellular differentiation as indicated by the phenotypes of gene disruption or overexpression mutants. The Gα2

subunit is required for cellular responses to the extracellular cAMP signal that directs the aggregation process during development (91). These responses include the accumulation of cGMP and cAMP, inositol triphosphate production, and chemotactic movement to cAMP. The Gα3 subunit is also required for aggregation but only in absence of exogenous cAMP, suggesting that Gα3 subunit is not directly involved with responses to extracellular cAMP (13). While not required for aggregation, the Gα4 and Gα5 subunits play important roles in the development of spores and the anterior prestalk cells, respectively, consistent with the increased expression of these subunits upon aggregate formation (71). The Gα4 subunit is required for proper multicellular morphogenesis, spore production, and chemotactic responsiveness to folic acid (68, 69). Overexpression of the Gα4 subunit inhibits prestalk cell development, as does exogenous folic acid, and promotes cell-autonomous localization of cells to the prespore region of development. Conversely, the Gα5 subunit promotes formation of the anterior prestalk cell region (70) and inhibits folic acid responsiveness indicating that it plays an antagonistic role to that of the Gα4 subunit during development. Loss of the Gα2 subunit affects only cAMP chemotaxis and loss of the Gα4 subunit only affects folic acid chemotaxis, even though both subunits are required for the accumulation of cGMP and cAMP and cell movement (69, 91). Similarly, the loss of other Gα subunits does not appear to affect these chemotactic responses implying that these pathways are mediated by single Gα subunits.

In addition to gene disruption and overexpression mutants, Gα subunit function has been characterized by the analyses of Gα subunits that contain a leucine for glutamine residue replacement within a conserved region designated as G-3 (11). The expression of a Gα1 subunit with this alteration in wild type cells produces large aggregates even

though Gα1 function does not appear to be required for multicellular development (41). Expression of a Gα2 subunit with this same alteration in wild type cells inhibits Gα2-mediated responses to cAMP, including the accumulation of cGMP, cAMP, and IP3 and the chemotactic movement of cells to cAMP (121). The analogous alteration in the Gα5 subunit appears to be detrimental to vegetative growth when overexpressed as a result of increased gene dosage even though the loss or overexpression of the wild type Gα subunit does not noticeably alter vegetative growth (70). In this study we examined the phenotypes associated with an analogous alteration in the Gα4 subunit and determined that this mutant subunit interferes with responses to both folic acid and cAMP stimulation. We also found that analogous alterations in the Gα5 or Gα2 subunits also inhibit folic acid and cAMP responses, indicating that these mutant subunits are capable of inhibiting multiple signal transduction pathways.

Materials and Methods

Strains and Media

All of the strains used in this study were isogenic to the axenic strain KAx3 except at the loci noted. The construction of *gα4* null cells (*Gα4* locus disrupted with the *THY1* gene, strain JH142) or $G\alpha4^{HC}$ cells with the *Gα4* expression vector pJH154 (strain JH384 or strain JH202) have been previously described (69). Electroporation of DNA into *Dictyostelium* cells was performed as previously described by Dynes and Firtel (44), and multiple *Dictyostelium* transformants were isolated and characterized from each electroporation unless otherwise noted. Vector copy number was verified by genomic

DNA blots and the expression of Gα subunit genes were determined by RNA blot analysis.

RNA blot analysis

Total cellular RNA was isolated as described previously (116). Briefly, cells were developed either for 5 hours or 14 hours (as appropriate) and then lysed with diethylpropyl carbonate (DEPC) and 20% sodium dodecyl sulphate (SDS). RNA was purified in a series of steps employing phenol/chloroform (1:1). RNA yields were determined by measuring absorbance at 260 nm. Samples were electrophoresed in a 1.3% agarose gel with 37% formaldehyde and subsequently blotted onto nitrocellulose membrane (MSI Inc.) by overnight capillary transfer in 10X SSC as described (109). Standard amounts of RNA (2-6 μg) were run on each lane of the gel, and blots were fixed by U.V. crosslinking. DNA restriction fragements used for making probes were a part of the *Gα4* ORF i.e a 1.5 Kb *EcoRI/BamHI* fragment from plasmid 317 (68), discoidin-I (130), ecmA, cotC and cAR1. The fragments used to make the *ecmA* probe were a kind gift from Dr. Jeffrey Williams while the fragment used to make the *cotC* probe was received from Dr. Chris Gaskins. The *cAR1* probe was prepared from plamid p6B (a gift from Dr. Dale Hereld) by digesting with *Eco*RI which gave a 1.2 Kb fragment. DNA probes were labeled with [α ^{32}P] -dATP using the protocol described earlier (51). Hybridization was performed in hybridization solution (20X SSC, 0.1M sodium phosphate buffer, pH 7.0; 0.4M EDTA, 100X Denhardts solution and 20%SDS) at 65°C in a Techne hybridizing oven. The blots were washed once with 2X SSC, 0.1% SDS at 37°C for about 30 minutes and then with 0.1X SSC, 0.1% SDS twice for 15 minutes each.

All blots were exposed to X-ray film. Densitometry was used to quantitate bands on the autoradiograms. For RNA blots, the concentration of each sample was normalized using the corresponding ribosomal RNA band.

Southern blot analysis

Genomic DNA from *Dictyostelium discoideum* was purified as described previously (116). Samples were digested with individual restriction endonucleases that either did not cut or cut once within the *Gα4* gene. Samples were then electrophoresed in a 0.7% agarose gel and then blotted to nitrocellulose membrane (MSI Inc.) by overnight capillary transfer as described earlier (116).

Random primer DNA probes employing radiolabeled ^{32}P were generated as described (51). A 1.5 Kb *Eco*RI/*Bam*HI fragment comprising a part of the *Gα4* ORF from plasmid pJH317 (68) was used for preparation of the *Gα4* DNA probe. Hybridization of the labeled probe to the membrane was carried out as described eariler for RNA blot analysis. After hybridization, the blot was washed twice for 20 minutes in 2X SSC, 0.1% SDS at 37°C, followed by one wash for 15 minutes at 1X SSC, 0.1% SDS also at 37°C.

Development and Chemotaxis Assays

Cells were grown in shaking cultures to mid log phase (~2 x 10^6 cells/ml) and then washed in 12 mM Na/K phosphate buffer before plating on non-nutrient plates (phosphate buffer, 15% agar) at 10^7 or 10^8 cells/ml for development or chemotaxis assays.

For chemotaxis assays, cells were grown to mid-log phase and washed in phosphate buffer. For gradient chemotaxis assays of cells from multicellular aggregates, cell suspensions at a density of $1x10^8$ cells/ml were plated on non-nutrient plates and allowed to develop to the desired stage of development (3, 6 and 9 hrs or as indicated). Cell aggregates were then dispersed in phosphate buffer by repeated pipetting before being used for gradient chemotaxis assays. Chemotaxis assays were conducted as described by Hadwiger and Srinivasan (67) by spotting 1 μl of the cell suspension onto a nonnutrient plate and then spotting 1 μl of chemoattractant solution (1 mM folic acid or 100 μM cAMP) approximately 2 mm away from the cell suspension droplet. In some cases, cells were starved on nonnutrient plate for indicated time, harvested in phosphate buffer, and then spotted onto fresh nonnutrients plates for chemotaxis assays. The number of cells that migrated beyond the perimeter of the original droplet 3 hrs after spotting was measured and taken as reflecting their chemotactic responsiveness. Droplets were photographed 2.5 hrs after spotting the chemoattractant next to them.

cAMP pulsing assays

The procedure followed was a modification of the one decribed previously (146). Cells were shaken in suspension cultures at a density of $1x10^7$/ml at 125 rpm for 6 hrs so that cell-cell contacts could form. After 6 hrs, 100 μM cAMP was added at every subsequent 2 hrs interval up to 12 hrs. After two additions of cAMP, cells were plated on nonnutrient agar plates for development at a density of $5x10^8$/ml.

Temperature shift assays

The procedure followed has been previously reported in literature (121). Cells were grown to mid-logarithmic phase ($2x10^6$/ml) after which they were spun down and washed twice with phosphate buffer. Cells were then plated at a density of $1x10^8$/ml at 10°C. After 24 hr of incubation, these cells were placed at 20°C for another 24 hr before being photographed.

Cyclic nucleotide assays

cAMP assays:

Cells were starved on nonnutrient medium at 22°C for 3 h before being treated with folic acid or starved for 6 h before being treated with cAMP. Cell lysates were then prepared for cyclic nucleotide assays as described previously (91). For the assay of cAMP response, 2' -deoxy cAMP was used as stimulant instead of cAMP. Cyclic AMP concentrations were determined using a radioimmunoassay kit (Amersham Corp.). Briefly, [^{3}H]-cAMP was added to cell extracts along with the cAMP binding protein. Separation of protein bound cyclic AMP from the unbound nucleotide was achieved by adsorption of the free nucleotide onto charcoal followed by centrifugation. An aliquot of the supernatant was then removed for determining activity by liquid beta scintillation counting.

cGMP assays:

Cells were prepared as described for cAMP assays by prestarving them for 3 hrs before treatment with folic acid or 6 hrs before treatment with cAMP. Cell lysates were collected as described previously (69). Cyclic GMP concentrations were determined

using a radioimmunoassay kit from Amersham as per the manufacturer's instructions. Briefly, [^{3}H]-cGMP was added to cell extracts along with an antiserum having a high affinity and specificity for cGMP. The amount of bound labeled cyclic GMP is inversely related to the amount of cyclic GMP present in the assay sample. Ammonium sulfate precipitation followed by centrifugation separated bound cGMP-antibody complexes which were then dissolved in water and radioactivity determined by liquid beta-scintillation counting.

Stimulus solutions used for cyclic nucleotide assays:

cAMP stimulus solution: 15 µl 10 mM cAMP

30 ml 0.5 M DTT

15 µl 0.1 M PB pH 6.5

90 µl H_2O

dcAMP solution: 30 µl 10^{-3} M dcAMP

120 µl 0.5 M DTT

60 µl 0.1 M PB pH 6.5

390 µl H_2O

Folic acid stimulus: 45 µl 1 mM Folic acid

84 µl 5 mM 8-azaguanine

15 µl 1 M DTT

All assays were repeated several times.

Construction of expression vectors and mutagenesis

Site directed *in-vitro* mutagenesis of the *Gα4* gene was performed as outlined by

Kunkel (121). In brief, the process involved annealing a mutagenic oligonucleotide (for replacement of amino acid glutamine at position 200 with a leucine) to a DNA template containing uracil residues in place of thymine and synthesis of the mutant strand with T4 DNA polymerase and T4 DNA ligase. The oligonucleotide 5' CTTCTTTGAGATCTTAGACCACCGACATCTAC 3' (*Gα 4(Q200L)*) was used as to mutagenize a 0.8 Kb *ScaI/BspDI* fragment of the *Gα4* gene. The uracil rich DNA template was prepared by standard procedures after growth in an *E. coli dut⁻ung⁻* strain, CJ236. This template is not biologically active upon transformation into a wild type (i.e., *ung*$^{+}$) *E. coli* host, NM522. Expression of the desired change, present in the newly synthesised non uracil-containing covalently closed circular complementary strand, is thus strongly favoured. The resulting mutagenized fragment was used to replace a *ScaI/BspDI* fragment of the wild type gene in the plasmid pJH56 (71). The sequence of the mutated fragement in the plasmid thus created (pJS004) was verified by DNA sequencing using the dideoxy chain termination method as per the manufacturer's instructions (Amersham).

DNA constructs

The mutated *Gα4* gene was subcloned into various vectors with marker genes. The *PYR5-6* marker gene (3.7 Kb *BHI* fragment) from plasmid pJH58 (69) was inserted in the *Gα 4* open reading frame (ORF) to create plasmid pJS007 lacking the *Gα4* promoter. The construct was linearized using the *NcoI* site internal to the *Gα4* gene and targeted at the endogenous *Gα4* gene. Plasmid pJS010 was created by cloning the blasticidin resistance gene as a *KpnI/EcoRI* fragment from plasmid pUCBsrδBam (161)

onto plasmid pJS004.

Plasmid pJS011 containing the G-418 resistance gene was created by cloning the drug resistance gene as a *Sal/EcoRI* fragment onto the multiple cloning site of plasmid pJS004.

Plasmid pJS023 was created by fusing the discoidin promoter (initially taken from plasmid pVEII and cloned into the multiple cloning site of the bluescript SK^+) as a *SalI/XbaI* fragment to the *Gα4* ORF bearing the mutation Q200L in the promoterless construct pJS015. This allowed expression of the mutant *Gα4* gene from the discoidin promoter rather than its endogenous promoter. Plasmid pJS032 was created by substituting the *Gα4* ORF bearing the Q200L mutation with the wildtype *Gα4* fragment (from pJH56) to allow for expression of the wildtype *Gα4* gene from the discoidin promoter.

Plasmid pJS038 was created by deletion of 0.3 Kb *NdeI/BglII* fragment internal to the *Gα4* ORF. This plasmid served as a control to examine regulation of expression of the discoidin promoter by folic acid without the complication of the mutant *Gα4* gene altering expression.

The plasmids *Gα2(Q208L)* and *Gα5(Q199L)* plasmids have been previously described (70, 121).

Staining for detection of apoptotic cells

The procedure followed was a modification of one described earlier (28). Transformed cells were plated on plastic trays and drug resistant colonies were selected. After the medium was removed, cold medium containing 20 μg/ml propidium iodide was

added to cells and the flourescent cells were counted. These red flourescent cells comprised the necrotic cells. After allowing the dye to drain off, cells are allowed to dry and then treated with cold phosphate buffer containing 0.4 μg/ml Hoechst 33342 and incubated in refrigerator for 15-30 min. After draining off the dye, the cells were allowed to dry before being examined under the flourescence microscope.

Results

Analysis of *Gα4(Q200L)* mutants

To characterize the effects of an activated Gα4 subunit on chemotactic movement and development, site-directed mutagenesis was used to create a *Gα4* mutant that encodes a Gα4 subunit containing the substitution Q200L within the G-3 region of the Gα4 subunit. A *Gα4(Q200L)* expression vector containing a selectable marker conferring resistance to the drug G-418 was transformed into wild type *Dictyostelium* cells but relatively few transformants were obtained as compared to transformation with the control vector expressing the wild type Gα4 subunit. Careful examination of cultures transformed with the *Gα4(Q200L)* expression vector revealed many small G-418-resistant colonies, usually less than 50 cells, that disappeared several days after the transformation, presumably due to inviability. The inability to obtain viable transformants upon electroporating the *Gα4 (Q2008L)* (neo^r) plasmid into wild-type *Dictyostelium* cells pointed towards the likelihood of the mutant gene being lethal in high copy numbers. Upon analysing the few surviving mutants by Southern analysis, it was found that the DNA fragments obtained by restriction enzyme analysis did not match up with the expected size fragments and appeared to be products of genomic rearrangement.

The plasmid map of the vector is shown in Figure 13. The enzymes used for Southern analysis were *ClaI/PstI* and the expected band size which would be detected using the *Gα4* probe was 2 Kb. However, smaller size bands were seen which could have arisen due to DNA rearrangement (Figure 14). The mutant plasmid probably undergoes rearrangement driven by recombination between regions of homology in the plasmid. This may be followed by amplification which could explain the identical band sizes obtained by running out digested DNA isolated from several transformants with the mutant plasmid.

In order to determine if the *Gα4(Q200L)* mutant plasmid was lethal in high copies alone, or if single insertion mutants could be obtained, the *Gα4(Q200L)* gene with the *PYR5-6* marker gene was electroporated into the JH8 *(PYR5-6⁻)* strain. The efficiency of electroporation appeared to be low, but the transformants obtained were used for Southern analyses. The fragment sizes observed upon restriction enzyme digest with *ClaI/KpnI* appeared to be lower than would be expected (data not shown). These transformants were however not analysed further. Instead, the *Gα4(Q200L)* gene with the Bs^r gene was electroporated into wild-type *Dictyostelium* cells. This was done in order to obtain transformants with low copy numbers of the plasmid. Upon checking these transformants for phenotypic analysis, these mutants appeared to develop like wild-type cells. These mutants were then checked further using Southern analysis. The plasmid map of *Gα4 (Q200L)* gene with the Bs^r gene is shown in Figure 15. The enzymes used for restriction analysis were *ClaI/KpnI*. The expected size of the fragments that would be detected by the *Gα4* probe was 2 Kb. However, in addition to the expected band size, smaller bands were seen which could not be explained and which

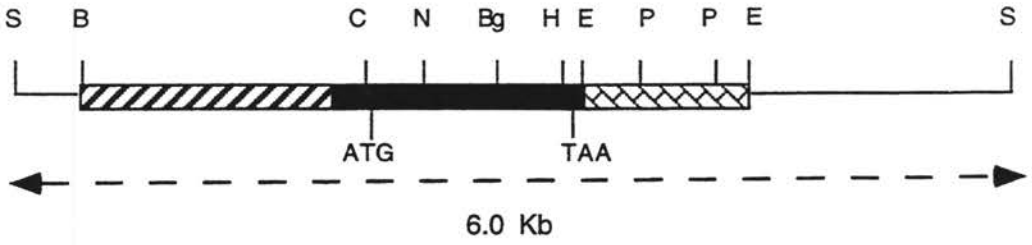


Figure 13: Plasmid contruct pJS011, carrying the *Gα4(Q200L)* gene with the neomycin resistance gene. A detailed description of the construct is given in Materials and Methods. The initiation and termination codons are represented by ATG and TAA, respectively. The shaded segments represent the *Gα4* gene sequences, the striped segments represent the *Gα4* promoter sequences and the patterned segments represent the neomycin resistance gene (*G-418*^{r}). Restriction enzymes shown are *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Hind*III (H), *Nco*I (N), *Pst*I (P) and *Sal*I (S).

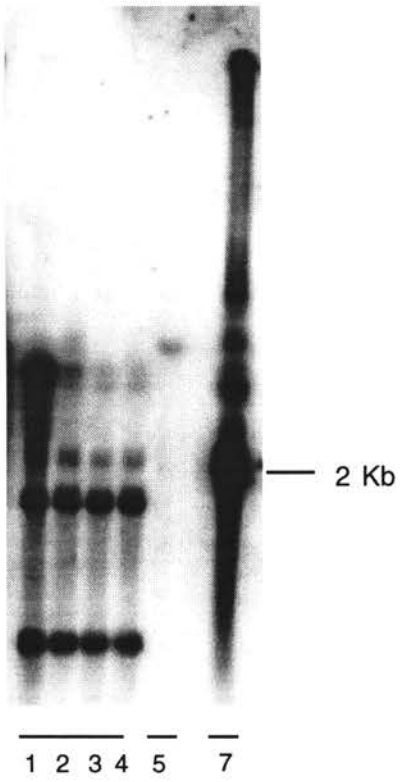


Figure 14: Southern blot of the cells transformed with the plasmid pJS011 i.e. *Gα4(Q200L)* gene with the neomycin resistance gene (*G-418*^{r}). Cells were grown to mid-log phase and genomic DNA was isolated as described in Materials and Methods. The DNA was then digested with *Cla*I/*Pst*I and run on a 0.7% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a 1.5 Kb *Eco*RI/*Bam*HI fragment of the *Gα4* open reading frame from pJH317 as described in Materials and Methods. Lanes 1-4 represent DNA isolated from the mutants, lane 5 represents DNA from untransformed wild type strain and lane 7 is the plasmid pJS011 digested with *Cla*I/*Pst*I (control). The *Gα4* probe is expected to hybridize a 2.0 Kb fragment of the *Gα4* gene in the mutants. The lower bands may be products of genomic rearrangement (see text).

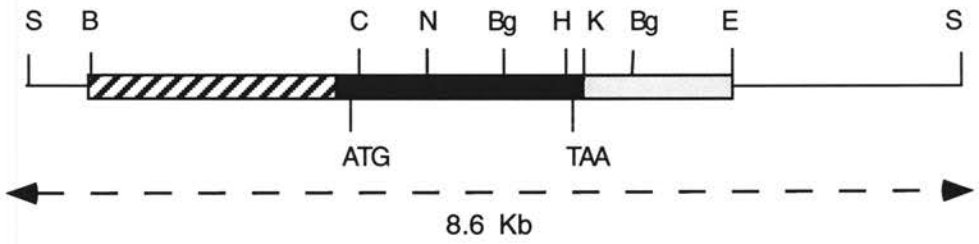


Figure 15: Plasmid contruct pJS010 carrying the *Gα4(Q200L)* gene with the blasticidin resistance gene (Bs^r). A detailed description of the construct is given in Materials and Methods. The initiation and termination codons are represented by ATG and TAA respectively. The dark shaded segments represent the *Gα4* gene sequences, the striped segments represent the *Gα4* promoter sequences and the light shaded segments represent the blasticidin (Bs^r). Restriction enzymes shown are *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Nco*I (N) and *Sal*I (S).

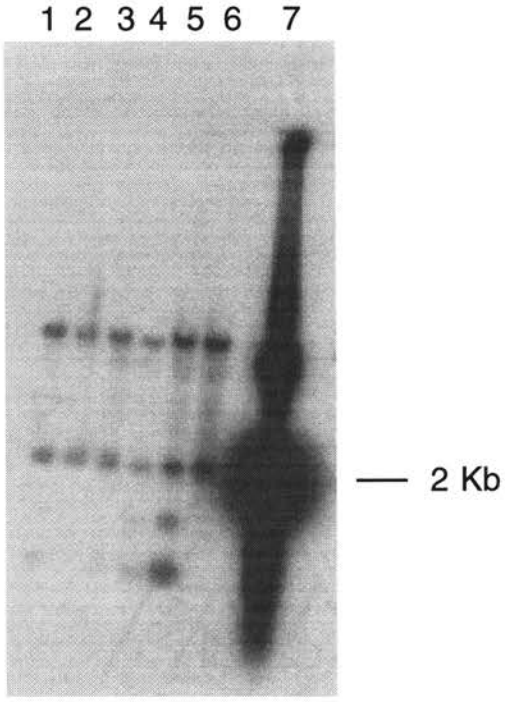


Figure 16: Southern blot of cells transformed with the *Gα4(Q200L)* gene with the blasticidin resistance gene (*Bs*^{r}). Cells were grown to mid-log phase and genomic DNA was isolated as described in Materials and Methods. The DNA was then digested with *Cla*I/*Kpn*I and run on a 0.8% agarose gel and blotted onto a nitrocellulose membrane. The blot was hybridized with a 1.5 Kb *Eco*RI/*Bam*HI fragment of the *Gα4* open reading frame from pJH317 as described. The *Gα4* probe hybridizes a 2.0 Kb fragment of the *Gα4* gene in the mutants. Lanes 1-6 represent DNA isolated from wild type strain with the plasmid carrying the *Gα4(Q200L)* gene with the Bs^r marker (pJS010). Lane 7 represents plasmid pJS010 digested with *Cla*I/*Kpn*I which is the control.

might be due to DNA rearrangement (Figure 16).

Since the *Gα4(Q200L)* plasmid appeared to be lethal to the cells, we wanted to investigate if the cell death observed could be programmed cell death or apoptosis. Since programmed cell death in several systems to be accompanied by DNA fragmentation (28), we sought to investigate if this was the case in our system. Earlier reports in literature have indicated that no clear cut ladder pattern of DNA fragmentation could be seen in the case of *Dictyostelium* at the multicellular stage of development involving differention to stalk cells which ultimately die out (28). We weren't able to determine if DNA fragmentation was occurring in the transformants carrying the *Gα4(Q200L)* gene, since these transformants appeared to die out and we did not have sufficient cells to run out genomic DNA on gels. Therefore, we attempted to detect DNA fragmentation using flourescent dyes as described in literature (28). This was done using a combination of two dyes i.e., propidium iodide which would stain the nuclei of cells which no longer retained membrane integrity (necrotic cells) and Hoechst 33342 which would stain the nuclei of apoptotic cells that lose membrane integrity only after treatment with ethanol. Staining of apoptotic cells on plastic petri plates did not however, show markedly flourescent DNA when examined under the fluorescent microscope. Although some flourescence was seen, it wasn't clear enough to enable us to take photographs. The analyses of these mutants was further rendered more difficult due to the rapid demise of these mutants as all analyses needed to be performed very rapidly. The poor staining of the apoptotic *Dictyostelium* cells could be attributed to the presence of insufficient cells. Therefore, it was decided that the expression of the mutant *Gα4* gene from a repressible promoter, such as the discoidin promoter might help us overcome this obstacle (see

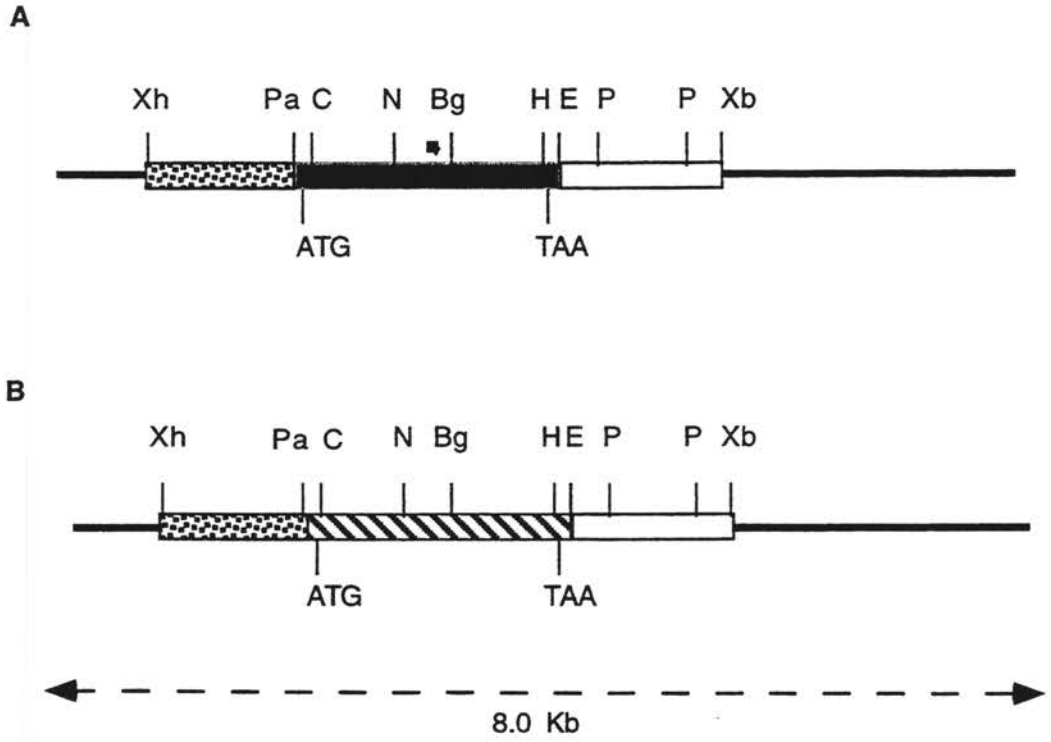


Figure 17: Inducible constructs with the discoidin-I promoter. (A). Map of the inducible *Gα4(Q200L)* gene (*pdisc-Gα4(Q200L)*) and (B) Map of the inducible wild type *Gα4* gene (*pdisc-Gα4*). A detailed description of the constructs is given in Materials and Methods. The initiation and termination codons are represented by ATG and TAA respectively. The light patterned segments represent the discoidin promoter sequences, the dark spotted segments (A) represent the mutant *Gα4(Q200L)* gene sequences, the light and dark hatched segments (B) represent the wild type *Gα4* gene sequences, and the clear segments represent the *pACT6::Neo*^{r} gene which confers resistance to G-418. The restriction enzyme sites shown are *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Hind*III (H), *Pac*I (Pa), *Pst*I (P), *Xba*I (Xb) and *Xho*I (Xh). The dark shaded box upstream of the *Bgl*II site (A) denotes the site of the Q200L mutation.

Figure 17 for vector constructs) as the discoidin promoter is capable of being regulated by folic acid (9). The rationale behind the use of this promoter to control the expression of the mutant *Gα4* gene was that this would enable us to grow sufficient numbers of cells by turning off the expression of the mutant gene. Repression could then be removed and the mutant cells could be analyzed. However, the mutants obtained bearing the*Gα4(Q200L)* gene under the control of the discoidin promoter did not show any difference in phenotype whether they were grown in the presence or absence of folic acid. This pointed towards the possibility of expression of the discoidin promoter not being subjected to tight regulation by folic acid. This was confirmed using Northern blots (Figure 18) where two distinct transcripts were seen which appeared to be the *Gα4* transcript and the discoidin transcript. The control used for comparison was untransformed wild type strain probed with the *Gα4* gene. However, some of the transformants obtained by electroporating the *pDis::Gα4(Q200L)* gene exhibited an aggregation deficient phenotype. Expression of the wild type Gα4 subunit from the discoidin promoter did not affect the developmental phenotype of wild type cells but did rescue folic acid chemotaxis and early developmental morphology in *gα4* null cells, indicating that the expression from the *discoidin-I* and *Gα4* promoters overlap during early development. From the analyses using the discoidin promoter, it seemed likely that the expression of the *Gα4(Q200L)* mutant gene at low levels could result in viable transformants. In order to confirm this, it was decided that wild-type cells would be electroporated using the *Gα4(Q200L)* gene with the Bs^r marker in order to select for transformants bearing low copies of the mutant gene.

Transformants obtained by electroporating *pGα4::Gα4(Q200L)* gene into wild-

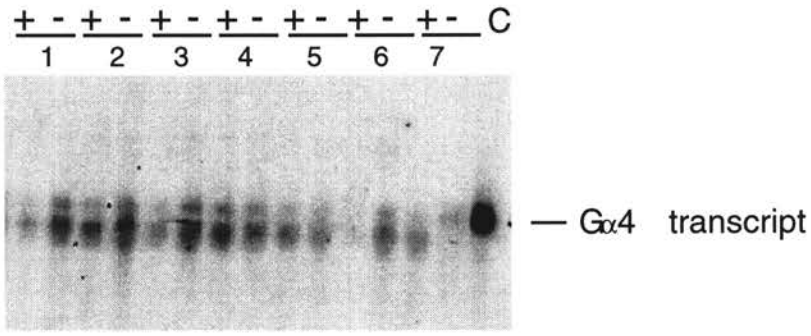


Figure 18: Expression of the repressible *pDisc::Gα4(Q200L)* gene constructs in cells. Cells were grown with and without folic acid (denoted by the signs + and – respectively) and were harvested for isolation of RNA as described in Materials and Methods. Total RNA was isolated from the vegetative cells and 4 µg of RNA was run per lane on formaldehyde gels as described in Materials and Methods. The last lane is the control which is untransformed wild type cells expressing the endogenous *Gα4* gene. Lanes 1-14 represent RNA from wild type cells transformed with the repressible mutant construct.

type cells at this stage were selected with low level of the drug blasticidin(<5μg/μl) and were seen to exhibit an aggregation deficient phenotype. These mutants were subjected to further analyses (as described earlier in the results section). In order to rule out the possibility of the mutants having arisen as a result of another compensating mutation which could allow them to survive, several electroporations were done with the mutant plasmid and the efficiency of obtaining mutants exhibiting aggregation deficient phenotype was noted. It was seen that the efficiency of obtaining transformants was between 30-50% with the mutant *Gα4(Q200L)* plasmid in each case when compared to control electroporations carried out with the wild-type *Gα4* gene (Table 4). It is of interest that nearly 40% of these mutants exhibited an aggregation deficient phenotype.

Similar electroporations were also carried out with the *pGα4:Gα4(Q200L)* gene where the drug used for selection was G-418 (neomycin sulfate)(Table 4). Although, the efficiency of electroporation was markedly reduced with this plasmid, a large majority of the transformants obtained were aggregation deficient. Care was taken to allow viability of transformants carrying few copies of the mutant *Gα4* gene by using low levels of the drug. Since the possibility of transformants of the mutant *Gα4(Q200L)* gene with the neomycin resistance gene with a large number of copies was higher as compared to those carrying the blasticidin resistance gene(also confirmed by Southern blot analysis, shown in Figure 19), all analyses were carried out with the latter mutants. This was done to avoid analyzing mutants that might have lost copies of the mutant gene in order to maintain viability.

A

Electroporation #	Number of Transformants in wild type cells with plasmids			
	pJS010	pJH287	pJS011	pJH154
1	65	125	52	102
2	115	>200	82	162
3	84	130	50	125

B

Electroporation #	Number of Transformants in *gα4* null cells with plasmids			
	pJS010	pJH287	pJS011	pJH154
1	81	>200	63	>200
2	132	>200	85	>200
3	92	>200	70	185

Table 4: Results of electroporation of wild type and *gα4* null cells with plasmids carrying the *Gα4(Q200L)* gene with G-418 resistance gene (pJS011) or the *Gα4(Q200L)* gene carrying the blasticidin resistance marker (pJS010). The controls for the above plasmids are pJH154 and pJH287 respectively which have the wild type *Gα4* gene without the Q200L mutation.

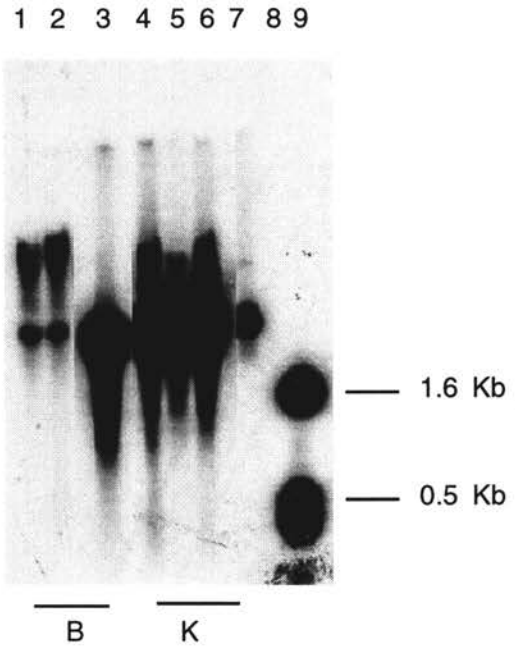


Figure 19: Southern blot of wild type cells transformed with plasmid carrying the *Gα4(Q200L)* gene with the *Bs*^{r} gene (lanes 1 and 2) and wild type cells carrying the *Gα4(Q200L)* gene with the *G-418*^{r} gene (lanes 4, 5 and 6). Restriction enzyme digests were carried out on genomic DNA as specified and a 1.5 Kb *Eco*RI/*Bam*HI *Gα4* ORF fragment was used as the probe. The untransformed plasmids digested with the appropriate restriction enzymes as specified were used as controls (lane 3 is pJS010 and lane 7 is pJS011). The *Gα4* probe hybridizes a 2.0 Kb fragment in the mutant cells. Lane 9 represents the 1 Kb molecular weight marker. The restriction enzymes indicated at the bottom are *BamH*I (B) and *Kpn*I (K).

Aggregation defects associated with the Gα4(Q200L) subunit

The lack of viable transformants with a high copy number of the *Gα4(Q200L)* expression vector was similar to the inability to obtain high copy number transformants with *Gα5(Q199L)* expression vectors (70). *Dictyostelium* cells transformed with a high copy number of the control vector, expressing the wild type Gα4 subunit, displayed no obvious defects in vegetative growth but these Gα4 overexpression transformants did have altered developmental morphology as described in earlier reports.

When the *Gα4(Q200L)* expression vector using a selectable marker gene that confers resistance to the drug blasticidin S was transformed into wild type cells, the electroporation produced approximately half the number of transformants as did a control vector expressing the wild type *Gα4* gene (Table 4)). The majority of transformants displayed an aggregation defective phenotype when plated on nonnutrient plates or bacterial lawns (Figure 20). On the bacterial lawns, *Dictyostelium* cells consume the bacteria forming plaques and then typically aggregate for multicellular development when starved for nutrients in the interior of the plaque. However, cells expressing the Gα4(Q200L) subunit were only capable of forming loose aggregates at the plaque perimeters where cell density is high due to the accumulation of cells searching for more bacteria. Aggregates that formed on the plaque perimeters typically failed to proceed any further through development. The *Gα4(Q200L)* vector was also transformed into *gα4* null cells that do not produce the Gα4 subunit, and $G\alpha4^{HC}$ cells that overexpress the wild type Gα4 subunit due to increased gene dosage. In both strains, the majority of transformants were defective in the aggregation phase of development. The defective aggregation phenotype was also observed in wild type cells or *gα4* null mutants

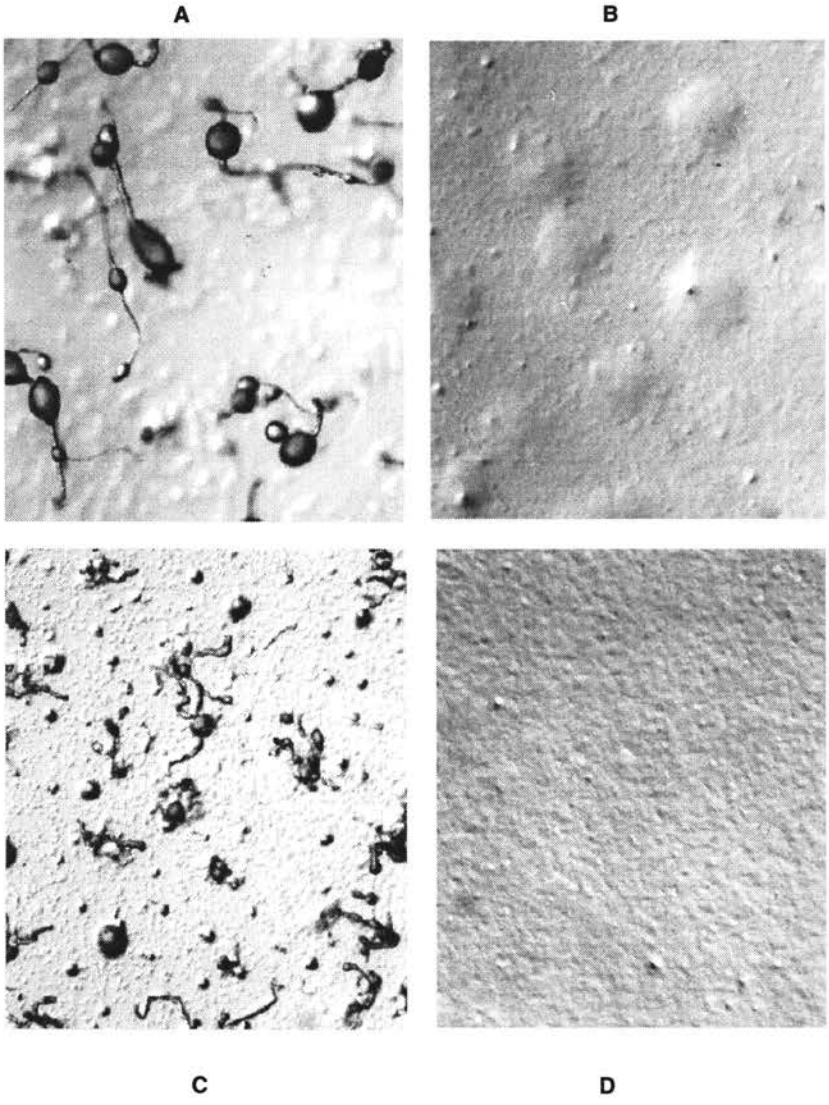


Figure 20: Morphological phenotypes of wild type cells (A), wild type cells expressing the *Gα4(Q200L)* gene (B), *gα4* null cells (C) and *gα4* null cells expressing the *Gα4(Q200L)* gene. Cells were grown to mid-log phase in shaking culture, washed in phosphate buffer, and then plated for development on nonnutrient plates as described in the Materials and Methods section. Photographs were taken 25 hours after starvation.

transformed with the *Gα4(Q200L)* vector conferring resistance to the drug G-418 if the drug selection was decreased significantly (< 5 μg/ml G-418) to allow for transformants with a low vector copy number. Aggregation defects were also observed when the Gα4(Q200L) subunit was expressed from the folic acid-repressible discoidin-I promoter in wild type or $G\alpha4^{HC}$ cells. Attempts to repress *Gα4(Q200L)* expression from the discoidin promoter with 1 mM exogenous folic acid did not rescue the ability to aggregate, suggesting that the discoidin promoter was not completely repressed. All subsequent analyses of *Gα4(Q200L)* mutants were conducted with cells carrying the low copy number vector conferring resistance to blasticidin S.

The Gα4(Q200L) subunit inhibits responses to folic acid

Cells containing the Gα4(Q200L) expression vector that confers blasticidin S resistance were examined for responsiveness to external folic acid since Gα4 function is essential for all known responses to folic acid signaling. Wild type cells expressing Gα4(Q200L) subunit were inhibited in their chemotactic movement to folic acid during early development with the inhibition being more apparent at 3 hours post-starvation versus 0 or 6 hours post-starvation (Figure 21). The expression of the Gα4(Q200L) subunit in $G\alpha4^{HC}$ cells did not rescue the ability to chemotax to folic acid at any time after starvation, indicating that the *gα4* null cells are not complemented by the *Gα4(Q200L)* allele with respect to chemotaxis. The Gα4(Q200L) subunit was not capable of inhibiting folic acid chemotaxis in $G\alpha4^{HC}$ cells suggesting that increased expression of the wild type Gα4 subunit prevents the inhibition of the Gα4(Q200L) subunit with respect to folic acid responsiveness.

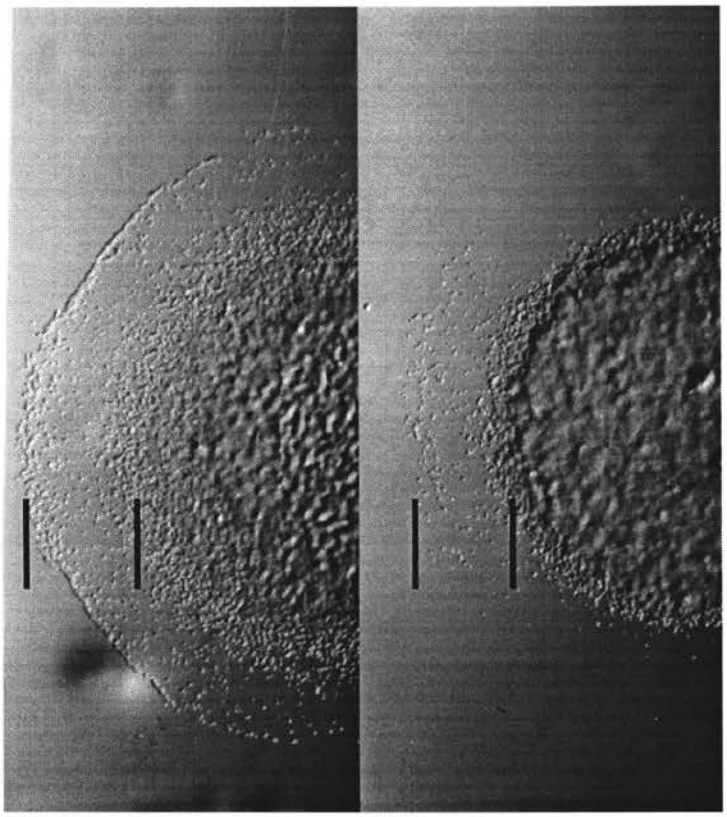


Figure 21: Chemotaxis of wild type (A) and wild type cells expressing the *Gα4(Q200L)* gene to 1 mM folic acid. Cells were prepared for development as described in Materials and Methods and were plated at a concentration of $1x10^8$ cells/ml for chemotaxis assays as described. Photographs were taken 2.5 hours after the cells were plated. The folic acid droplet was placed to the left of the cell droplets. Bars represent the leading edge of the chemotaxing cells and the cell droplet perimeter.

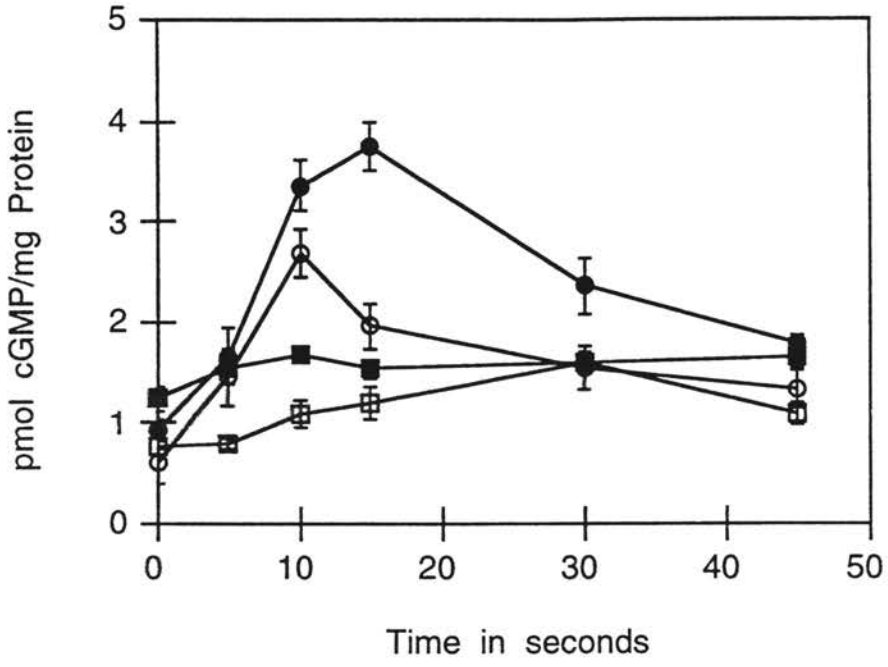


Figure 22: Accumulation of cGMP in folic acid stimulated wild type (closed circles), *gα4* null cells (closed squares), wild type cells expressing the *Gα4(Q200L)* gene (open circles) and *gα4* null cells expressing the *Gα4(Q200L)* gene (open squares). Cells were grown and washed as described in the Materials and Methods section. After stimulation with folic acid, cells were collected at the times indicated and assayed for cGMP concentration. The cGMP concentrations were normalized to the protein concentration in each stimulated cell culture. The data represent one of the several independent experiments that produced similar results. Each extract was assayed in duplicate and the vertical error bars represent the deviation from the mean value.

The effects of the Gα4(Q200L) subunit on cyclic nucleotide accumulation in response to folic acid were also examined since these responses are dependent on Gα4 function. Wild type cells expressing the Gα4(Q200L) subunit displayed a reduced accumulation of cGMP in response to folic acid compared to wild type cells without the vector (Figure 22). Cyclic GMP accumulation was absent in *gα4* null cells that express the mutant Gα4(Q200L) subunit, suggesting that the mutant Gα4(Q200L) subunit is not capable of rescuing this response. The Gα4(Q200L) subunit in wild type cells also inhibited the accumulation of cAMP as compared to wild type cells without the mutant subunit (Figure 23). Expression of the Gα4(Q200L) subunit in *gα4* null mutants did not rescue cAMP in response to folic acid, consistent with the inability of this Gα4(Q200L) subunit to provide wild type Gα4 function.

The Gα4(Q200L) subunit inhibits responses to extracellular cAMP

The aggregation defective phenotype of cells expressing the Gα4(Q200L) subunit suggested that the mutant Gα subunit also inhibits the cAMP responses needed during aggregation. Therefore, we examined the ability of *Gα4(Q200L)* expressing cells to chemotax to cAMP after 6 hours of starvation when cells typically initiate the aggregation process. Wild type cells expressing the *Gα4(Q200L)* vector were severely inhibited in their ability to chemotax to cAMP in comparison to wild type cells without the Gα4(Q200L) subunit (Figure 24). Even after 12 hours of starvation, cells expressing the Gα4(Q200L) subunit were still unable to exhibit cAMP chemotaxis, indicating that the inhibition of cAMP chemotaxis was not merely delayed (data not shown). Cells expressing the Gα4(Q200L) subunit were transformed with a high copy number vector

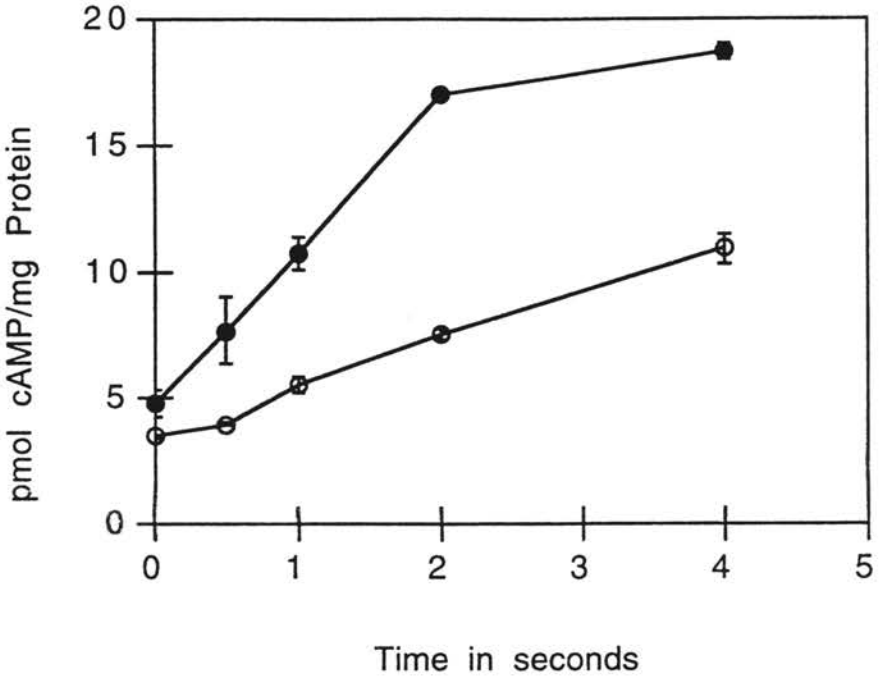
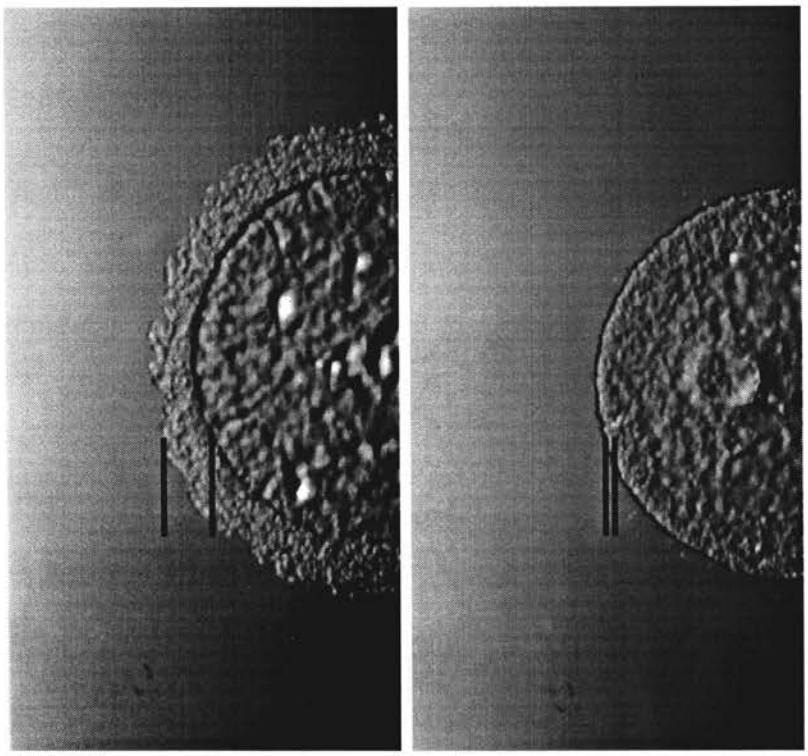


Figure 23: Accumulation of cAMP in folic acid stimulated wild type (closed circles), and wild type cells expressing the *Gα4(Q200L)* gene (open circles). Cells were grown and washed as described in the Materials and Methods section. After stimulation with folic acid, cells were collected at the times indicated and assayed for cAMP concentration. The cAMP concentrations were normalized to the protein concentration in each stimulated cell culture. The data represent one of the several independent experiments that produced similar results. Each extract was assayed in duplicate and the vertical error bars represent the deviation from the mean value.



A B

Figure 24: Chemotaxis of wild type cells (A) and wild type cells expressing the *Gα4(Q200L)* gene (B) to 100 μM cAMP. Photographs were taken 2.5 hours after the cells were plated. Cells were prepared as described in Materials and Methods and were plated at a density of $1x10^8$ cells/ml for chemotaxis assays as outlined. The source of cAMP is to the left of cell droplets. Bars represent the leading edge of the chemotaxing cells and the cell droplet perimeter.

expressing the wild type Gα2 subunit but none of the resulting transformants werecapable of aggregation, suggesting that Gα2 subunit overexpression is not sufficient to overcome the inhibitory effects of the Gα4(Q200L) subunit. Wild type cells expressing the Gα4(Q200L) allele were also tested for the ability to accumulate cGMP and cAMP in response to extracellular cAMP since these responses are important in responding to and relaying the cAMP signal during aggregation. Wild type cells expressing the Gα4(Q200L) subunit were not capable of accumulating cGMP in response to extracellular cAMP in contrast to that observed for wild type cells without the mutant subunit. This inability to elevate cGMP concentrations is consistent with the Gα4(Q200L) subunit preventing chemotaxis since cGMP is thought to be a secondary messenger necessary for chemotactic movement (Figure 25). The Gα4(Q200L) subunit also inhibited the ability of wild type cells to accumulate cAMP which is necessary for aggregating cells to relay the cAMP signal to other cells (Figure 26). The inability of *Gα4(Q200L)* expressing cells to accumulate either cAMP or cGMP suggests that the mutant Gα4 subunit affects cAMP signal transduction early in the pathway, similar to the inhibition to folic acid responses.

Rescue of aggregation in *Gα4(Q200L)* mutants requires Gα4 function.

Aggregation defects have been previously associated with the loss or alteration of the Gα2 subunit since this G-protein subunit is necessary for the aggregative response to cAMP signaling. In some cases, the ability to form multicellular aggregates can be restored by exogenous cAMP treatments or temporary incubation of cells at lower temperatures. To determine if exogenous cAMP treatment is capable of rescuing

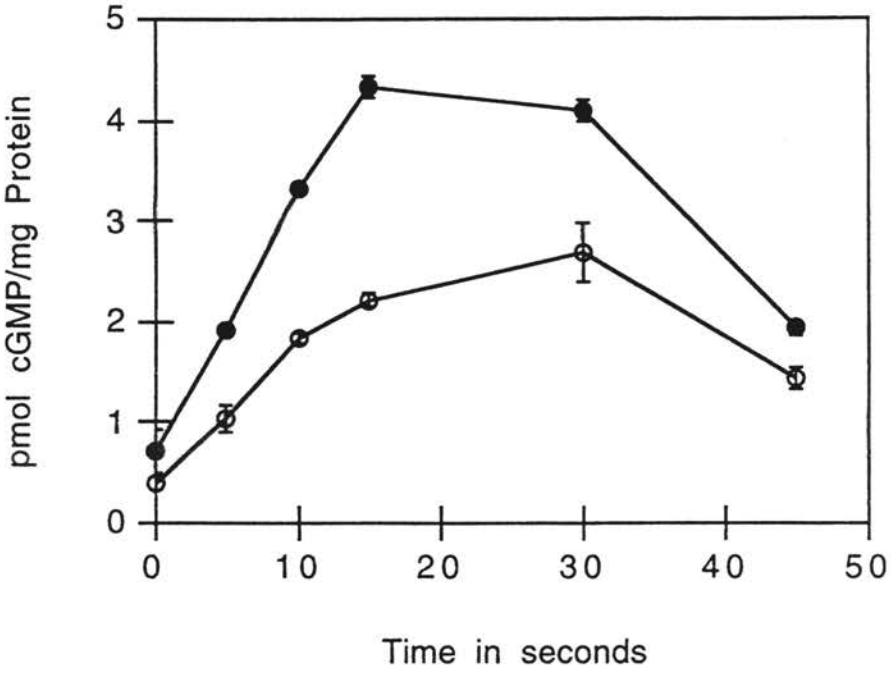


Figure 25: Accumulation of cGMP in cAMP stimulated wild type (closed circles), and wild type cells expressing the *Gα4(Q200L)* gene (open circles). Cells were grown and washed as described in the Materials and Methods section. After stimulation with cAMP, cells were collected at the times indicated and assayed for cGMP concentration. The cGMP concentrations were normalized to the protein concentration in each stimulated cell culture. The data represent one of several independent experiments that produced similar results. Each extract was assayed in duplicate and the vertical error bars represent the deviation from the mean value.

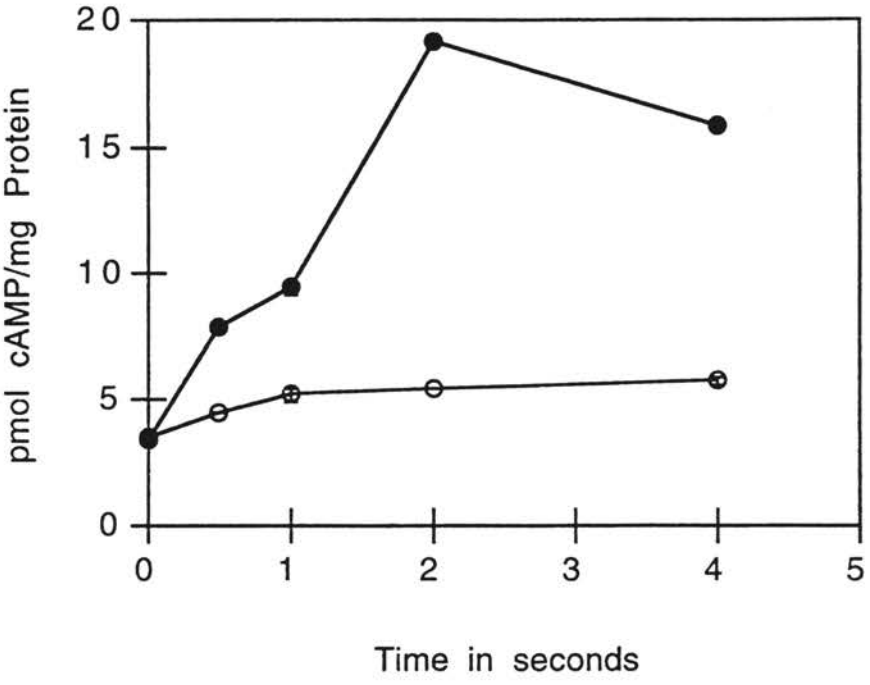


Figure 26: Accumulation of cAMP in cAMP stimulated wild type cells (closed circles), and wild type cells expressing the *Gα4(Q200L)* gene (open circles). Cells were grown and washed as described in the Materials and Methods section. After stimulation with cAMP , cells were collected at the times indicated and assayed for cAMP concentration. The cAMP concentrations were normalized to the protein concentration in each stimulated cell culture. The data represent one of several independent experiments that produced similar results. Each extract was assayed in duplicate and the vertical error bars represent the deviation from the mean value.

aggregate formation, cells expressing the Gα4(Q200L) subunit were treated with exogenous cAMP while shaken in phosphate buffer and then plated for development on nonnutrient plates. Small fruiting bodies developed from these shaken cultures (Figure 27). However, *gα4* null cells expressing the Gα4(Q200L) subunit displayed no multicellular development after cAMP treatment, indicating a requirement for wild type Gα4 function for bypassing the aggregation defect associated with the Gα4(Q200L) subunit.

The ability of Gα4(Q200L) subunit expressing cells to undergo multicellular development after incubation at lower temperatures was also tested by incubating nutrient-deprived cells at 4° C on nonnutrient plates and then returning them to 22° C. Wild type cells expressing the Gα4(Q200L) subunit were capable of forming aggregates that often developed into fruiting bodies after the cold temperature treatment, indicating that this temperature treatment was capable of bypassing the Gα4(Q200L) subunit impairment of multicellular development (Figure 28). However, the same temperature treatment of *gα4* null cells expressing the Gα4(Q200L) subunit failed to produce aggregates, consistent with the requirement of wild type Gα4 function for multicellular development.

Southern and Northern analyses of the *Gα4(Q200L)* mutants

Southern blots of the Gα4(Q200L) mutants showed insertion of the mutant plasmid in multiple copies (Figure 29). In order to determine whether expression of developmental genes was significantly high in these mutants, Northern blots were performed. As seen in Figure 30, the level of *Gα4(Q200L)* expression seemed to be

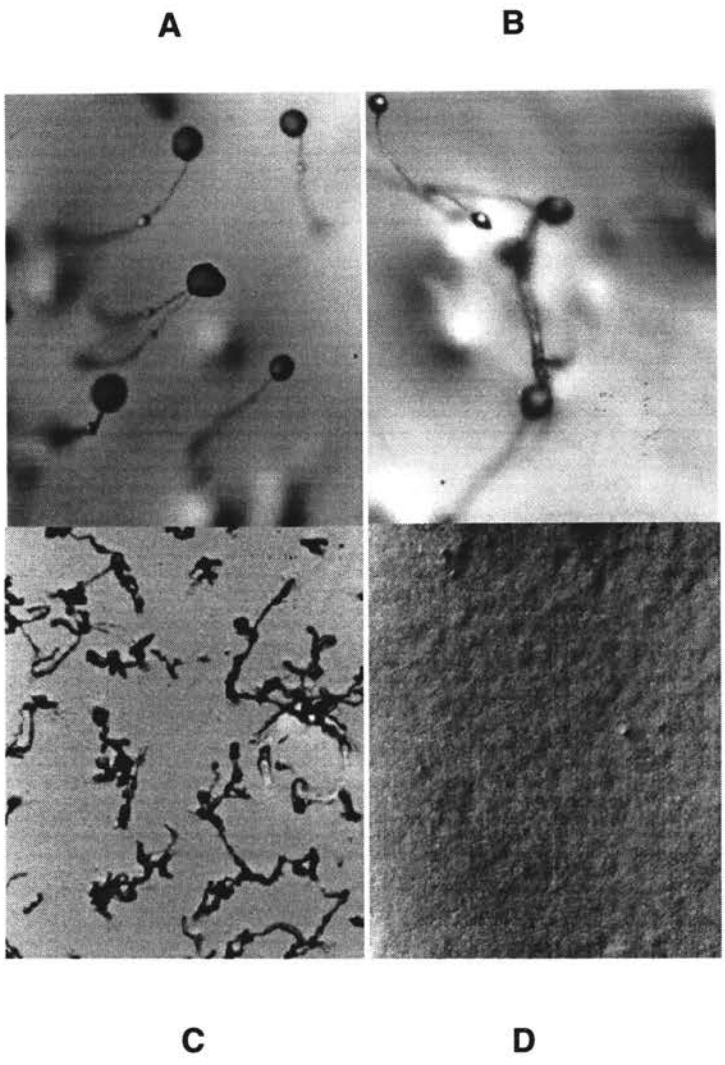


Figure 27: Morphological phenotypes of wild type cells (A), wild type cells expressing the *Gα4(Q200L)* gene (B), *gα4* null cells (C) and *gα4* null cells expressing the *Gα4(Q200L)* gene (D) after treatment with cAMP pulses. Cells were grown and washed as described in Materials and Methods and resuspended at $1x10^7$ cells/ml in phosphate buffer. Cells were then shaken at 125 rpm for 6 hours, pulsed with 100 μM cAMP every 2 hours and plated at $5x10^7$ cells/ml. Photographs were taken 24 hours after cells were plated.

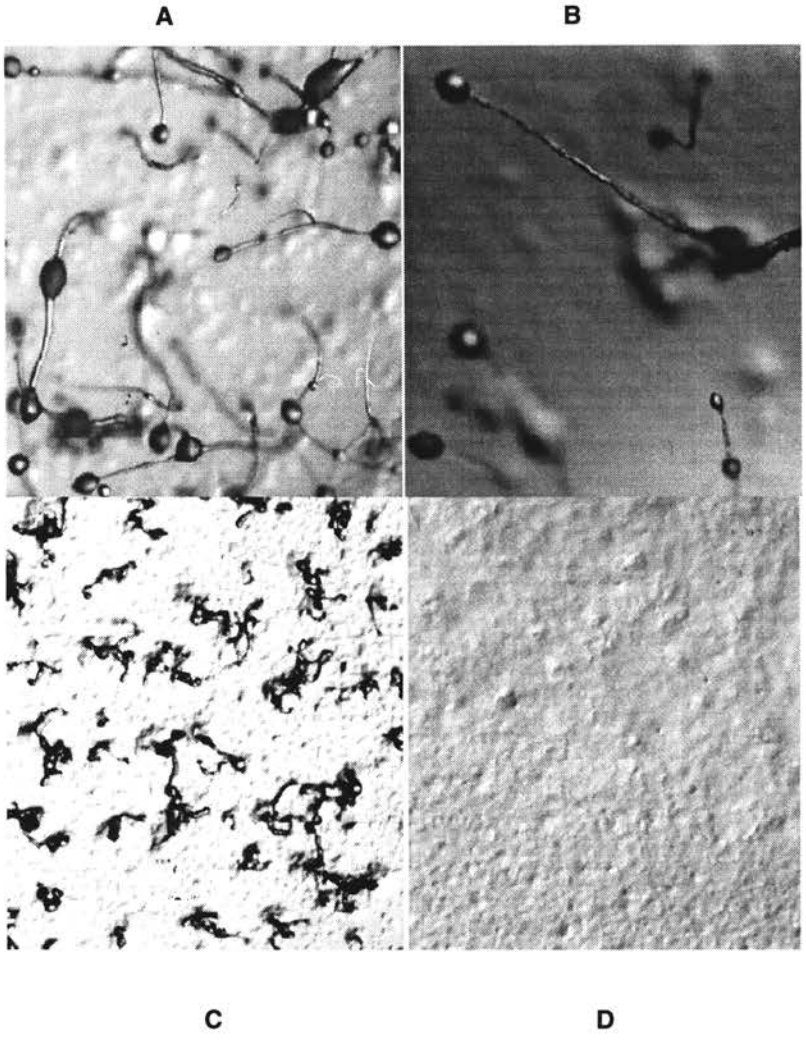


Figure 28: Morphological phenotypes of wild type cells (A), wild type cells expressing the *Gα4(Q200L)* gene (B), *gα4* null cells (C) and *gα4* null cells expressing the *Gα4(Q200L)* gene (D) after incubation at 4°C for 24 hours followed by incubation at 22°C. Cells were grown and washed as described in Materials and Methods. Photographs were taken 24 hours after cells were transferred from 4°C to 22°C.

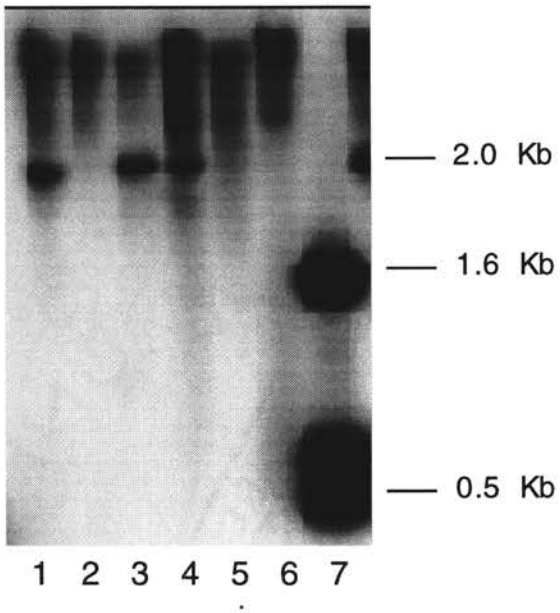


Figure 29: Southern blot of cells transformed with the *Gα4(Q200L)* gene with the blasticidin resistance gene (*Bs*^{r}). Cells were grown to mid-log phase and genomic DNA was isolated as described in Materials and Methods. The DNA was then digested with *Bam*HI and run on a 0.7% agarose gel and blotted onto a nylon membrane. The blot was hybridized with a 1.5 Kb *Eco*RI/*Bam*HI fragment of the *Gα4* open reading frame from pJH317 as described. The *Gα4* probe is expected to hybridize a 2.0 Kb fragment of the *Gα4* gene in the mutants. DNA was run in lanes 1-7 in the following order:

Lane 1: Wild type strain with the *Gα4(Q200L)* gene with *Bs*^{r}.

Lane 2: Wild type strain with the control plasmid carrying the *Gα4* gene without the Q200L mutation.

Lane 3: *gα4* null strain with the *Gα4(Q200L)* gene with *Bs*^{r}.

Lane 4: *gα4* null strain with the control plasmid carrying the *Gα4* gene without the Q200L mutation.

Lane 5: Untransformed wild type strain as control.

Lane 6: Untransformed *gα4* null strain as control.

Lane 7: 1 Kb molecular weight marker.

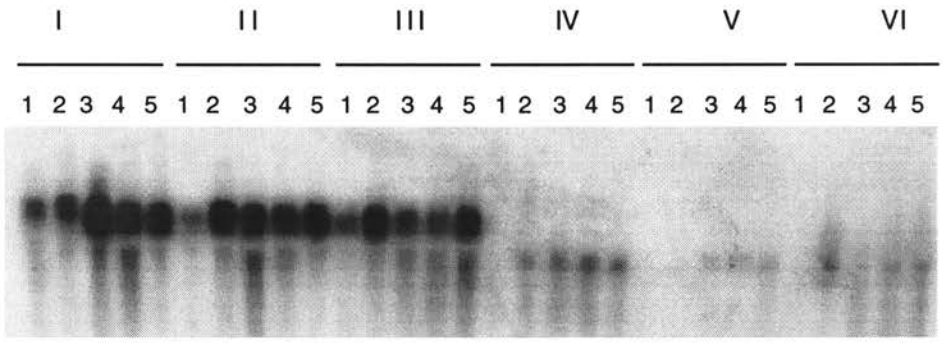


Figure 30: Expression of *Gα4* transcripts in wild type cells and *gα4* null cells transformed with plasmids carrying the *Gα4(Q200L)* gene with either the G-418 resistance (pJS011) or the blasticidin resistance (pJS010) gene. RNA was isolated from developing cells at times indicated (0, 5, 10, 15 and 20 h) and then analysed as described in Materials and Methods. The RNA blot was hybridized with a 1.5 Kb *Eco*RI/*Bam*HI *Gα4* probe. The numbers 1 through 5 represent RNA isolated at 0, 5, 10, 15 and 20 hours. The numbers above the time points indicate the strain from which the RNA was collected and are as follows:

I. Wild type strain.

II. Wild type carrying the control plasmid (*Gα4* gene without the Q200L mutation).

III. Wild type strain carrying the *Gα4(Q200L)* plasmid with the Bs^r gene.

IV. *gα4* null strain.

V. *gα4* null strain carrying the control plasmid (*Gα4* gene without the Q200L mutation).

VI. *gα4* null strain carrying the *Gα4(Q200L)* gene with the Bs^r gene.

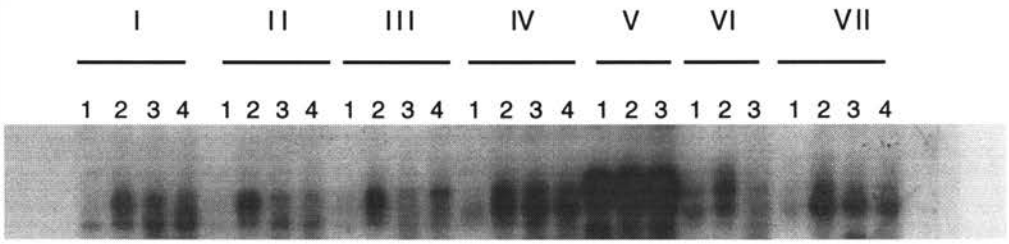


Figure 31: Expression of *Gα2* transcripts in wild type cells and *gα4* null cells transformed with plasmids carrying the *Gα4(Q200L)* gene with the blasticidin resistance (pJS010) gene. RNA was isolated from developing cells at times indicated (0, 5, 10 and 15 h) and then analysed as described in Materials and Methods. The RNA blot was hybridized with a 1.2 Kb *Eco*RI *Gα2* probe. The numbers 1 through 4 represent RNA isolated at 0, 5, 10, and 15 hours. The numbers above the time points indicate the strain from which the RNA was collected and are as follows:

I: Wild type carrying the control plasmid (*Gα4* gene without the Q200L mutation).

II: Wild type strain carrying the *Gα4(Q200L)* plasmid with the Bs^r gene.

III: *gα4* null strain carrying the *Gα4(Q200L)* gene with the Bs^r gene.

IV: *gα4* null strain.

V: Wild type strain with the *Gα4(Q200L)* gene transformed with plasmid carrying the wild type *Gα2* gene.

VI: *gα4* null strain with the *Gα4(Q200L)* gene transformed with plasmid carrying the wild type *Gα2* gene.

VII: Wild type strain.

sufficiently low as to not show up clearly. The expression of the *Gα2* gene on the other hand could be detected (Figure 31), although the later stages of development (15 h) showed lower levels of expression when compared to the wild-type cells (control). This was also observed in the case where these mutants were probed with the prespore and prestalk specific genes, *ecmA* and *cotC,* respectively (data not shown). This is consistent with the observation that these mutants are unable to reach later stages of development as they exhibit inability to aggregate upon starvation. In order to determine if the mutant *Gα4(Q200L)* gene affects the expression of the cAR1 gene, RNA extacted from the mutants at various stages of development was probed with a fragment containing the cAMP receptor gene, cARI. The levels of *cARI* expression were seen to be about the same as in wild-type cells (Figure 32).

Therefore, Northern analysis indicates that the mutant *Gα4(Q200L)* gene does not appear to affect expression of developmental genes such as *cotC*, *ecmA*, *Gα2* or *Gα4*.

Analogous mutations in other *Gα* mutants inhibit cAMP and folic acid chemotactic movement

The ability of the Gα4(Q200L) subunit to inhibit chemotactic responses to extracellular cAMP resembles the phenotype previously reported for cells expressing an analogous alteration in the Gα2 subunit, suggesting that the mutant Gα4 and Gα2 subunits might affect common signaling mechanisms. Wild type cells were transformed with a Gα2(Q208L) subunit expression vector in which the mutant subunit is expressed from the *actO* (*actin 15*) promoter. Most of the transformants displayed partial or complete inhibition of aggregation and cAMP chemotaxis similar to that previously

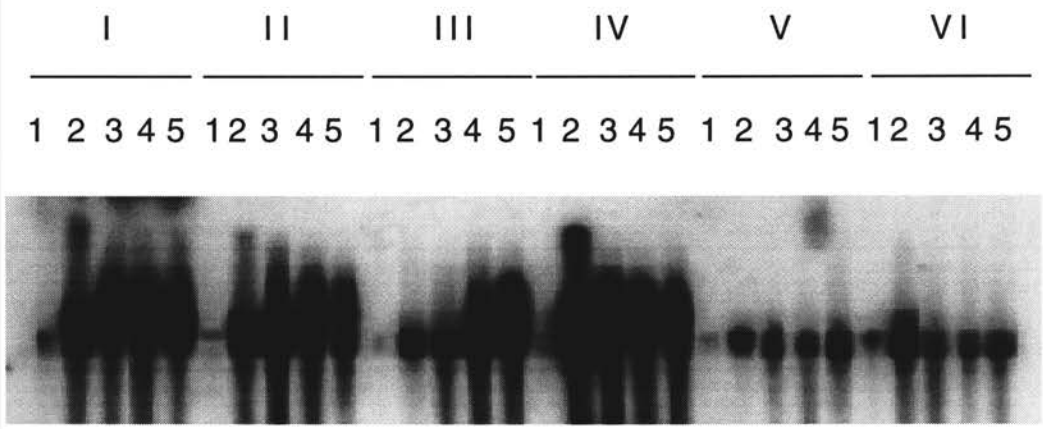


Figure 32: Expression of *cAR1* transcripts in wild type cells and *gα4* null cells transformed with plasmids carrying the *Gα4(Q200L)* gene with the blasticidin resistance gene (pJS010). RNA was isolated from developing cells at different times (0, 5, 10, 15 and 20 h) and 4 µg of RNA run per lane on formaldehyde gels as described in Materials and Methods. The RNA blot was hybridized with a 1.2 Kb *Eco*RI *cAR1* probe. The numbers 1-5 represent RNA isolated at 0, 5, 10, 15 and 20 hours. The numbers above the time points indicate the strain from which the RNA was collected and are as follows:

I. Wild type strain.

II. Wild type carrying the control plasmid (*Gα4* gene without the Q200L mutation).

III. Wild type strain carrying the *Gα4(Q200L)* plasmid with the Bs^r gene.

IV. *gα4* null strain.

V. *gα4* null strain carrying the control plasmid (*Gα4* gene without the Q200L mutation).

VI. *gα4* null strain carrying the *Gα4(Q200L)* gene with the Bs^r gene.

described for the expression of the Gα2(Q208L) subunit from its endogenous promoter. Aggregation defective Gα2(Q208L) subunit expressing cells were examined for the ability to chemotax to folic acid and found to be slightly impaired in their chemotactic response in comparison to wild type cells without the Gα2(Q208L) subunit (Figure 33).

Since both Gα4 and Gα2 mutants are capable of inhibiting chemotactic responses to both folic acid and cAMP, we predicted that other structurally-related mutant Gα subunits, such as the Gα5(Q199L) subunit, might also be capable of inhibiting these chemotactic responses. Previous studies of cells transformed with Gα5(Q199L) subunit expression vectors had not revealed defects in chemotaxis but aggregation defective transformants might have been overlooked if the selection for vector copy number had been too high as was initially the case for Gα4(Q200L) expression vector transformants. Wild type cells were transformed with a vector expressing the Gα5(Q199L) subunit from its endogenous promoter and selection for transformants conducted using a low level of drug selection. Many of the transformants were found to be defective in aggregation and cAMP chemotaxis similar to that described for cells expressing the Gα4(Q200L) or Gα2(Q208L) subunit (Figure 34). These Gα5(Q199L) subunit expressing transformants appeared to be more severely inhibited in their ability to chemotax to folic acid after three hours of starvation as compared to the *Gα4(Q200L)* or *Gα2(Q208L)* mutants (Figure 35). These results suggest that Gα5(Q199L) allele can inhibit chemotactic responses to both cAMP and folic acid which is similar to the observation in the *Gα4(Q200L)* and *Gα2(208L)* mutants.

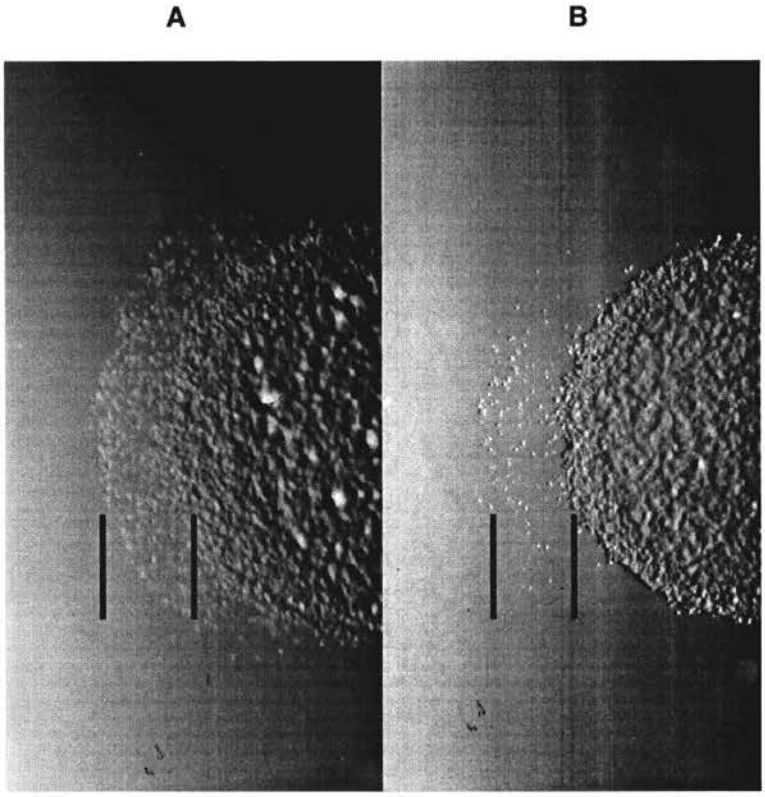


Figure 33: Chemotaxis of wild type cells (A), and wild type cells expressing the *Gα2(Q208L)* gene (B) to 1 mM folic acid. Chemotaxis assays were performed as described in Materials and Methods. Photographs were taken 2.5 hours after cells were plated. Folic acid source is located to the left of cell droplets. Bars represent the leading edge of the chemotaxing cells and the cell droplet perimeter.

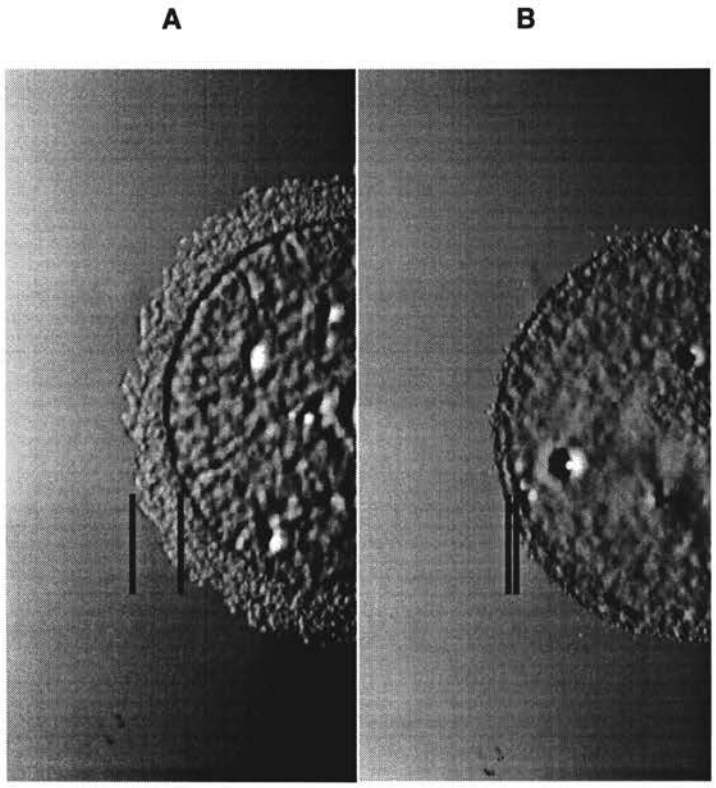


Figure 34: Chemotaxis of wild type cells (A), and wild type cells expressing the *Gα5(Q199L)* gene (B) to 100 μM cAMP. Chemotaxis assays were performed as described in Materials and Methods. Photographs were taken 2.5 hours after cells were plated. cAMP source is located to the left of cell droplets. Bars represent the leading edge of the chemotaxing cells and the cell droplet perimeter.

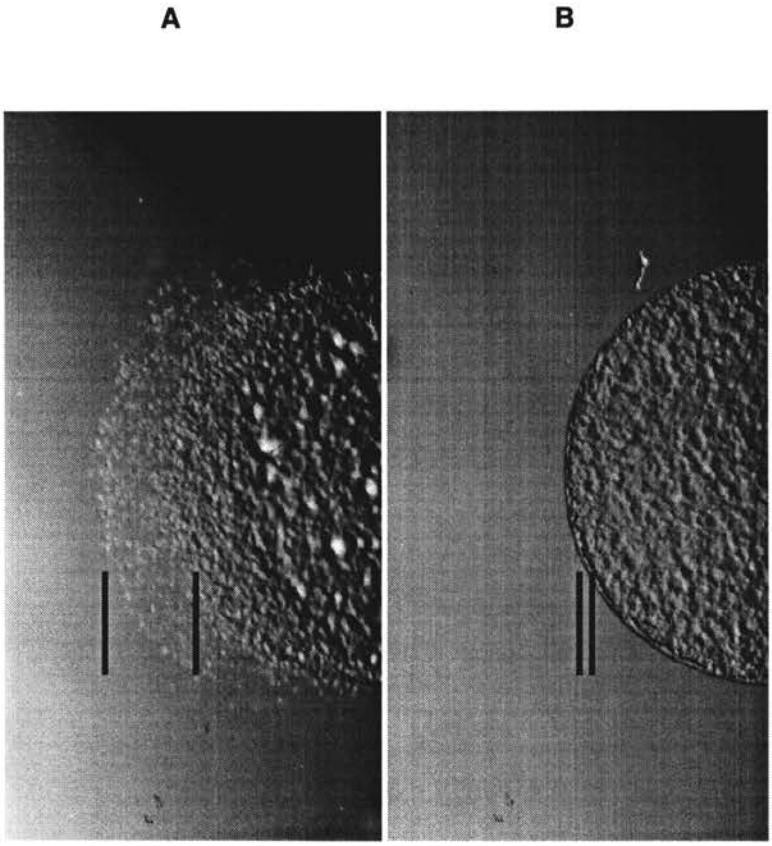


Figure 35: Chemotaxis of wild type cells (A), and wild type cells expressing the *Gα5(Q199L)* gene (B) to 1 mM folic acid. Chemotaxis assays were performed as described in Materials and Methods. Photographs were taken 2.5 hours after cells were plated. Folic acid source is located to the left of cell droplets. Bars represent the leading edge of the chemotaxing cells and the cell droplet perimeter.

Multicellular development of aggregation defective *Gα* mutants requires Gα4 function

Since multicellular development was observed for wild type cells expressing Gα4(Q200L) subunit when they were incubated at 4°C for 24 hours, we tested the ability of Gα4(Q208L) and Gα5(Q199L) expressing cells to develop under these conditions. Wild type cells expressing either the Gα2(Q208L) or the Gα5(Q199L) subunits were capable of multicellular development after the incubation at the lower temperature (Figure 36), suggesting that these mutant subunits might inhibit aggregation and development in a manner similar to the Gα4(Q200L) subunit. However, *gα4* null cells expressing these Gα2(Q208L) or Gα5(Q199L) subunits were not able to aggregate and undergo multicellular development, indicating that wild type Gα4 function is required for multicellular development of these *Gα* mutants under these conditions.

Discussion

The inability to obtain transformants with a high copy number of the Gα4(Q200L) expression vector suggests that the Gα4(Q200L) subunit is detrimental to vegetative growth. This adverse effect on growth was not observed with the overexpression of the wild type Gα4 subunit indicating that only the altered form of the Gα4 subunit negatively impacts cell viability. A similar phenotype has been reported for the expression of the Gα5(Q199L) subunit but not for the expression of the Gα1 (Q206L) or Gα2(Q208L) subunits, indicating that Gα4 and Gα5 subunits belong to a different class of Gα subunits. This distinction in mutant Gα subunit function might be related to similarities in Gα4 and Gα5 subunit structure or expression patterns. A potential mechanism by which the

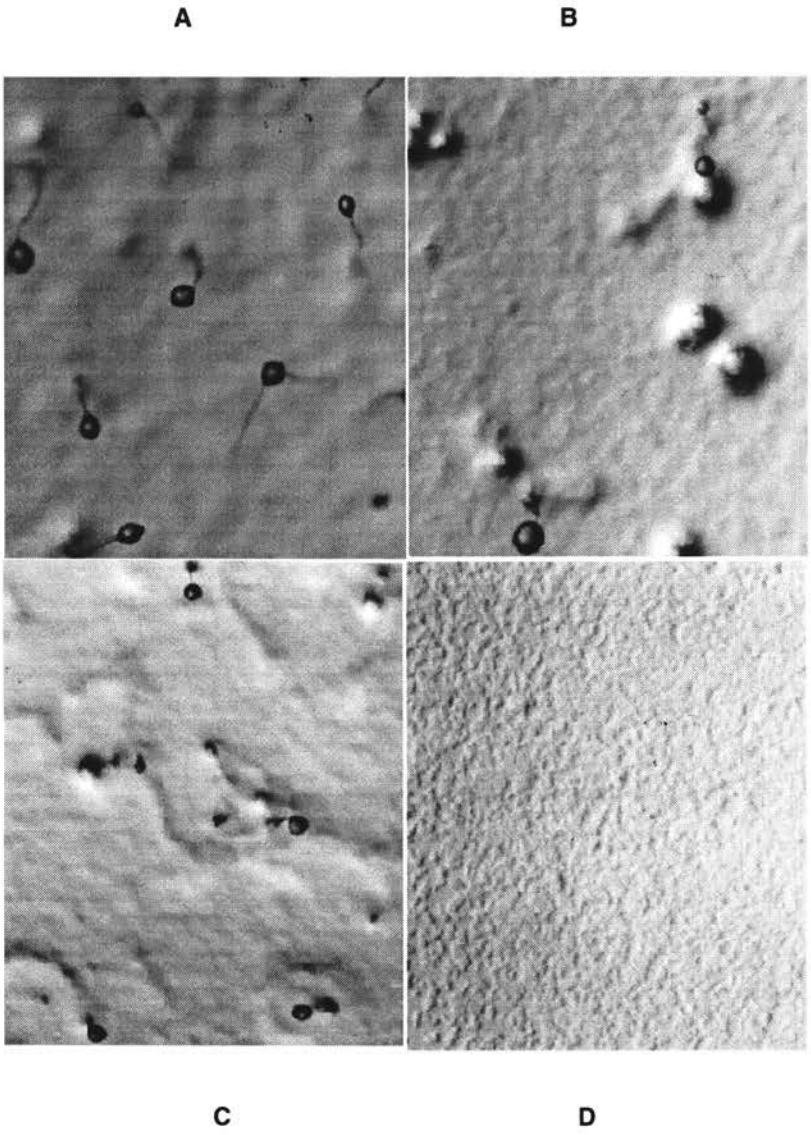


Figure 36: Morphological phenotypes of wild type cells (A), wild type cells expressing the *Gα2(Q208L)* gene (B), wild type cells expressing the *Gα5(Q199L)* gene (C), and *gα4* null cells expressing the *Gα2 (Q208L)* gene (D) after incubation at 4°C for 24 hours followed by incubation at 22°C. Cells were grown and washed as described in Materials and Methods. Photographs were taken 24 hours after cells were transferred from 4°C to 22°C.

mutant Gα4 or Gα5 subunits affect cell viability might be due to their interference with one or more G-protein signaling pathways essential for viability. However, all known Gαsubunit genes, except for Gα6, have been shown to be nonessential for vegetative growth. The Gα6 subunit does not show any greater similarity to the Gα4 or Gα5 subunits than to the Gα1 or Gα2 subunits (Natarajan and Hadwiger, unpublished data).

The inability of the Gα4(Q200L) subunit to rescue folic acid-stimulated cAMP and cGMP accumulation and chemotactic movement indicates that this altered subunit cannot provide wild type Gα4 function. Furthermore, the moderate inhibition in the accumulation of cGMP and cAMP and chemotactic movement in response to folic acid soon after nutrient deprivation suggests that the Gα4(Q200L) subunit can actually inhibit the Gα4-mediated signal transduction pathway. While not yet understood, this mechanism of inhibition by the Gα4(Q200L) or other mutant Gα subunits might be related to the mechanism by which these subunits inhibit cAMP responses. These mutant subunits could potentially interact with a common signaling component (e.g., the Gβ subunit, guanylyl cyclase, or adenylyl cyclase) that is shared by multiple pathways or with pathway specific components that are capable of binding multiple mutant Gα subunits. If the mutant Gα subunits did interact with a common component, then this component must impact different pathways differently because cAMP responses are more severely blocked than folic acid responses. The difference in inhibition might also be related to signaling parameters since cells typically respond to continuous folic acid signals and pulsatile cAMP signals. Based on analogous mutants in other systems, these mutant subunits are presumably defective in GTPase activity and likely to remain in an active state, and so these subunits are not likely to remain associated with their receptors

or Gβγ dimers. Furthermore, the inhibitory mechanism does not appear to be due to altered expression of known signaling components such as the cAR1 receptor or the Gα2 and Gα4 subunits because these components are expressed in developing Gα4(Q200L) mutants. However, the inhibition is likely to occur at a relatively early step in the signaling pathway, perhaps at the level of G-protein regulation, because both cAMP and cGMP accumulations are affected. Previous studies have indicated that the folic acid- and cAMP-stimulated signaling pathways converge before the activation of guanylyl cyclase because the cGMP responses to these stimuli are not cumulative.

The requirement of wild type Gα4 function for the multicellular development of aggregation defective Gα mutant cells after temperature shift or cAMP treatment suggests that Gα4 function has a role in this aggregation process. These mutant aggregates are relatively small and do not display aggregation streams typical of wild type cells, indicating distinctions from the normal aggregation process. Folic acid, a stimulant of the Gα4-mediated signal transduction pathway, has not been previously implicated in the aggregation of *Dictyostelium discoideum* but this compound has been reported to mediate the aggregation of *Dictyostelium minutum*. Therefore, Gα4-mediated aggregation might be a cryptic mechanism of *Dictyostelium discoideum* cells to bypass defects in Gα2-stimulated aggregation. However, Gα4 function is important for the positioning of cells within the multicellular aggregate and so the mutant aggregates might form from cell movement mechanisms normally associated with cell movement within the aggregates. This latter explanation is consistent with the enhancement of development by exogenous cAMP treatment. The mechanism by which cold temperature shift affects this aggregation is not known but cold temperature shifts have been reported to help

synchronize cells with respect to the cell cycle (120). The ability of *gα2* null cells expressing the Gα2(Q208L) subunit to aggregate after incubation at 4°C had previously led others to speculate that Gα2(Q208L) might provide some Gα2 function under these conditions (121). However, the dependency of this aggregation on Gα4 function suggests that this process might involve Gα4-mediated signal transduction.

All of the identified *Dictyostelium* subunits, including the three in this study, share relatively little identity (maximum 51% amino acid sequence identity) compared to identity observed among G-protein classes in mammalian systems. The ability of mutant *Dictyostelium* Gα subunits to interfere with multiple G-protein-mediated signaling pathways suggests similar pathway interactions might occur in other organisms where the similarity between Gα subunits is even greater. Therefore, the changes in cell growth characteristics, such as tumor formation, associated with analogous mutations mammalian Gα subunits could possibly result from the alteration of signaling in multiple transduction pathways rather than a single pathway.

CHAPTER 5

CONCLUSIONS

Cellular localization and differentiation are processes often regulated by cell-cell interactions during the development of eukaryotic organisms. This is particularly important in the case of the slime mold, *Dictyostelium discoideum* where cells respond to extracellular cAMP during the aggregation stage of development.

The aim of the first part of this study was to understand the role of the Gα4 subunit in localization of cells which can affect cell fate choices during multicellular development. Earlier studies had indicated a role for Gα4 in spore development based on the analyses of *Gα4* mutants. However, the mechanism by which this might be occurring was largely unknown. This study has attempted to establish the role of Gα4 subunit in prespore and prestalk cell differentiation through the use of mutants overexpressing the *Gα4* gene ($G\alpha4^{HC}$ cells), and *gα4* null mutants which do not express a functional *Gα4* gene. Expression of developmental genes such as *Gα4*, prestalk specific *ecmA*, *ecmB* and prespore specific *cotC* were examined by Northern analysis of *gα4* null and $G\alpha4^{HC}$ cells. While the expression of the prestalk and prespore specific genes in *gα4* null cells was not very different from the wild type cells, the expression of the prestalk specific genes was severely reduced in $G\alpha4^{HC}$ cells. This is consistent with the fact that the $G\alpha4^{HC}$ lack a distinct prestalk region and are blocked in development due to inability of the prespore

cells to follow the extending prestalk region in the developing aggeregate. *Gα4*^{HC} cells were also observed to have greatly reduced levels of the prespore specific gene, *cotC*. In order to determine if prestalk and prespore gene expression is rescued in *Gα4*^{HC} mutants by wild type cells, the mutants carrying reporter constructs were developed with wild type cells and developmental gene expression was monitored through the use of quantitative β-galactosidase assays. Also, it had been reported earlier that spore production in *Gα4*^{HC} cells is greatly increased during development when these mutants are developed in chimeras composed of wild type and *Gα4*^{HC} cells. However, it wasn't established whether this was due to changes in developmental gene expression or other developmental parameters. This study demonstrates that although expression of prestalk genes remained unaffected in *Gα4*^{HC} mutants when developed with wild type cells, the expression of the prespore specific gene *cotC* increased nearly eight fold in the presence of wild type cells. This is a significant finding which implies that the wild type cells may be rescuing development in *Gα4*^{HC} cells by specifically rescuing prespore gene expression through the production of a missing intercellular signal. Since, the *Gα4*^{HC} mutant aggregates lack a defined prestalk region, it was necessary to determine if transplantation of the prestalk region from developing aggregates of wild type cells onto mounds of *Gα4*^{HC} cells would rescue development. The transplanted anterior tips were found to rescue development and remained segregated in the chimeric fruiting bodies. This suggests that although the production of the missing intercellular signal requires anterior prestalk cells, the signal might be diffusible. Furthermore, this signal does not appear to be dependent on Gα4 function as *gα4* null cells can fulfill this role in chimeras with *Gα4*^{HC} cells.

In order to examine the role of Gα4 subunit in cell localization, spatial distribution of the *gα4* null cells and $G\alpha4^{HC}$ cells was examined in chimeras with wild type cells. The distribution of *gα4* null cells in the chimeric organisms was like those exhibited by anterior-like cells which could mean that the *gα4* null cells are precursors to prespore and prestalk cells. The $G\alpha4^{HC}$ cells, on the other hand, were found to reside primarily in the central prespore region and were conspicuously absent from the anterior prestalk region. This could mean that *Gα4* gene, which is overexpressed in $G\alpha4^{HC}$ cells, could be promoting localization of these mutants in the prespore region and/or it could be preventing localization of the mutant cells in the prestalk region.

Responses to folic acid and other pterin compounds had previously been reported to be dependent on Gα4 function. In order to examine the role of the Gα4 subunit in cell localization in response to a pterin compound such as folic acid, the chemotactic responsiveness of the *Gα4* mutants was examined. The responses of $G\alpha4^{HC}$ mutants to folic acid were seen to be consistently greater at all stages of development as compared to wild type cells, which stresses the importance of Gα4 function for responses to folic acid. Since the $G\alpha4^{HC}$ mutant aggregates lack a defined tip region, it was important to determine if stimulation of wild type cells with exogenous folic acid would produce similar effects on phenotype. Studies performed by others in the lab provided answers in this regard. When wild type cells were treated with folic acid, they showed a substantial delay in tip formation and this effect was dosage dependent. Cells treated with lower concentrations of folic acid or developed at higher densities exhibited shorter delays which pointed towards the fact that inactivation of folic acid could be means of overcoming the delay in development. Expression of prespore and prestalk specific

genes was also delayed in these cells after treatment with folic acid. This phenomenon was not observed in the case of the *gα4* null cells which indicates that the observed delay must be mediated through the Gα4 signal transduction pathway. This could be through interference of other G-protein pathways in the $G\alpha4^{HC}$ mutants.

This study demonstrates that the Gα4 subunit is required in a cell autonomous fashion during spore development and appears to function by promoting spore development and preventing prestalk cell development. Folic acid may therefore regulate cell fate choice by stimulating the Gα4 signal transduction pathway.

The other part of this research project was to characterize activated G-protein mutants of *Dictyostelium*. Certain residues in the G-3 region of the Gα subunit have been shown to be critical for the Gα-subunit GTPase activity. Arginine and glutamine residues (201 and 227, respectively, in the long form of $G_s\alpha$) are conserved in all Gα-subunits. The glutamine residue is analogous to the Q61 of *ras*, known to be an oncogenic 'hotspot'. In the case of $G\alpha_s$, the mutation leads to constitutive activation of the adenylyl cyclase pathway leading to accumulation of high levels of cAMP in human pituitary tumors.

The aim of this study was to characterize *Gα4(Q200L)* mutants to determine if they exhibit an activated phenotype due to overstimulation of the signaling pathway that might be a consequence of impairment of the GTPase activity of the Gα subunit.

Although, the Gα subunits of *Dictyostelium* do not appear to share any functions with the Ras proteins, they do share conserved GTPase regions and therefore, it was important to study the effect of the Q->L mutations in these genes. Analogous mutations in the Gα5 subunit had been reported earlier to cause cell death. Transformants with high

copy numbers of the mutant *Gα4* allele seemed to rapidly die out. Due to lack of sufficient cells for analysis, we could not determine if these cells exhibited characteristics associated with apoptosis. The use of the discoidin promoter (which is repressible by folic acid) to drive the expression of the mutant *Gα4(Q200L)* gene did not help us with the analysis due to inadequate regulation. However, it did indicate that viable transformnats could be obtained with the mutant *Gα4(Q200L)* gene when it wasn't expressed to a great extent. Transformation of wild type and *gα4* null mutant cells with the mutant *Gα4(Q200L)* allele were performed again with care being taken to allow viable transformants to survive through use of lower levels of the drug. A third of these transformants were found to exhibit deficiency in aggregation during development. This was consistently observed with all drug selection vectors available. These mutants were further characterized with respect to chemotaxis to cAMP and folic acid, cyclic nucleotide accumulation, ability to develop when pulsed with exogenous cAMP as well as under cold (4°C) conditions. The wild type transformants with the mutant *Gα4(Q200L)* allele were found to exhibit a slight inhibition to folic acid at about 3 hours of development which appeared to be alleviated by 6 hours and beyond. This implies that the mutant Gα4 subunit is not capable of completely inhibiting folic acid chemotaxis. Chemotaxis to cAMP, on the other hand, was found to be completely inhibited by the presence of the mutant *Gα4* gene in wild type cells. This is a significant finding as it indicates that the mutant Gα4 subunit may be affecting other signaling pathways such as the Gα2, which is required for aggregation in response to cAMP during normal development of the organism. *The Gα4(Q200L)* mutant subunit expressed in wild type cells did not appear to completely block aggregate formation when pulsed with

exogenous cAMP as well as when the mutant cells were developed under cold temperature conditions (4°C). This could be due to the ability of these cells to undergo aggregation under these conditions using a pathway other than the Gα2 pathway. In order to determine whether this aggregation was dependent on Gα4 function, *gα4* null cells with the mutant *Gα4(Q200L)* allele were developed under identical conditions and were found to be incapable of undergoing aggregation. Analogous *Gα2(Q208L)* mutant subunits expressed in *gα2* null cells have been reported to overcome their aggregation defect partially under cold conditions. These mutants are believed to be defective in their adaptation responses to cAMP which might be the cause of their aggregation deficient phenotype. However, it is believed that these mutants might be be able to overcome this defect partially through the use of an unidentified mechanism under cold conditions. Therefore, it was important for us to test if the mutant *Gα2(Q208L)* allele expressed in *gα4* null cells could rescue aggregation. However, *gα4* null cells expressing the *Gα2(Q208L)* subunit did not show even partial rescue of aggregation under cold conditions. This could mean that Gα4 function is absolutely required for aggregation under these conditions. The presence of the *Gα2(Q208L)* gene in *gα2* null cells could perhaps alleviate some of the aggregation defects and could be accountable for the partial rescue which had been reported earlier in literature. Although analogous *Gα5* mutants bearing the mutant *Gα5(Q199L)* allele were observed to die out, we attempted to select for transformants in low copy numbers which were viable. These mutants also exhibited similar aggregation and chemotaxis defects which may be due to the similarity shared between the Gα4 and Gα5 subunits. The failure of the mutant Gα4(Q200L) subunit to inhibit expression of developmental genes such as *Gα2*, *Gα4* or the cAMP receptor, *cAR1*

suggests that the inhibition mechanism may not involve inhibition of the expression patterns of these signaling elements. However, the inhibition of accumulation of cyclic nucleotides (cAMP and cGMP), suggests that the effects of the mutant *Gα4 (Q220L)* may be at an early step in the signaling pathway. The *Gα4(Q200L)* subunit may have altered properties with respect to binding to the receptor, the Gβγ subunits, a GAP or even a downstream element such as an effector. It is however unlikely that the Gα4(Q200L) mutant subunit may be specific for a G-protein receptor as it seems to affect two different pathways. Elucidation of the components of the Gα4 pathway and the elements interacting with the Gα4 subunit could help us understand the mechanism of action of these mutant subunits. Studies involving purified *Gα4(Q200L)* subunits such as binding assays might help guide us in the right direction

The prospect of mutant G-protein subunits affecting other related G-protein signaling pathways could be particularly important in the case of other systems where analogous mutations in conserved GTPase regions of the G-protein result in altered or diseased states such as tumor formation in mammalian systems.

BIBLIOGRAPHY

1. **Abe, K., and K. Yanagisawa.** 1983. A new class of rapidly developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. Developmental Biology **95:**200-210.

2. **Anjard, C., C. Zeng, W. Loomis, and W. Nellen.** 1998. Signal transduction pathways leading to spore differentiation in *Dictyostelium discoideum*. Developmental Biology **193:**146-55.

3. **Araki, T., H. Nakao, I. Takeuchi, and Y. Maeda.** 1994. Cell-cell dependent sorting in the development of *Dictyostelium* cells. Developmental Biology **162:**221-8.

4. **Barbacid, M.** 1987. *ras* Genes. Annual Review of Biochemistry **56:**779-827.

5. **Berks, M., and R. Kay.** 1990. Combinatorial control of cell differentiation by cAMP and DIF-1 during development of *Dictyostelium discoideum*. Development **110:**977-84.

6. **Berman, D., T. Wilkie, and A. Gilman.** 1996. GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein α subunits. Cell **86:**445-452.

7. **Birnbaumer, L.** 1992. Receptor to effector signaling through G proteins: roles for $\beta\gamma$ dimers as well as α subunits. Cell **71:**1069-1072.

8. **Birnbaumer, L., J. Abramowitz, and A. M. Brown.** 1990. Receptor-effector coupling by G-proteins. Biochimica Biophysica Acta **1031:**163-224.

9. **Blusch, J., P. Morandini, and W. Nellen.** 1992. Transcriptional regulation by

folate: Inducible gene expression in *Dictyostelium* transformants during growth and early development. Nucleic Acids Reaserch **20:**6235-6238.

10. **Boguski, M., and F. Mc Cormick.** 1993. Proteins regulating Ras and its relatives. Nature **366:**643-654.

11. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:**117-127.

12. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72:**248-254.

13. **Brandon, M., S. Voglmaier, and A. Siddiqui.** 1997. Molecular characterization of a *Dictyostelium* G-protein alpha-subunit required for development. Gene **200:**99-105.

14. **Breitweiser, G., and G. Szabo.** 1985. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion ion cahnnels by a guanine nucleotide analogue. Nature **317:**538-40.

15. **Brown, A.** 1990. Ionic channels and their regulation by G protein subunits. Annual Review of Physiology **52:**197-213.

16. **Buhl, B., K. Fischer, and H. MacWilliams.** 1993. Cell sorting within the prespore zome of *Dictyostelium discoideum*. Developmental Biology **156:**481-9.

17. **Carty, D. J., E. Padrell, J. Codina, L. Birnbaumer, J. D. Hildebrandt, and R. J. Iyengar.** 1990. Distinct guanine nucleotide binding and release properties of the three G proteins. Journal of Biological Chemistry **265:**6268-6273.

18. **Chen, M., R. Insall, and P. Devreotes.** 1996. Signaling through chemoattractant receptors in *Dictyostelium*. Trends in Genetics **12:**52-57.

19. **Clapham, D. E., and E. J. Neer.** 1993. New roles for G protein βγ dimers in transmembrane signaling. Nature **365:**403-406.

20. **Clarke, M., W. L. Bazari, and S. C. Kayman.** 1980. Isolation and properties of calmodulin from *Dictyostelium discoideum*. Journal of Bacteriology **141:**397-400.

21. **Clarke, M., and R. H. Gomer.** 1995. PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. Experentia .

22. **Clarke, M., S. C. Kayman, and K. Riley.** 1987. Density-dependent induction of discoidin-I synthesis in exponentially growing cells of *Dictyostelium discoideum*. Differentiation **34:**79-87.

23. **Cohen, G., T. Yang, P. Robinson, and D. Oprian.** 1993. Constitutive activation of opsin: influence of charge at position 134 and size at position 296. Biochemistry **32:**6111-15.

24. **Cohen, J., R. Duke, V. Fadok, and K. Sellins.** 1992. Apoptosis and programmed cell death in immunity. Annual Review of Immunology **10:**267.

25. **Cole, G. M., D. E. Stone, and S. I. Reed.** 1990. Stoichiometry of G protein subunits affects the Saccharomyces cerevisiae mating pheromone signal transduction pathway. Molecular and Cellular Biology **10:**510-7.

26. **Coleman, D. E., A. M. Berghuis, E. Lee, M. E. Linder, A. G. Gilman, and S. R. Sprang.** 1994. Structures of active conformations of Gia1 and the mechanism of GTP hydrolysis. Science **265:**1405-1412.

27. **Conklin, B. R., and H. R. Bourne.** 1993. Structural elements of Gα subunits that interact with Gβγ, receptors, and effectors. Cell **73:**631-641.

28. **Cornillon, S., C. Foa, J. Davoust, N. BUonavista, and J. D. Gross.** 1994. Programmed cell death in Dictyostelium. Journal of Cell Science **107:**2691-2704.

29. **Cotter, T. G., and M. Al-Rubeai.** 1995. Cell death (apoptosis) in cell culture systems. Trends in Biotechnology **13:**150.

30. **Coussen, F., J. Haiech, J. D'Alayer, and A. Monneron.** 1985. Identification of the catalytic subunit of brain adenylate cyclase: a calmodulin binding protein of 135 KDa. Proceedings of the National Academy of Sciences **82:**6736-40.

31. **Crespo, P., N. Xu, W. F. Simonds, and J. S. Gutkind.** 1994. Ras-dependent activation of MAP kinase pathway mediated by G-protein $\beta\gamma$ subunits. Nature **369:**418-420.

32. **Crowley, T. E., W. Nellen, R. H. Gomer, and R. A. Firtel.** 1985. Phenocopy of discoidin-I-minus mutants by antisense transformation of *Dictyostelium*. Cell **43:**633-641.

33. **Darzynkiewicz, Z., S. Bruno, G. Del Bino, W. Gorczyca, M. Hotz, P. Lassota, and F. Traganos.** 1992. Features of apoptotic cells measured by flow cytometry. Cytometry **13:**795-808.

34. **De Wit, R. J. W., and R. Bulgakov.** 1986. Folate Chemotactic Receptors in *Dictyostelium discoideum.* II. Guanine Nucleotides Alter the Rates of Interconversion and the Proportioning of Four Receptors States. Biochim. Biophys. Acta **886:**88-95.

35. **De Wit, R. J. W., and R. Bulgakov.** 1985. Guanine Nucleotides Modulate the Ligand Binding Properties of Cell Surface Folate Receptors in *Dictyostelium discoideum.* FEBS Lett. **179:**257-261.

36. **De Wit, R. J. W., and T. M. Konijn.** 1983. Identification of the acrasin of

Dictyostelium minutum as a derivative of folic acid. Cell Differentiation **12:**205-210.

37. **Detterbeck, S., P. Morandini, B. Wetterauer, A. Bachmair, K. Fisher, and H. MacWilliams.** 1994. The 'prespore-like cells' of *Dictyostelium* have ceased to express a prespore geen: Analysis using short -lived β-galactosidases as reporters. Development **120:**2847-2855.

38. **Devreotes, P.** 1994. G-protein linked signaling pathways control the developmental program in *Dictyostelium discoideum*. Neuron **12:**235-41.

39. **Devreotes, P.** 1989. A model system for cell-cell interactions in development. Science **245**.

40. **Devreotes, P., and S. Zigmond.** 1988. Chemotaxis in eukayotic cells: a focus on leukocytes and *Dictyostelium*. Annual Review of Cell Biology **4:**649-686.

41. **Dharmawardhane, S., A. Cubitt, A. Clark, and R. Firtel.** 1994. Regulatory role of the Gα1 subunit in controlling cellular morphogenesis in *Dictyostelium*. Development **120:**3549-3561.

42. **Dingermann, T., N. Reindl, H. Werner, M. Hildebrandt, W. Nellen, A. Harwood, J. Williams, and K. Nerke.** 1989. Optimization and in situ detection of Escherichia coli beta-galactosidase gene expression in Dictyostelium discoideum. Gene **85:**353-62.

43. **Dohlman, H. G., J. Thorner, M. C. Caron, and R. J. Lefkowitz.** 1991. Model systems for the study of seven-transmembrane-segement receptors. Annual Review of Biochemistry **60:**653-688.

44. **Dynes, J. L., and R. A. Firtel.** 1989. Molecular complementation of a genetic marker in Dictyostelium using a genomic DNA library. Proceedings of the National

Academy of Sciences **86:**7966-70.

45. **Early, A., T. Abe, and J. Williams.** 1983. Evidence for positional differentiation of prestalk cells and for a morphogenetci gradient in *Dictyostlium*. Cell **83:**91-99.

46. **Ellis, R., J. Yuan, and H. Horvitz.** 1991. Mechanisms and functions of cell death. Annual Review of Cell Biology **7:**663-698.

47. **Esch, R. K., and R. A. Firtel.** 1991. cAMP and cell sorting control the spatial expression of a developmentally essential cell-type-specific ras gene in *Dictyostelium*. Genes Dev **5:**9-21.

48. **Evan, G.** 1994. Old cells never die, they just apoptose. Trends in Cell Biology **4:**191-192.

49. **Faure, M., A. Voyno-Yasenetskaya, and H. R. Bourno.** 1994. cAMP and βγ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. Journal of Biological Chemistry **269:**7851-7854.

50. **Feig, L., B. Pan, T. Roberts, and G. Cooper.** 1986. Isolation of ras GTP-binding mutants using an in situ colony binding assay. Proceedings of the National Academy of Sciences **83:**4607-4611.

51. **Feinberg, A. P., and B. Vogelstein.** 1984. A Technique for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity. Analytical Biochemistry **137:**1984.

52. **Firtel, R. A., and Chapman.** 1990. A role for cAMP-dependent protein kinase in early *Dictyostelium* development. Genes and Development **4:**18-28.

53. **Firtel, R. A., H. P. Van, A. R. Kimmel, and P. N. Devreotes.** 1989. G protein linked signal transduction pathways in development: *Dictyostelium* as an experimental

system. .

54. **Franke, J., and R. H. Kessin.** 1992. The cyclic nucleotide phosphodiesterases of *Dictyostelium discoideum*: molecular genetics and biochemistry. Cell Signalling **4:**471-478.

55. **Fung, B. K. K., and C. R. Nash.** 1983. Characterization of transducin from bovine retinal rod outer segments. II. Evidence for distinct binding sites and conformational changes revealed by limited proteolysis with trypsin. Journal of Biological Chemistry **258:**10503-10510.

56. **Gao, B., and A. Gilman.** 1991. Cloning and expression of a widely distributed (type IV) adenylate cyclase. Proceedings of the National Academy of Sciences **88:**10178-10182.

57. **Giershik, P., and K. Jakobs.** 1992. ADP-ribosylation of signal-transducing guanine-nucleotide binding proteins by cholera and pertussis toxin, vol. 102. Springer, Berlin, Heidelberg, New York.

58. **Gilman, A. G.** 1987. G proteins: transducers of receptor-generated signals. Annual Review of Biochemistry **56:**615.

59. **Gomer, R., and R. Ammann.** 1996. A cell-cycle phase-associated cell-type choice mechanism monitors the cell cycle rather than using an independent timer. Developmental Biology **174:**82-91.

60. **Gomer, R., and R. Firtel.** 1987. Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. Science **237:**758-762.

61. **Gomer, R. H., I. S. Yuen, and R. A. Firtel.** 1991. A secreted $80x10^3$ M_R protein mediates sensing of cell density and the onset of development in *Dictyostelium*.

Development **112:**269-278.

62. **Gross, J. D.** 1994. Developmental decisions in *Dictyostelium discoideum.* Microbiological Reviews **58:**330-351.

63. **Gudermann, T., F. Kalkbrenner, and G. Schultz.** 1996. Diversity and selectivity of receptor-G protein interaction. Annual Review of Pharmacology and Toxicology **36:**429-59.

64. **Gudermann, T., B. Nurnberg, and G. Schultz.** 1995. Receptors and G proteins as primary components of transmembrane signal transduction. 1. G-protein-coupled receptors: structure and function. Journal of Molecular Medicine **73:**51-63.

65. **Haberstroh, L., and R. A. Firtel.** 1990. A spatial gradient of expression of a cAMP-regulated prespore cell-type-specific gene in *Dictyostelium.* Genes Dev **4:**596-612.

66. **Hacker, G., and D. Vaux.** 1995. The medical significance of physiological cell death. Medical Research Reviews **15:**299-311.

67. **Hadwiger, J., and J. Srinivasan.** 1999. Folic acid stimulation of the Gα4 G protein-mediated signal transduction pathway inhibits anterior prestalk cell development in *Dictyostelium.* Differentiation **64:**195-204.

68. **Hadwiger, J. A., and R. A. Firtel.** 1992. Analysis of G alpha 4, a G protein required for multicellular development in Dictyostelium. Genes and Development **6:**38-49.

69. **Hadwiger, J. A., S. Lee, and R. A. Firtel.** 1994. The G alpha subunit, G alpha 4 couples to pterin receptors and identifies a signaling pathway that is essential for multicellular development in Dictyostelium. Proceedings of the National Academy of

Sciences **91:**10566-70.

70. **Hadwiger, J. A., K. Natarajan, and R. A. Firtel.** 1995. The Dictyostelium heterotrimeric G protein a subunit Ga5 regulates the kinetics of tip morphogenesis. Development .

71. **Hadwiger, J. A., T. M. Wilkie, M. Strathmann, and R. A. Firtel.** 1991. Identification of *Dictyostelium* Gα genes expressed during multicellular development. Proc Natl Acad Sci U S A **88:**8213-7.

72. **Halliday, K.** 1983-1984. Regional homology in GTP-binding proto-oncogene products and elongation factors. Journal of Cyclic Nucleotide Protein Phosphorylation Research **9:**435-448.

73. **Helmreich, E., and K. Hofmann.** 1996. Structure and function of proteins in G-protein-coupled signal transfer. Biochimica Biophysica Acta **1286:**285-322.

74. **Hepler, J., and A. Gilman.** 1992. G proteins. Trends in Biochemical Sciences **17:**383-87.

75. **Hildebrandt, J.** 1997. Role of subunit diversity in signaling by heterotrimeric G proteins. Journal of pharmacology **54:**325-339.

76. **Holbrook, S. R., and S. H. Kim.** 1989. Molecular model of the G protein α subunit based on the crystal structure of the HRAS protein. Proceedings of the National Academy of Sciences **86:**1751-1755.

77. **Huang, H., D. Takagawa, G. Weeks, and C. Pears.** 1997. Cells in the center of *Dictyostelium* aggregates become spores. Developmental Biology **192:**564-571.

78. **Insall, R. H., Soede, R.D., Schaap, P. & Devreotes, P.N.** 1994. Two cAMP receptors activate common signaling pathways in *Dictyostelium*. Molecular and Cellular

Biology **5:**703-711.

79. **Iyengar, R.** 1997. There are GAPS and GAPS. Science **275:**42-43.

80. **Johnson, G. L., and N. Dhanasekaran.** 1989. The G-protein family and their interaction with receptors. Endocr Rev **10:**317-31.

81. **Johnson, R., P. Van Haastert, A. Kimmel, C. Saxe, B. Jastorff, and P. Devreotes.** 1992. The cyclic nucleotide specificity of three cAMP receptors in *Dictyostelium*. Journal of Biological Chemistry **267:**4600-7.

82. **Kalinec, G., A. J. Nazarali, S. Hermouet, N. Xu, and J. S. Gutkind.** 1992. Mutated alpha subunit of the Gq protein induces malignant transformation in NIH 3T3 cells. Mol Cell Biol **12:**4687-93.

83. **Kalpaxis, D., I. Zundorf, H. Werner, N. Reindl, E. Boy-Marcotte, M. Jacquet, and T. Dingermann.** 1991. Positive selection for *Dictyostelium discoideum* mutants lacking UMP synthase activity based on resistance to 5-flouroorotic acid. Molecular General Gentics **255:**492-500.

84. **Kay, R. R.** 1992. Cell differentiation and patterning in *Dictyostelium*. Current Opinions in Cell Biology **4:**934-938.

85. **Kayman, S. C., R. Birchman, and M. Clarke.** 1988. *motA*1552, a mutation of *Dictyostelium discoideum* having pleiotropic effects on motility and discoidin-I regulation. .

86. **Kesbeke, F., B. E. Snaar-Jagalska, and P. J. M. van Haastert.** 1988. Signal transduction in Dictyostelium fgdA mutants with a defective interaction between surface cAMP receptors and a GTP-binding regulatory protein (published erratum appears in J Cell Biol 1988, Dec;107 (6 pt1): following 2463). Journal of Cell Biology **107:**521-8.

87. **Kesbeke, F., P. Van Haastert, R. De Wit, and B. Snaar Jagalska.** 1990. Chemotaxis to cAMP and folic acid is mediated by different G-proteins in *Dictyostelium discoideum*. Journal of Cell Science **96:**669-73.

88. **Khosla, M., G. B. Speigelman, and G. Weeks.** 1996. Overexpression of an activated *rasG* gene during growth blocks the initiation of *Dictyostelium* development. Molecular and Cellular Biology **16:**4156-4162.

89. **Kleuss, C., J. Hescheler, C. Ewel, W. Rosenthal, G. Schultz, and B. Wittig.** 1991. Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature **353:**43-48.

90. **Kontani, K., K. Taksunobu, A. Inanobe, M. Ui, and T. Katada.** 1992. Molecular heterogeneity of the bg subunits of GTP binding proteins in bovine brain membranes. Archives of Biochemistry and Biophysics **294:**527-33.

91. **Kumagai, A., J. A. Hadwiger, M. Pupillo, and R. Firtel.** 1991. Molecular Genetic Analysis of Two Ga Protein subunits in Dictyostelium. Journal of Biological Chemistry **266:**1220-1228.

92. **Kumagai, A., M. Pupillo, R. Gunderson, L. Miake, P. Devreotes, and R. Firtel.** 1989. Regulation and function of G alpha protein subunits in *Dictyostelium*. Cell **57:**265-75.

93. **Kurjan, J., and C. Dietzel.** 1988. Analysis of the role of SCG1, a G alpha homolog, and SST2 in pheromone response and desensitization in yeast. Cold Spring Harb Symp Quant Biol **2:**577-84.

94. **Kuspa, A., and W. F. Loomis.** 1994. Transformation of *Dictyostelium*. Gene disruptions, insertional mutagenesis, and promoter traps., vol. 3. Academic Press, New

York.

95. **Landis, C. A., S. B. Masters, A. Spada, A. M. Pace, H. R. Bourne, and L. Vallar.** 1989. GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. Nature **340:**692-696.

96. **Laugwitz, K., A. Allgeier, S. Offermanns, K. Spicher, and J. Van Sande.** 1996. The human thyrotropin receptor: a heptahelical receptor capable of stimulating all four G-protein families. Proceedings of the National Academy of Sciences .

97. **Lee, E., R. Taussig, and A. Gilman.** 1992. The G226A mutant of G_S-alpha highlights the requirement for dissociation of G-protein subunits. Journal of Biological Chemistry **267:**1212-1218.

98. **Linder, M. E., D. A. Ewald, R. J. Miller, and A. G. Gilman.** 1990. Purification and characterization of Goc and G1c after expression in *Escherichia coli*. Journal of Biological Chemistry **265:**8243-8251.

99. **Liu, T., J. G. Williams, and M. Clarke.** 1992. Inducible expression of calmodulin antisense RNA in *Dictyostelium* cells inhibits the completion of cytokinesis. Molecular Biology of the Cell **3:**1403-1413.

100. **Lochrie, M. A., and M. I. Simon.** 1988. G protein multiplicity in eukaryotic signal transduction systems. Biochemistry **27:**4957-4965.

101. **Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham.** 1987. The βγ subunits of GTP-binding proteins activate the muscarinic K^+ channel in the heart. Nature **325:**321.

102. **Loomis, W. F.** 1992. Antisense inactivation of developmental genes in Dictyostelium. Raven Press, New York.

103. **Loomis, W. F. (ed.).** 1982. The Development of *Dictyostelium discoideum*. Academic Press, New York.

104. **Loomis, W. F.** 1975. Dictyostelium discoideum. A Developmental System.

105. **Luo, Q., C. Michaelis, and G. Weeks.** 1994. Overexpression of a truncated cyclin B gene arrests *Dictyostelium* cell division during mitosis. Journal of Cell Science **107:**3105-3114.

106. **Lupas, A. N., J. M. Lupas, and J. B. Stock.** 1992. Do G protein subunits associate via a three-stranded coiled coil? FEBS Letters **314:**105-108.

107. **Lyons, J., C. Landis, and G. Harsh.** 1995. Two G protein oncogenes in human endocrine tumors. Science **249:**655-659.

108. **Lyons, J., C. A. Landis, G. Harsh, L. Vallar, K. Grunewald, H. Feichtinger, Q.-Y. Duh, O. H. Clark, E. Kawasaki, H. R. Bourne, and F. McCormick.** 1990. Two G protein oncogenes in human endocrine tumors. Science **249:**655-659.

109. **Mann, S. K. O., and R. A. Firtel.** 1987. Cyclic AMP regulation of early gene expression in Dictyostelium discoideum: Mediation via the cell surface cAMP receptor. Molecular and Cellular Biology **7:**458-69.

110. **Markby, D., R. Onrust, and H. Bourne.** 1993. Separate GTP binding and GTPase domains of a Gα subunit. Science **262:**1895-901.

111. **McRobbie, S. J., and P. C. Newell.** 1983. Changes in actin associated with the cytoskeleton following chemotactic stimulation of *Dictyostelium discoideum*. Biochemical and Biophysical Research Communications **115:**351-359.

112. **Mehdy, M. C., and R. A. Firtel.** 1985. A secreted factor and cyclic AMP jointly regulate cell-type specific gene expression. Molecular and Cellular Biology **5:**705-713.

113. **Morishita, R., Y. Fukada, K. Kokame, T. Yoshizawa, K. Masuda, M. Niwa, K. Kato, and T. Asano.** 1992. Identification and isolation of common and tissue-specific geranylated g subunits of guanine-nucleotide-binding proteins in various tissues. European Journal of Biochemistry **210:**1061-69.

114. **Neer, E.** 1995. Heterotrimeric G proteins: organizers of transmembrane signals. Cell **80:**249-57.

115. **Neer, E. J., and D. E. Clapham.** 1988. Roles of G protein subunits in transmembrane signalling. Nature **333:**129-34.

116. **Nellen, W., S. Datta, C. Reymond, A. Siversten, S. Mann, T. Crowley, and R. Firtel (ed.).** 1987. Molecular Biology in Dictyostelium:tools and applications in Dictyostelium discoideum., vol. 28. Academic Press, Orlando, FL.

117. **Nishizuka, Y.** 1995. Protein kinase C and lipid signaling for sustained cellular response. FASEB Journal **9:**484-496.

118. **Noel, J. P., H. E. Hamm, and P. B. Sigler.** 1993. The 2.2A^{o} crystal structure of transducin-α complexed with GTPγs. Nature **366:**654-663.

119. **Oberhammer, F., J. Wilson, C. Dive, I. Morris, J. Hickman, A. Wakeling, P. Walker, and M. Sikorska.** 1993. Apoptotic death in epithelial cells: Cleavage of DNA to 300 and /or 50 Kb fragments prior to or in the absence of intrernucleosomal fragmentation. EMBO. Journal **12:**3679-3684.

120. **Ohmori, T., and Y. Maeda.** 1986. Implications of differential chemotaxis and cohesiveness for cell sorting in the development of *Dictyostelium discoideum*. Development and growth differentiation **28:**169-175.

121. **Okaichi, K., A. B. Cubitt, G. S. Pitt, and R. A. Firtel.** 1992. Amino acid

substitutions in the *Dictyostelium* Gα subunit Gα2 produce dominant negative phenotypes and inhibit the activation of adenylyl cyclase, guanylyl cyclase and phospholipase C. Molecular Biology of the Cell **3:**735-747.

122. **Osawa, S., and G. Johnson.** 1991. A dominant negative Gas mutant is rescued by secondary mutation of the alpha chain amino terminus. Journal of Biological Chemistry **266:**4673-4676.

123. **Pace, A., Y. Wong, and H. Bourne.** 1991. A mutant α subunit of G_{i2} induces neoplastic transformation of Rat-1 cells. Proceedings of the National Academy of Sciences **88:**7031-7035.

124. **Pan, P., E. M. Hall, and J. T. Bonner.** 1972. Folic Acid as Second Chemotactic Substance in the Cellular Slime Moulds. Nature **237:**181-182.

125. **Parent, C., and P. Devreotes.** 1996. Molecular genetics of signal transduction in *Dictyostelium*. Annual Review of Biochemistry **65:**411-440.

126. **Pfeuffer, E., R. Dreher, H. Metzger, and T. Pfeuffer.** 1985. Catalytic unit of adenylate cyclase: Purification and identification by affinity crosslinking. Proceedings of the National Academy of Sciences **82:**3086-90.

127. **Pupillo, M., A. Kumagai, G. S. Pitt, R. A. Firtel, and P. N. Devreotes.** 1989. Multiple alpha subunits of guanine nucleotide-binding proteins in *Dictyostelium*. Proceedings of the National Academy of Sciences **86:**4892-4896.

128. **Rahmatullah, M., and J. D. Robishaw.** 1994. Direct interaction of the α and γ subunits of the G proteins. Journal of Biological Chemistry **269:**3574-3580.

129. **Rao, V., G. Cohen, E. Zhukovsky, and D. Oprian.** 1994. Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. Nature **367:**639-42.

130. **Rathi, A., and M. Clarke.** 1992. Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by autocrine-dependent mechanism. Mechanisms in Development **36:**173-182.

131. **Rathi, A., S. C. Kayman, and M. Clarke.** 1991. Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. Developmental Genetics **12:**82-87.

132. **Rens-Dorniano, S., and H. Hamm.** 1995. Structural and functional relationship of heterotrimeric G-proteins. FASEB Journal **9:**1059-1088.

133. **Rhee, S., and K. Choi.** 1992. Multiple forms of phopholipase C isozymes and their activation mechanisms. Advances in Second Messenger Phosphoprotein Research **26:**35-60.

134. **Rhee, S., P. Suh, S. Ryu, and S. Lee.** 1989. Studies of inositol phospholipid-specific Phospholipase C. Science **244:**546-550.

135. **Ross, E., T. Haga, A. Howlett, J. Schwarzmeier, L. Scheleifer, and A. Gilman.** 1978. Hormone-sensitive adenylyl cyclase: Resolution and reconstitution of some components necessary for regulation of the enzyme. Advances in Cyclic Nucloetide Research **9:**53-68.

136. **Ross, E. M.** 1989. Signal sorting and amplification through G protein-coupled receptors. Neuron **3:**141-52.

137. **Roush, W.** 1996. Regulating G protein signaling. Science **271:**1056-58.

138. **Rudoph, U., K. Spicher, and L. Birnbaumer.** 1996. Adenylyl cycalse inhibition and altered G protein subunit expression and ADP-ribosylation patterns in tissues and cells from $G_{i2}\alpha$-/- mice. Proceedings of the National Academy of Sciences **93:**3209-

3214.

139. **Sakmann, B., A. Noma, and W. Trautwein.** 1983. Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of human heart. Nature **303:**250-53.

140. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: A laboratory Manual. Cold Spring Harbor Laboratory Press.

141. **Savarese, T., and F. CM.** 1992. *Invitro* mutagenesis and the search for structure-function relationships among G-protein coupled receptors. Journal of Biochemistry **283:**1-19.

142. **Saxe, C., G. Ginsburg, J. Louis, R. Johnson, P. Devreotes, and A. Kimmel.** 1993. CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. Genes and Development **7:**262-272.

143. **Saxe, C., Y. Yu, C. Jones, A. Bauman, and C. Haynes.** 1996. The cAMP receptor is restricted to a subset of prestalk cells during *Dictyostelium* development and displays unexpected DIF-1 responsiveness. Developmental Biology **174:**202-13.

144. **Schaap, P., and M. Wang.** 1984. The possible involvement of oscillatory cAMP signaling in multicellular morphogenesis of the cellular slime molds. Developmental Biology **105:**470-8.

145. **Schubert, B., A. Van Dongen, G. Kirsch, and A. Brown.** 1989. Modulation of cardiac Na channels by β-adrenoreceptors and the G protein, G_s. Journal of Biophysics **55:**229a.

146. **Scnitzler, G., C. Briscoe, J. Brown, and R. Firtel.** 1995. Serpentine cAMP receptors may act through a G-protein independent pathway to induce post aggregative

development in *Dictysotelium*. Cell **81:**737-745.

147. **Segall, J. E., A. Kuspa, G. Shaulsky, M. Ecke, M. Maeda, C. Gaskins, R. A. Firtel, and W. F. Loomis.** 1995. A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium*. Journal of Cell Biology **128:**405-13.

148. **Shaulsky, G., A. Kuspa, and W. Loomis.** 1995. A multidrug resistance transporter/serine protease gene is required for prestalk specialization in *Dictyostelium*. Genes and Development **9:**1111-22.

149. **Simon, M. I., M. P. Strathmann, and N. Gautam.** 1991. Diversity of G proteins in signal transduction. Science **252:**802-8.

150. **Smith, C. A.** 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. Cell **76:**959.

151. **Sobolewski, A., N. Neave, and G. Weeks.** 1983. The induction of stalk cell differentiation in submerged monolayers of *Dictyostelium discoideum*. Characterization of the temporal sequence for the molecular requirements. Differentiation **25:**93-100.

152. **Spiegel, A.** 1997. Inborn errors of signal transduction: Mutations in G proteins and G protein-coupled receptors as a cause of disease. Journal of Metabolic diseases **20:**113-121.

153. **Spiegel, A., T. Jones, W. Simonds, and L. Weinstein.** 1994. G proteins. Landes, Austin, Tx.

154. **Spudich, J. A.** 1987. *Dictyostelium discoideum*: Molecular approaches to cell biology. Academic Press., New York.

155. **Sternfeld, J., and C. David.** 1981. Cell sorting during pattern formation in *Dictyostelium*. Differentiation **20:**10-21.

156. **Strader, C., T. Fong, M. Tota, D. Underwood, and R. Dixon.** 1994. Structure and function of G protein-coupled receptors. Annual Review of Biochemistry **63:**101-32.

157. **Stryer, L.** 1986. Cyclic GMP cascade of vision. Annual reviews of Neuroscience **9:**87-119.

158. **Suarez, H., J. Du Villard, B. Caillou, M. Schlumberger, C. Parmetier, and R. Monier.** 1991. Gsp mutations in human thyroid tumors. Oncogene **6:**677-679.

159. **Sullivan, K. A., R. T. Miller, S. B. Masters, B. Beiderman, W. Heideman, and H. R. Bourne.** 1987. Identification of receptor contact site involved in receptor-G protein coupling. Nature **330:**758-760.

160. **Sun, T. J., and P. N. Devreotes.** 1991. Gene targeting of the aggregation stage cAMP receptor cAR1 in Dictyostelium. Genes and Development **5:**572-82.

161. **Sutoh, K.** 1993. A transformation vector in *Dictyostelium discoideum* with a new selectable marker Bsr. Plasmid **30:**150-154.

162. **Tada, S., K. Gomi, K. Kitamoto, K. Takahashi, G. Tamura, and S. Hara.** 1991. Construction of a fusion gene comprising the Taka-amylase A promoter and the *Escherichia coli* beta-glucuronidase gene and analysis of its expression in Aspergillus oryzae. Mol Gen Genet **229:**301-6.

163. **Tang, W., and A. Gilman.** 1992. Adenylyl Cyclases. Cell **70:**869-872.

164. **Tang, W. J., and A. G. Gilman.** 1991. Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Science **254:**1500-1503.

165. **Tatischeff, I., M. Bomsel, C. De Paillerets, H. Durand, B. Geny, D. Segretain, E. Turin, and A. Alfsen.** 1998. *Dictyostelium discoideum* cells shed vesicles with associated DNA and vital stain Hoechst 33342. Cellular and Molecular Life Sciences

54:476-487.

166. **Thomason, P., S. James, P. Casey, and C. Downes.** 1994. A G protein βγ-subunit-responsive phosphoinositide 3-kinase activity in human platelet cytosol. Journal of Biological Chemistry **269:**16525-28.

167. **Thornberry, N., H. Bull, J. Calaycay, K. Chapman, A. Howard, M. Kostura, D. Miller, S. Molineaux, J. Weidner, and T. Aunins.** 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature **356:**768.

168. **Tillinghast, H., and P. Newell.** 1987. Chemotaxis towards pteridines during development of *Dictyostelium*. Journal of Cell Science **87:**45.

169. **Vallar, L., A. Spada, and G. Giannasttasio.** 1987. Altered G_s and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature **330:**556-558.

170. **Van Haastert, P.** 1983. Relationship between adaptation of the folic acid and cAMP mediated cGMP response in *Dictyostelium*. Biochemical and Biophysical Research Communications **115:**130-136.

171. **Van Haastert, P., R. De Wit, and T. Konijn.** 1982. Antagonists of chemoattractants reveal separate receptors for cAMP, folic acid and pterin in *Dictyostelium*. Experimental Cell research **140:**453-456.

172. **Van Haastert, P., and H. Kuwayama.** 1997. cGMP as second messenger during *Dictyostelium* chemotaxis. FEBS Letters **410:**25-28.

173. **Van Haastert, P. J. M., P. M. W. Janssens, and C. Erneax.** 1991. Sensory transduction in eukaryotes: a comparison between *Dictyostelium* and vertebrate cells. European Journal of Biochemistry **195:**289-303.

174. **Vauti, F., P. Moradini, J. Blusch, A. Sachse, and W. Nellen.** 1990. Regulation of the Discoidin I g gene in Dictyostelium discoideum: Identification of individual promoter elements mediating induction of transcription and repression by cAMP. Molecular and Cellular Biology **10:**4080-88.

175. **Wang, M., R. Aerts, W. Spek, and P. Schaap.** 1988. Cell cycle phase in *Dictyostelium discoideum* is correlated with the expression of cyclic AMP production, detection, and degradation. Involvement of cyclic AMP signaling in cell sorting. Developmental Biology **125:**410-6.

176. **Watson, N., M. Linder, K. Druey, J. Kehrl, and K. Blumer.** 1996. RGS family members: GTPase-activating proteins for heterotrimeric G-protein α-subunits. Nature **383:**172-175.

177. **Wedegaetner, P., P. Wilson, and H. Bourne.** 1995. Lipid modifications of trimeric G-proteins. Journal of Biological Chemistry **270:**503-506.

178. **Weijer, C., G. Duschl, and C. David.** 1984. Dependence of cell-type proportioning and sorting on cell cycle phase in *Dictyostelium discoideum*. Journal of Cell Science **70:**133-145.

179. **Weinstein, L., A. Shenker, P. Gejman, M. Merino, E. Friedman, and A. Spiegel.** 1991. Activating mutations of the stimulatory G protein in the McCune-Albright syndrome. New England Journal of Medicine **325:**1688-1695.

180. **West, R. E., J. Moss, M. Vaughan, T. Liu, and T. Y. Liu.** 1985. Pertussis toxin-catalyzed ADP-ribosylation of transducin. Journal of Biological Chemistry **260:**14428-14430.

181. **Whitbread, J. A., M. Sims, and E. R. Katz.** 1991. Evidence for the presence of

a growth factor in *Dictyostelium discoideum*. Developmental Genetics **12:**78-81.

182. **Whiteway, M., L. Hougan, D. Dignard, D. Y. Thomas, L. Bell, G. C. Saari, F. J. Grant, P. O'Hara, and V. L. MacKay.** 1989. The STE4 and STE18 genes of yeast encode potential beta and gamma subunits of the mating factor receptor-coupled G protein. Cell **56:**467-77.

183. **Whiteway, M., L. Hougan, and D. Y. Thomas.** 1990. Overexpression of the STE4 gene leads to mating response in haploid Saccharomyces cerevisiae. Molecular and Cellular Biology **10:**217-22.

184. **Williams, J. G., D. P. Lane, S. J. Mcrobbie, A. J. Harwood, D. Traynor, R. R. Kay, and K. A. Jermyn.** 1989. Origins of the prestalk-prespore pattern in Dictyostelium development. Cell **59:**1157-63.

185. **Williams, J. G., A. S. Tsang, and H. Mahbubani.** 1980. A change in the rate of transcription of an eukaryotic gene in response to cyclic AMP. Proceedings of the National Academy of Sciences **77:**7171-7175.

186. **Williamson, E., M. Daniels, S. Foster, W. Kelly, P. Kendall-Taylor, and P. Harris.** 1994. $G_s\alpha$ and $G_{i2}\alpha$ mutations in clinically nonfunctioning pituitary tumors. Clinical Endocrinology **41:**815-820.

187. **Wong, Y., J. Chan, L. Yung, and H. Bourne.** 1995. Mutant a subunit of Gz transforms Swiss 3T3 cells. Oncogene **10:**1927-1933.

188. **Wong, Y., B. Conklin, and H. Bourne.** 1992. G_z-mediated hormonal inhibition of cyclic AMP accumulation. Science **255:**339-42.

189. **Wu, L., C. Gaskins, K. Zhou, R. Kessin, and P. Devreotes.** 1994. Cloning and targeted mutations of Gα7 and Gα8: Two developmentally regulated G protein α-subunits

in *Dictyostelium*. Molecular Biology of the Cell **5:**691-702.

190. **Wu, L., R. Valkema, P. Van Haastert, and P. Devreotes.** 1995. The G protein beta subunit is essential for multiple responses to chemoattractants in *Dictyostelium*. Journal of Cell Biology **129:**1667-75.

191. **Yamada, M., A. Jahangir, Y. Hosoya, A. Inanobe, and T. Katada, Kurachi, Y.** 1994. G_k and brain $G\beta\gamma$ activate muscarinic K^+ channel through the same mechanism. Journal of Biological Chemistry **268:**24551-54.

192. **Yatani, A., J. Codina, R. Sekura, L. Birnbaumer, and A. Brown.** 1987. Reconstitution of somatostatin and muscarinic receptor mediated stimulation of K^+ channels by isolated G_k protein in clonal rat anterior pituitary cell membranes. Molecular Endocrinology **1:**283-89.

193. **Yoshimoto, K., H. Iwahana, A. Fukuda, T. Sano, and M. Itakura.** 1993. Rare mutations of the $G_S\alpha$ subunit gene in human endocrine tumors. Cancer **72:**1386-1396.

194. **Yu, Y., and C. Saxe.** 1996. Differential distribution of cAMP receptors cAR2 and cAR3 during *Dictyostelium* development. Developmental Biology **173:**353-6.

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