CHARACTERIZATION OF THE Gα5 G PROTEIN SIGNAL TRANSDUCTION PATHWAY AND ITS SPECIFICITY IN THE DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

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NOMENCLATURE

Gα4	*Gα4* gene
Gα5	*Gα5* gene
Gα4	Gα4 protein
Gα5	Gα5 protein
gα4 / gα4 null	Cells in which the wild-type *Gα5* gene has been mutated by gene disruption (insertion mutagenesis) (*gα5* null mutant strain)
gα5 / gα5 null	Cells in which the wild-type *Gα5* gene has been mutated by gene disruption (insertion mutagenesis) (*gα5* null mutant strain)
$G\alpha4^{HC}$	Cells which carry a high copy number of the *Gα4* expression vector (*Gα4* overexpression strain)
$G\alpha5^{HC}$	Cells which carry a high copy number of the *Gα5* expression vector (*Gα5* overexpression strain)
cAMP	Cyclic adenosine 3', 5' monophosphate
cGMP	Cyclic guanosine 3', 5' monophosphate
WT	Wild-type axenic strain Kax-3
FA	Folic Acid
PAF	Platelet Activating Factor
LPA	Lysophosphatidic Acid
SP60 / cotC	Prespore specific gene
ecmA	Prestalk specific gene

lacZ	Gene that encodes for β-galactosidase activity
GFP	Green Fluorescent Protein
gusA	Gene that encodes for β-glucouronidase activity
G418^r	Gene that encodes for neomycin resistance
Bsr^r	Gene that encodes for blasticidin resistance
Hyg^r	Gene that encodes for hygromycin resistance
THY1	Gene encoding the thymidylate synthetase enzyme
PYR5-6	Gene necessary for synthesis of uracil

CHAPTER 1

INTRODUCTION

Of the numerous physical and chemical signals that constantly reach the surface of various cells in their environment, some bind to receptors on the cell membrane and initiate a cascade of events that transduces to the cell interior. The transduction of the signal to the interior of the cell evokes an intracellular response, which underlies the physiological response of tissues and organisms. Some of these signal transduction processes are facilitated by a group of heterotrimeric GTP-binding proteins known as G proteins. G proteins achieve cellular responses by bringing about a change in the activity of a target protein (effector molecules such as ion channels and enzymes), which modulates ionic composition or second messenger levels.

Three components are involved in G protein pathways – the receptors, G proteins and the effectors. Multitudes of all these three components have been identified in the different systems studied to date. The diversity of participants in G protein–coupled pathways seems to increase from effector to G protein to receptor (160). In addition, multiple subtypes of each of the heterotrimeric G protein subunits (α, β and γ) have been identified in the various systems studied, with the number of α subunits greatly overshadowing the number of β and γ subunits (17, 20, 53, 160). Despite the variety in terms of protein components, each signal is processed differently by the cell. Each of these signals is highly specific and regulated to allow the cell to clearly decode the information.

Many different G proteins are co-expressed by a single cell and multiple signals are processed simultaneously. Hence, the combinations of different G protein subunits as well

as other G protein–associated proteins within an individual cell, may have a considerable impact on the signaling networks that operate in response to external signals. This networking between these components in different signal transduction pathways remains to be fully understood.

G protein mediated signal transduction pathways are involved in nearly all physiological events and play important roles in determining the specificity and temporal characteristics of the cellular responses to the signals. G proteins take part in a variety of biological sensing and communication systems and help in controlling a variety of developmental processes from mating in yeast, to egg-laying in the nematode *C. elegans*, to immune responses, vision and olfaction in mammals (136).

A defect in any stage of a signal transduction pathway often gives rise to disease. A molecular defect in the structure of the G protein might induce an inappropriate signal and hence an altered response, thereby changing the specificity and the defined function of the G protein. Allowing inappropriate cell responses will contribute to abnormal cell function. For example, recent reports of Gα subunit mutations in mammalian tumors have given rise to the idea of *Gα* genes functioning as oncogenes (4). The relation between G proteins and cancerous growth suggests a possible impact of G protein mediated signal transduction on cell fate. An understanding of specific signaling pathways will help provide new opportunities to alter the systems when something has gone wrong. Thus studies on the functioning of G proteins will help unravel the mechanisms of cellular networks of complex inflow of information processing and response.

However, these signal transduction pathways and defects in them are difficult to study in complex multicellular organisms. Fortunately, studies done on microorganisms have shown that certain signaling strategies have been conserved throughout eukaryotic evolution. The soil amoeba *Dictyostelium discoideum* provides a convenient eukaryotic model system for study of intercellular communications and signal transductions. During *Dictyostelium* development, several developmental processes are mediated through signal

transduction pathways that initiate at G protein coupled cAMP receptors. Delineation of the signaling pathways and determining how extracellular signals act to induce cellular differentiation may significantly aid understanding of morphogenesis. The genetics and biochemistry of *Dictyostelium discoideum* provides powerful tools for the study of signal transduction and chemotaxis (31). In *Dictyostelium*, G protein–linked signal transduction events are essential for chemotaxis, cell aggregation, morphogenesis, gene expression and pattern formation. Many of the proteins involved in these events have mammalian counterparts (32) and hence *Dictyostelium* can serve as a model system to study G protein mediated signal transduction processes.

More than eight Gα subunits, one Gβ and one Gγ subunit have been identified in *Dictyostelium* (95, 126). Of the Gα subunits identified, two Gα subunits of *Dictyostelium discoideum*, Gα4 and Gα5, are the focus of the research described here. Gα4 and Gα5 are the most closely related Gα subunits in *Dictyostelium discoideum*, sharing about 51% amino acid sequence identity. The genes are both primarily expressed during multicellular development. Although very similar in terms of amino acid sequence and expression patterns, the Gα4 and Gα5 subunits seem to be essential for very different processes in development based on their mutant phenotypes (58, 62).

The focus of this research involves the Gα5 subunit and its similarity to the Gα4 subunit. The subunit encoded by the *Gα5* gene appears to play a pivotal role in tip morphogenesis. None of the intermediary players inclusive of the initial signal stimulating the Gα5 signal transduction pathway have been clearly defined. This study aims at defining the external signal(s) activating the Gα5 pathway, analyzing the effects of some well-studied site directed mutations in the Gα5 subunit, and determining the functional similarity between the Gα4 and Gα5 subunits.

In the first section, an attempt was made to elucidate the external signal that activates the Gα5 signal transduction pathway. Extracellular cAMP is a known regulator of tip formation in the developmental life cycle of *Dictyostelium discoideum*. Mutational

analysis suggests that Gα5 is required for tip formation as well. Given the hypothesis that extracellular cAMP activates the Gα5 mediated signal transduction pathway, Gα5 mutants were tested for responses to cAMP. Also, since cAMP is a known activator of the Gα2 signal transduction pathway in *Dictyostelium*, responses to cAMP were also studied in *gα2* null and *gα2/gα5* double null cells to ascertain the role of Gα5 in response to cAMP in Gα2 independent pathways.

Given the similarity between Gα4 and Gα5, there is a possibility that the Gα5 subunit triggers cellular responses similar to Gα4. One such response is chemotaxis to an external signal. Chemotaxis assays involving wild-type cells and Gα5 mutants were done to determine responses triggered by Gα5. The accumulation of possible secondary messengers, cAMP and cGMP were also determined by radioimmunoassays in wild type cells and mutants of Gα5 that had been treated with cAMP. In addition, responses to other biomolecules like folic acid, lysophosphatidic acid (LPA) and platelet activating factor (PAF) were tested because these biomolecules have been reported as possible external signals that activate G protein coupled receptors in *Dictyostelium.*

Mutations that impair the GTPase activity of Gα subunits have been reported to affect downstream signaling of the respective signal transduction pathways and in some cases promote cancerous growth in mammals. Mutational analysis was used to create and analyze the effects of two mutant *Gα5* genes, $G\alpha5^{Q199L}$ and $G\alpha5^{G197T}$, for alterations in developmental morphology. These mutations were in the conserved G-3 region (as described by Bourne *et. al.* (12)) of the Gα subunit. The glutamine to leucine mutation (analogous to $G\alpha5^{Q199L}$) has been associated with an impairment of GTPase activity, thereby leading to an always "activated" Gα subunit. The glycine to threonine mutation (analogous to $G\alpha5^{G197T}$) in the mammalian $G\alpha_s$ subunit has been associated with a decrease in downstream effector activation which may lead to a decrease in G protein function. The study of these two mutations in the Gα5 subunit may provide insight into the alterations of signal transduction and its regulation of different parameters such as cell division,

differentiation and cell migration, that are associated with analogous Gα mutants in mammalian cancer cells.

The Gα4 and Gα5 subunits are the most closely related Gα subunits in *Dictyostelium*. Another purpose of the study outlined here was to test the specificity of the Gα5 subunit and its functional similarity to the Gα4 subunit. To determine whether the subunits can function interchangeably, cross complementation studies were done. The ability of the *Gα4* gene to phenotypically complement a *gα5* null mutation and the ability of a *Gα5* gene to complement a *gα4* null mutation were tested using morphology and chemotaxis assays. Chimeric analysis of intercellular signaling between the mutant cells of Gα4 and Gα5 was done to determine if the phenotypic differences between the Gα4 and Gα5 mutants was due to common or distinct developmental defects. Potential epistatic relationships between the subunits were also studied by creating double mutants. Finally, the expression of the Gα4 and Gα5 subunits from heterologous promoters was studied to determine functional specificity and similarity in terms of expression of the Gα4 subunit and the Gα5 subunit. The results obtained might provide an insight into similarities and redundancies, and potential interactions and cross-talks, between signal transduction pathways that are mediated through related G proteins.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

Cells are continually responding to environmental signals of both cellular and non-cellular origins. Much of this information is provided to cells as a change in concentration of a biomolecule. When these biomolecules interact with their specific transmembrane receptors, the interaction is transduced into an intracellular signal. Occupation of cell-surface receptors by a specific ligand or biomolecule triggers a sequence of reactions between proteins in the cell membrane. This causes a change in the activity of effectors such as adenylyl or guanylyl cyclases, or ion-pores. This process is called signal transduction. One such signal transduction pathway is that which is mediated through membrane bound, guanine nucleotide binding proteins known as G proteins (54).

G Protein Mediated Signal Transduction:

G proteins are found in all eukaryotes and act as switches that regulate information processing circuits connecting the receptor at the cell surface to a variety of effector molecules such as adenylyl and guanylyl cyclases, phospholipases, phosphodieserases, receptor kinases and various ion channels. G proteins play important roles in determining the specificity and temporal characteristics of the cellular responses to signals. They are also important in determining cell fate by regulating a number of developmental processes such as cell division, differentiation and cell (47, 76, 117, 143).

Several hormones, neurotransmitters, chemokines, local mediators and sensory stimuli exert their effect on cells and organisms by binding to G protein coupled receptors (66). In addition, altered G proteins can also trigger oncogenic growth in mammalian cells (78, 93, 102). Therefore G proteins provide an important connection between signal transduction pathways and the regulation of cell growth. A thorough understanding of the regulation and function of G proteins in developmental processes may help provide insights into the mechanisms by which signal transduction pathways can change the fate and/or responses of cells with respect to the environment.

G protein mediated signaling pathways consist of three main components: receptors, G proteins and effectors as shown in Figure 1.

Receptors: Molecular cloning and biochemical studies have revealed a large family of G protein associated transmembrane receptor glycoproteins that are coupled to effectors via transducers. In Figure 1, R represents the receptor protein. All of these proteins have considerable amino acid similarity and are predicted to have seven hydrophobic helical segments that comprise the membrane spanning domains (134). No high resolution structure of G protein coupled receptors has yet been determined. Recently, a low resolution electron diffraction structure of rhodopsin, a model G protein coupled receptor shows the position and orientation of these seven transmembrane α-helices (3, 157). The receptors have three loop sequences and the carboxyl terminus on the cytoplasmic surface of the membrane. They also have characterized consensus sequences for N-linked glycosylation near the amino terminus. Sequence homology between the receptors is concentrated in the membrane spanning regions and, to a lesser extent, in the shorter connecting loops (135). In most cases an individual receptor, when activated by an appropriate ligand (L in Figure 1), can recognize and activate only a limited set of the many structurally related G proteins expressed within a cell (117). Many approaches have been used to study how this selectivity is achieved. Hybrid receptors constructed between

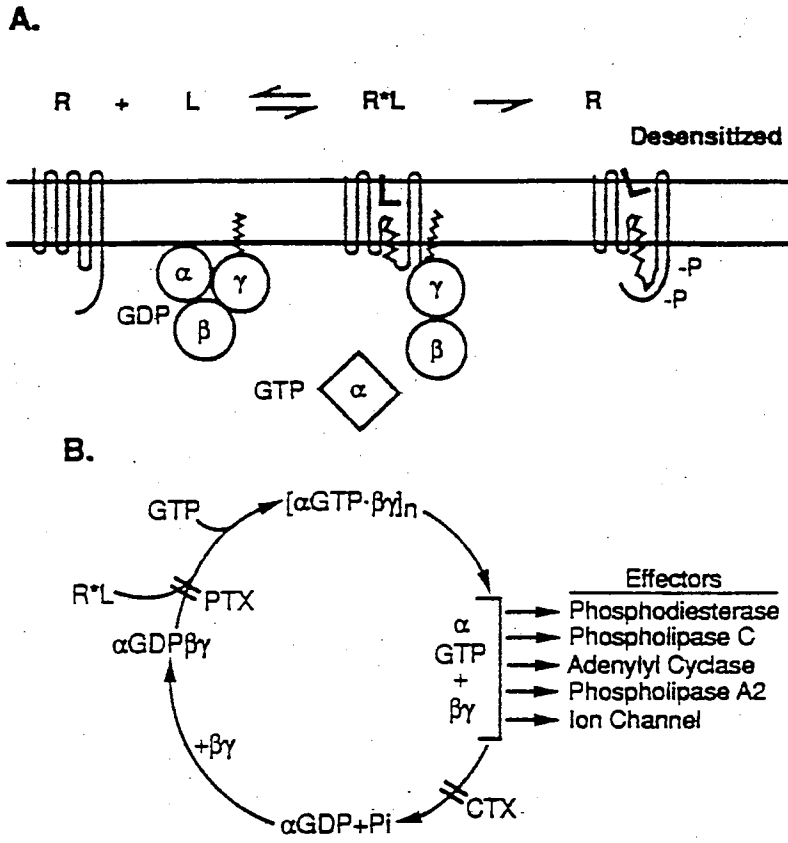

Figure 1. G protein-mediated signal transduction (A) Receptor (R) associates with a specific ligand (L). This stabilizes an activated form of the receptor (R*), which then catalyzes the exchange of GTP for the GDP bound to the α subunit of a specific G protein. The receptor is desensitized by specific phosphorylation. (B) The G protein cycle. Activated α subunits (αGTP) and βγ dimers can interact with different effectors (143). PTX represents pertussis toxin, while the site of action of cholera toxin is designated as CTX.

functionally distinct members of the G protein receptor subfamily have led to a wealth of information regarding receptor/ G protein coupling selectivity. A majority of such receptor chimera studies indicate that the selectivity of G protein recognition is mainly determined by the amino acids in the second intracellular loop and the amino and carboxy terminal portions of the third intracellular loop. Biochemical experiments done with peptides that mimic or inhibit receptor interactions and mutagenesis studies done on the receptors support the data obtained with the chimeric receptors (162).

A number of different G protein coupled receptors have been found in various eukaryotic systems, including distinct receptors that bind the same ligand (8). Receptors that respond to the same ligand can be further differentiated into subtypes depending on the intracellular responses they elicit. These responses are coupled to different second messenger pathways and hence to the regulation of different effectors. G protein coupled interactions thus form complicated networks as a single receptor subtype can be coupled to multiple effectors (divergent signaling) and multiple receptor forms can activate a single effector (convergent signaling) (54, 134, 135). A pictoral representation of these convergent and divergent signaling pathways is shown in Figure 2.

G Proteins: Heterotrimeric G proteins are part of a larger GTPase superfamily that also consist of small GTP binding proteins, Ras and Ras-like proteins, and many factors involved in protein synthesis.

The "small G proteins" are usually single polypeptides that are about 2000 amino acids long. These small G proteins function in regulating cell growth, protein secretion and intracellular vesicle interaction (65). In the last few years, a novel class of small (21-24kDa) monomeric, guanine nucleotide binding proteins have been characterized, the prototype of which is Ras. *ras* genes were first discovered as viral oncogenes harbored by two closely related transforming retroviruses. These proteins are very similar to classical G proteins and have a resting GDP form and an active GTP form. The Ras proteins are

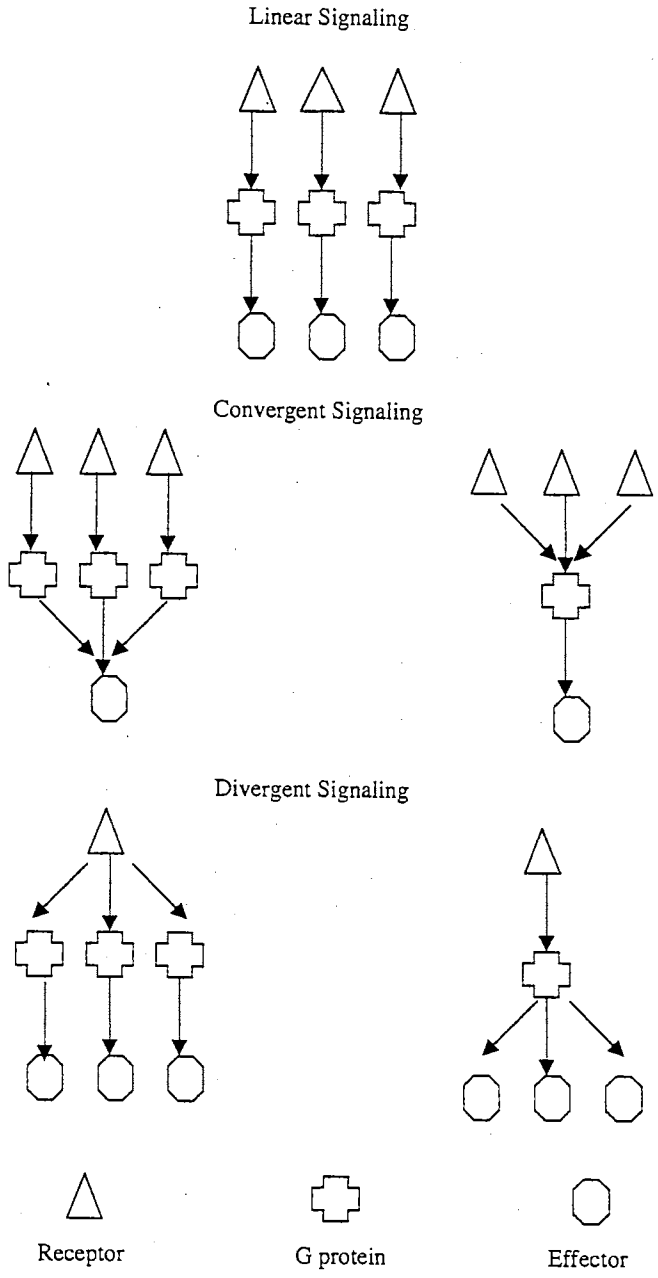

Figure 2. Signal Routing in G protein networks. Signals can converge or diverge at each level of the G protein network. In the native membrane, these patterns will be combined with one another to coordinate multiple secondary messenger outputs from multiple receptor inputs (69, 135)

capable of hydrolyzing GTP, although they have low intrinsic GTPase activity. Hence, the Ras proteins rely on GTPase activating proteins (GAPs) to convert their active GTP-bound forms to their inactive GDP-bound counterparts. A significant portion of human malignancies contain a constitutively expressed *ras* oncogene. Homologs of the *ras* and *ras*-related genes have since been discovered in many organisms including *Saccharomyces cerevisiae*, *Neurospora crassa*, *Dictyostelium discoideum*, *C. elegans* and maize (19).

Heterotrimeric G proteins are also members of the GTPase superfamily and these G proteins and are composed of α, β and γ subunits (54). Specific transient interactions between these subunits generate the pathways that modulate cellular responses to complex chemical signals.

Gα Subunits: In general, G proteins are named for their α subunits. The guanyl-nucleotide binding α chain is a polypeptide that encodes a 32-59kDa protein. Multiple G protein α subunits have been identified in all the eukaryotic organisms that have been studied. When the deduced amino acid sequences of all known α subunits are aligned, there is about 20% identity among them (97). Because of their similar structures, the α subunits are difficult to separate by classic biochemical techniques. In addition to amino acid similarity, there is also conservation at the level of gene structure. Sequence homology among these α subunits and similarity of their biochemical properies suggest that their tertiary structures will also be similar and that they will share definable functional domains (97, 108). Structural analysis of the α subunit shows five different regions. They are classified as follows:

(1) P or G-1 region – This region is thought to be important for interaction of the α subunit with GTP, GDP and guanine nucleotides

(2) E or G-2 region – Sequences in this region are thought to be important for α subunit interaction with the effector

(3) G-3 region – Amino acid sequences in this highly conserved region contribute to the intrinsic GTPase activity of the α subunit.

(4) G-4 region - Amino acids in this region interact with the guanine ring and stabilize the guanine nucleotide binding site.

(5) G-5 region – This region is not well conserved between the different members of the GTPase family.

These sequences are also found in the small G proteins including Ras, and in the protein synthesis factors like elongation factor Tu (128).

The crystal structure was not known for any α subunit of heterotrimeric G proteins until very recently. Earlier models for an approximate three dimensional structure of the Gα subunit had been developed on the basis of the crystal structures of the small G protein Ras and of elongation factor Tu (108). The recent resolution of the crystal structure of transducin-α complexed with the GTP homologue, GTPγS and GDP provide a structural framework for understanding the role of heterotrimeric G proteins as conformational switches in signaling pathways (91, 121).

Gα subunits contain two domains. The first domain is involved in binding and hydrolysing GTP (the G domain) and is structurally identical to the superfamily of GTPases including small G proteins and elongation factors (84). The second domain is a unique helical domain that buries the GTP in the core of the protein (25, 92, 121).

Gβ and Gγ Subunits: In contrast to the individuality of the α subunits, the β and γ subunits are relatively interchangeable among the G proteins. In combination, the β and γ subunits are a functional unit, and are not dissociable except by denaturation. Three functions can be ascribed to the βγ complex. They help anchor the G protein to the membrane, attenuate the α–subunit stimulation of effector enzymes or channels and are required for the efficient interaction of α with the receptor and the catalytic activation of guanine nucleotide exchange (76). The β (35-36kD) and γ (6-10kD) subunits are tightly associated and are purified as a complex under non-denaturing conditions. The βγ subunits of different G proteins are highly homologous and, at least *in vitro*, functionally

interchangeable so that they may associate with any of the different α subunits. Hence, subunit dissociation and interchange among G proteins at the surface of the membrane have the potential to modulate the activity of different signal transduction pathways (7, 54).

The β subunits studied to date are made up of a seven membered β-propeller structure containing a water-filled pore. This structure is formed by segments of a 40 amino acid repetitive sequence motif that is characterized by certain amino acids (such as a tryptophan- aspartic acid pair) known as the WD-40 repeat (92). This motif has recently been discovered in a number of other proteins besides the Gβ proteins. However, nothing is known about the function of this sequence motif (48, 51). Recent studies have suggested that the β subunit is necessary to hold the α subunit rigidly in place for GDP release to occur when the receptor is activated by ligand binding(49).

The number of γ subunits and differences among them are unknown. Up to three bands in the 6kD to 10kD range, with different electrophoretic mobilities, can be seen with highly purified samples of G proteins. The γ subunit has been predicted to be largely alpha helical and has been shown to extend along the repeat units of the β subunit (101). These γ subunits cofractionate with β subunits under a number of different conditions which suggests that each γ subunit is a part of a βγ dimer. The γ subunit interacts with the β through an N-terminal coiled coil and then makes extensive contacts all along the base of β (147).

Conclusive sequence data are available only for the γ subunits of transducin, a G protein from the brain and a subunit in yeast (52, 70, 165). These proteins are most divergent at their amino terminus sequence. At their carboxy terminus, they share a considerable amount of homology. A characteristic cysteine is present in the carboxy termini of the γ subunits, four residues from the end of the protein. All the γ subunits are modified by the carboxymethylation and isoprenylation of the terminal cysteine residue and by the removal of three carboxy terminal amino acids adjacent to the cysteine (23). In

addition, the C-terminus of the γ subunit of G proteins have been shown to be involved in receptor coupling and specificity (171).

G protein βγ dimers regulate several downstream effectors. Recently, Yan and Gautam suggested that βγ dimer effector specificity resides in the β subunit (170). Protein modifications of βγ are important for effector interactions just as in the case of receptor interactions. Prenylation of the γ subunit appears to be required for effector regulation as well (72, 116).

The extensive modification of the γ subunits, the diversity of the β and γ subunits, as well as the cross-talk between βγ subunits that are associated with different α subunits, all indicate that the βγ subunits are important for establishing specific receptor-G protein associations. They also point to the fact that the βγ subunits are important for integrating the effects and timing of various G protein-mediated circuits.

Effectors: The third component involved in transmembrane signaling is the effector molecules, which include enzymes and ion channels in several protein families. G proteins are responsible for organizing the signals they receive from multiple receptors and for directing them to an appropriate array of effector molecules. The enzymes mediated by G proteins include adenylyl cyclase, phosphodiesterases, phospholipase A2 and C and receptor kinases. Adenylyl cyclase is an integral membrane glycoprotein composed of a single subunit with multiple membrane spanning regions (129, 145). The phophodiesterase is a water-soluble, peripheral membrane protein composed of two non-identical subunits required for catalytic activity and two inhibitory subunits (2, 71). Phospholipases are effector molecules that regulate the arachidonic acid production, IP_3, and diacyl glycerol (DAG) in the cell and hence transduce the external signal.

Adenylyl cyclase, phosphodiesterases and phopholipase C can be regulated by both Gα and Gβγ subunits. Gα sbunits have also been shown to regulate phospholipase A2

activity, while the Gβγ subunit has been shown to regulate receptor function by controlling receptor phosphorylation and subsequent dimerization (138).

G proteins also regulate a wide variety of ion channels such as potassium channels, calcium channels and possibly sodium channels (16, 172, 173). The regulation of ion channels by G proteins involves modulation of ionic concentrations in the cells through soluble intermediary second messengers such as cGMP, inositol triphosphate (IP_3) and arachidonic acid (138).

Specificity of G Protein Mediated Signal Transduction Pathways:

The family of G protein-coupled receptors consists of several hundred proteins while the less numerous effectors include enzymes and ion channels in several protein families. G proteins transmit signals with high fidelity such that each G protein directs the flow of information from a distinctive subset of receptors to a limited array of effectors. This array of receptors and effectors raises questions about the nature of the information processing circuits that are formed. There is little information available about how many different G proteins are required to couple the receptor and effector subtypes and how specific receptors are linked through G proteins to form autonomous circuits that interact with each other during cellular growth and differentiation. Hence, in order to understand G protein function, it is necessary to identify the components of G protein mediated networks and the nature of their specific interactions.

Network specificity can be controlled by feedback processes. For example, activation of a specific G protein pathway can generate second messengers to activate protein kinases. These kinases could then influence the information processing system. In *Dictyostelium*, the Gα2 G protein is rapidly phosphorylated when the ligand binds the cAMP receptor coupled to the G protein (56). This may be a mechanism of inactivation of the pathway, leading to an adaptation process.

Some of the circuitry mediated by G proteins can serve the function of signal distribution. For example, an activated $G\alpha_s$ is capable of both opening Ca^{2+} channels and increasing the concentration of intracellular cAMP. Thus, two responses can be coordinated by the activation of a single G protein (174).

Also, G-protein similarities can generate cross-talk, resulting in signal integration. Both α and $\beta\gamma$ can convey information during signaling through the interaction of G proteins with one another. One such case, where there are G protein interactions through the exchange of their $\beta\gamma$ dimers, has been observed with the mammalian $G\alpha_s$ and $G\alpha_i$ subunits, both of which regulate adenylyl cyclase activity as shown in Figure 3. The $\beta\gamma$ subunits from $G\alpha_i$ inhibit adenylyl cyclase by suppressing the activation of $G\alpha_s$ (69). If two G proteins are activated by different receptors and characteristically deliver signals to different effectors, but are capable of interacting at low efficiency with other effectors, then the activation of either one of these pathways could elicit an activation of the other. Hence, cross-activation could be an essential part of the information transducing circuit. On the other hand, parts of the intracellular system may be set up so as to avoid this cross-talk. By confining specific G proteins and, perhaps their effectors, to local regions of the cell, their ability to cross-talk or interact could be effectively curbed. For example, in polarized renal epithelial cells, $G\alpha_{i2}$ is localized to the basal lateral membrane of the cell, while $G\alpha_{i3}$ is found in the Golgi and the apical membranes (41).

Experiments conducted to identify G proteins in various systems have revealed multiple $G\alpha$, $G\beta$ and $G\gamma$ subunits within cells. If all of these different subunits associated combinatorially at random, there would be a very large number of different kinds of heterotrimers. Different combinations could have different affinities for different receptors and this in turn, could affect the direction of flow of information to the effectors (69). Reconstitution experiments performed with mammalian G protein subunits have shown that different $G\alpha$ subunits have different affinities for different $\beta\gamma$ dimers (17). However, a $\beta\gamma$

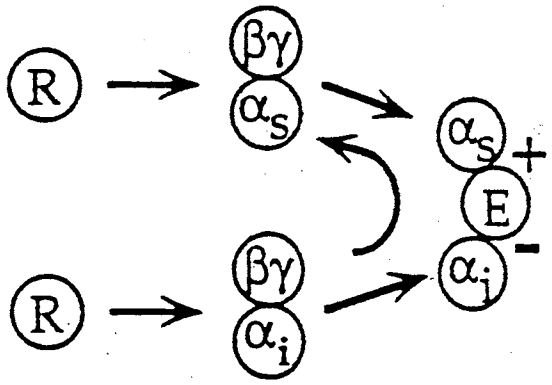

Figure 3. Signaling mechanisms based upon subunit dissociation as a mechanism of G protein activation. This example uses the action of $G\alpha_s$ and $G\alpha_i$ to regulate the effector adenylyl cyclase (69).

complex from the placenta has been shown to have distinctive properties and different γ subunits were found to be associated in heterotrimers with the same α subunit when isolated from different tissues (53). Thus the specificity of the interactions between the subunits seems to be important in the transduction of a signal into a cell.

The stoichiometry of G protein subunits is also important for the fidelity of signal transduction. The loss or overproduction of a single subunit can result either in the loss of signal transduction or in enhanced stimulation of downstream effectors, depending on which subunit expression is altered (24, 58, 86, 89, 166). Thus, subunit specificity plays a role in the formation of heterotrimers. This may also be important for G protein regulation, but is not well understood. The association of Gβγ dimers with different Gα subunits has been demonstrated *in vitro*, but whether these multiple associations are permissible *in vivo* has not been determined (17, 68). A better understanding of subunit specificity *in vivo* is needed to clearly define these stoichiometric interactions.

Mechanism of G Protein Mediated Signal Transduction:

Signal transduction by a receptor is initiated by ligand binding. This stabilizes an alternate conformational state of the receptor by changing the relative orientations of two of the transmembrane helices. These changes then alter the conformation of the intracellular loops of the receptor that interacts with the G protein and thus uncover previously masked sites to which a specific G protein can bind (162). The ligand bound receptor initiates two processes: one leads to desensitization and occurs through receptor modification and the other is a signal generating process that begins with the activation of a heterotrimeric G protein. Interaction of the G protein with the activated receptor promotes the exchange of GDP, that is bound to the α subunit, for GTP and the subsequent disassociation of the α-GTP complex (Gα*GTP) from the βγheterodimer. It is thought that the receptor contact sites on the G protein are distant from the GDP-binding pocket and hence the receptor must work "at a distance" to change the conformation of the protein (11). Since GDP is buried

between the two domains of Gα within protein, this must necessarily involve changing some interdomain interactions.

In the active, GTP-bound conformation, a new surface is formed on the Gα subunits. Hence, they interact with effectors with a 20-100 fold higher affinity than in their GDP-bound state (144). Once Gα*GTP has dissociated from the Gβγ, free βγ acts as an activator of a large number of proteins (21). The conformation of the free Gβγ is identical to the Gβγ in the heterotrimer (147). This suggests that the Gα inhibits Gβγ interactions with its effectors through the Gα binding site on Gβ. The α-GTP complex and the free βγ heterodimer may interact with effector proteins such as adenylate and guanylate cyclases that further amplify the signal by modulating low molecular weight secondary messengers such as cyclic AMP (cAMP), cyclic GMP (cGMP) or inositol triphosphate (IP). These secondary messengers, in turn, generate dramatic intracellular changes such as selective protein phosphorylation, gene transcription, cytoskeletal reorganization, secretion or depolarization of the membrane.

During effector stimulation, the intrinsic GTPase activity of the Gα subunit converts GTP to GDP allowing the α and βγ subunits of the G protein to reassociate, thereby completing the activation cycle as shown in Figure 1 (6). In some cases, hydrolysis of GTP is extremely slow and requires interaction with another protein, the GTPase Activating protein (GAP), to accelerate hydrolysis and in turn the GTPase activity of the Gα subunit. (6, 76, 143).

In many signal transduction pathways, the Gα subunit appears to be the primary transducer of the external signal. In other cases, Gβγ subunits can regulate effectors by assuming the role of the primary transducer of an external signal (165) as shown in Figure 4. Gβγ has well-defined effects on some isoforms of the classical second messenger enzymes, such as phospholipase C and adenylate cyclase (113). In addition, Gβγ serves as the direct activator of certain G protein responsive K^+, Ca^{2+} and perhaps, also Na^+ channels

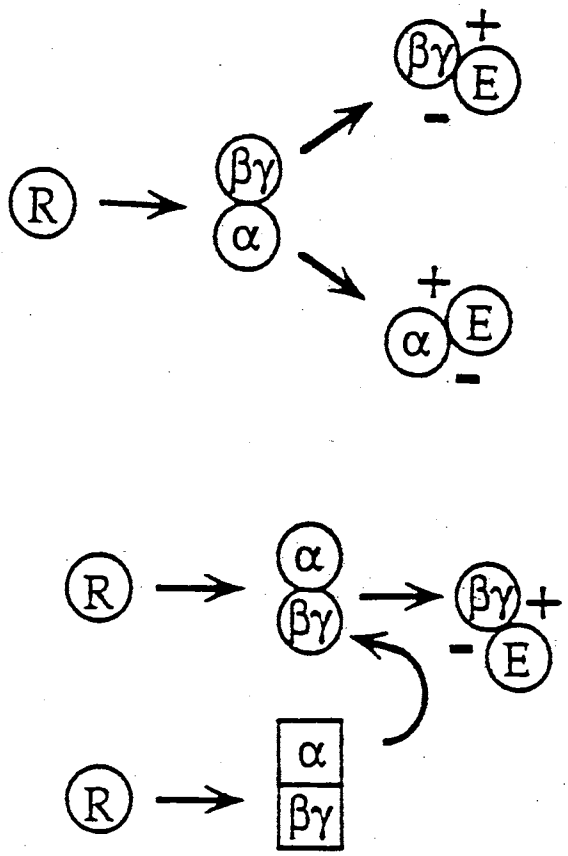

Figure 4. Signaling mechanisms of both activated α subunits and free βγ dimers. (A) Ability of a single G protein to activate dual signaling pathways (B) Suppression of the effects of βγ effects by the excess α subunits liberated from other activated G proteins.

(21). Gβγ also directly activates more than one phosphotidylinositol 3-kinase isoform and has also been reported to activate a number of other kinases (94, 132, 154). In yeast, Gβγ is the activator of a pheromone-stimulated MAP kinase pathway (165).

Recently, in yeast, Gβγ has also been reported to bind to members of the Rho family of GTPases such as Rho, Rac and Cdc42 as well as to the small G protein Arf (ADP-Ribosylation Factor), which is involved in coat formation and vesicular trafficking (50, 67, 142). Studies conducted on the muscaranic receptors and the β-adrenergic receptor kinase have shown that Gβγ is involved in the desensitization of receptors by certain G protein coupled receptor kinases. In this case, the Gβγ subunits not only anchor the kinase to the membrane via the isoprenylation site on the Gγ, but also act synergistically with the activated receptor to stimulate kinase activity (63, 64). Thus there is a rich list of Gβγ effectors and effector activation mechanisms.

The number of known forms of G protein α, β and γ subunits and also the enormous number of receptors they interact with, as well as their diverse effectors which affect all kinds of cellular processes make this an important and complicated field. Fortunately, there are similarities between the various structure and functional interactions of the G protein coupled receptors and the G protein subunits. This has helped to a vast extent in the study of G protein signal transduction pathways because it is often possible to apply information obtained in one G protein and/or receptor to the characterization of another system. However, the specificity in each of the protein-protein interactions must be understood before extrapolating results from one signal transduction pathway to another.

Study of G Proteins in Various Systems:

G proteins and hence G protein mediated signal transduction are an integral part of many developmental programs. As cells differentiate, their signaling characteristics change and new pathways are expressed and integrated into cell function. A number of regulatory

peptide factors and hormones can influence cell growth. Many of these molecules such as thrombin and angiotensin, and insulin-like growth factor bind specific G protein coupled receptors (143). G proteins also play a role in modulating cell movement, cytoskeletal structure and chemotaxis. The activation of G protein pathways in the appropriate context has been shown to initiate, facilitate or amplify changes required for cellular differentiation.

The role of G proteins in development has been studied genetically in a number of different organisms. In *Saccharomyces cerevisiae* (budding yeast) studies have shown that cell cycle arrest and cellular differentiation in response to a mating pheromone is dependent on G proteins (165). The mechanism of action in yeast seems different as compared to other multicellular organisms, but the overall features of the system are conserved, since mammalian Gα subunits can restore partial function to the yeast mutants (109). The two Gα subunits identified in yeast (GPA1 and GPA2) share a 60% identity and have highly conserved regions that are found in all G proteins. The overall similarities between yeast G proteins and mammalian G proteins are remarkable. However, two additional stretches of sequences exist in yeast G protein α subunits that do not bear any homology with any other proteins reported (115). The *STE4* and *STE18* genes of *S. cerevisiae* encode proteins homologous to mammalian G protein β and γ. The STE4 has a 49% identity in amino acid sequence to two types of mammalian Gβ subunits, while the deduced sequence of STE18 exhibited partial similarities with the mammalian g1 and g2 proteins (165). G protein subunit counterparts have also been identified in *Schizosaccharomyces pombe* (122).

Mammalian cell development also requires G protein mediated signal transduction pathways. One example is the regulated expression of G proteins during the differentiation process of hematopoeitic cells (1, 168). Based on amino acid sequence similarity, Gα subunits in mammals have been classified into four classes represented by $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$(110, 143). In *Drosophila melanogaster,* sequences that share 60-70% amino acid identity to all four α subunit classes of mammalian cells have been found (133). For example, a maternal gene called *concertina* (*cta*) that is required for appropriate

morphological development in developing larvae has been shown to be a Gα subunit (127). In *Caenorhabditis elegans* whose developmental program is well characterized, developmentally important G proteins ($G\alpha_s$ and $G\alpha_o$) have been identified by isolating genomic fragments using PCR. A Gα subunit is also essential for neuronal and developmental aspects of egg-laying in this creature (111). A gene encoding a G protein α subunit that has 63% amino acid sequence identity with mammalian $G\alpha_{i2\alpha}$ has also been cloned in this system (98). The nematode Gβ gene, which has a highly conserved amino acid sequence has also been identified and isolated (158). Two plant G protein alpha subunit genes, *GPA1* and *TGA1*, were isolated from *Arabidopsis thaliana* and tomato plants respectively (103, 104). The *GPA1* gene shows a high level of conserved residues at the intron-exon junctons and shares a 36% amino acid identity with the mammalian $G\alpha_i$.

G Protein Mutations:

Recently, the discovery of Gα subunit mutations in mammalian tumors has initiated the idea of the *Gα* genes functioning as oncogenes (78, 93, 102). A number of isolated pituitary tumors have been reported to have $G\alpha_s$ alleles with codon alternatives at position 201 (R201C/H- arginine converted to a cysteine or a histidine) or Q227R (93). The intrinsic GTPase activity of these Gα subunits is reduced, thus resulting in a greater level of signaling downstream of the Gα function. In support of these findings, all of the pituitary tumors examined were found to contain much higher levels of cAMP, the secondary messenger molecule regulated by $G\alpha_s$. Similar mutations have also been found in the $G\alpha_{i2}$ subunit isolated from tumors of other tissue types (102). However, due to a lack of knowledge of known effector molecules downstream of this subunit, no further analysis was possible (102).

Certain mutations like the Q227R of $G\alpha_s$ replace the conserved glutamine residues within the G-3 region of the Gα subunits (12). These changes in the conserved glutamine residue are of particular interest because an analogous mutation, glutamine to leucine, in the

mammalian *ras* gene, has been associated with oncogenesis (4). The introduction of the mutant $G\alpha_s$ or $G\alpha_i$ subunits into NIH3T3 cells or Swiss cells, has induced characteristics of oncogenesis in tissue culture, such as reduced serum requirements, reduced doubling time and loss of cell-contact inhibition. In these as well as in numerous other cases (Table 1), the substituted glutamine residue in Gα subunits and Ras proteins result in reduced GTPase activity. The inhibition of the GTPase activity is thought to prolong the activation states of these proteins, which in turn would result in an increase in signaling of downstream effectors. But in many cases, the level of expression of these mutant genes is amplified either by heterologous promoters or by a high copy number of the genes, which can also lead to increased signaling (24, 58, 166). The contribution of reduced or deficient GTPase activity on downstream signaling becomes more defined when these G proteins are studied at their normal physiological levels. This connection between G proteins and cancerous growth indicates that G protein mediated signal transduction can have a significant impact on cell-fate decisions, as has been implied by the requirement of G proteins for developmental processes.

In addition to the substitution of the glutamine residue in the conserved G-3 region, another substitution mutation (glutamine to threonine) in the same region, has also been previously studied in the mammalian $G\alpha_s$ gene and the *Dictyostelium Gα2* (123, 124). This mutation was associated with a decrease in adenylyl cyclase activity, termed as a dominant negative phenotype, which resulted in a decreased Gα response. Studies done with the same substitution mutation in the *Dictyostelium Gα1* gene also showed a significant reduction in adenylyl cyclase activity in response to cAMP (34).

Since mutant G proteins are associated with oncogenesis, it is possible that mutant G proteins may be associated with apoptosis as well because both oncogenesis and apoptosis are processes that involve cell growth and differentiation. Apoptosis or programmed cell death has been observed in a number of organisms and seems to be an

TABLE 1

G-3 regions of Gα subunits and Ras proteins

Gα subunit	*	Mutations
Mammalian $G\alpha_q$:	DVGGQRSERRKW	Q>L transforms NIH 3T3 cells
Mammalian $G\alpha_s$:	DVGGQRDERRKW	Q>L proliferation of Swiss 3T3 cells Q>R associated with pituitary tumors
Mammalian $G\alpha_{i2}$:	DVGGQRSERKKW	Q>L proliferation of NIH 3T3 cells
Mammalian Ras	DTAGQEEYSAMRW	Q>L oncogenic
Dictyostelium Gα4	DVGGQRSQRRKW	Q>L aggregation deficient/ cell death
Dictyostelium Gα1	DVGGQRSERKKW	Q>L abberant stalk differentiation
Dictyostelium Gα2	DVGGQRSERKKW	Q>L aggregation deficient

(Hadwiger *et. al.* 1996 *Development*, Srinivasan and Hadwiger unpublished data, Landis *et. al.* 1989 *Nature*, Hermouet *et al.* 1991 *PNAS*, Kalinec *et. al.* 1993 *Mol.Cell.Biol.*, Valler *et. al.* 1987 *Nature*, Lyons *et. al.* 1990 *Science*, Barbacid 1987 *Ann. Rev. Biochem.*,Dharmawardhane, S. *et. al.* 1994 *Development*)

important process in regulation of cell growth and differentiation in multicellular eukaryotic cells (163). The apoptotic death process is associated with pronounced morphological changes in the cell, as well as in intranucleosomal DNA fragmentation. A common characteristic associated with apoptosis is the early fragmentation of genomic DNA before the cell loses other cellular functions such as membrane integrity (43, 163). This helps differentiate between apoptosis and necrosis, which is cell death that occurs with a loss of membrane integrity.

Apoptosis has been observed in many systems. Failure to regulate apoptosis, either positively or negatively, leads to a discrepancy in maintaining homeostasis. An absence of negative regulation of apoptosis causes degenerative diseases and faulty positive regulation is associated with cancer and autoimmune disease. Such an example can be seen in the case of overexpression of *bcl-2*, which suppresses apoptosis (164). In the nematode*C. elegans*, *ced-3* and *ced-4* are involved in positively regulating developmental cell death, while the *bcl-2* homolog *ced-9* is involved in the negative regulation of apoptosis (40). Of the viral counterparts, the adenovirus E1B 19K protein is the best characterized and is required to inhibit apoptosis during an adenovirus infection. Recently, an African swine fever *bcl-2* homolog has also been identified (164). Examples of other apoptosis-related molecules include the *ced-3* equivalent ICE cysteine protease in mouse, some molecules that are preferentially expressed in dying thymocytes, the reaper molecule which regulates the death of a large number of cells during the embryonic development in *Drosophila*, and molecules that are inappropriately expressed during the cell cycle (26).

The timing of apoptosis and its regulation seems to be different in different eukaryotic organisms and tissue types, but common regulatory genes appear to be important for many systems. Some of these regulatory genes such as *c-myc* are proto-oncogenes or others like *p53* are tumor suppressor genes, indicating that common signal transduction pathways may regulate the opposing cellular processes of apoptosis and oncogenesis. The function of the wild-type subunit in cell growth and differentiation may

play a role in determining whether a particular mutant G protein promotes apoptosis or oncogenesis.

One characteristic, common to the apoptotic process in all eukaryotic cells, is the fragmentation of genomic DNA by nucleases during the process of cell death. This fragmentation of the genome in apoptosis occurs before the loss of cell membrane integrity. As opposed to this, in necrosis, genome fragmentation occurs after the loss of cell membrane integrity. Apoptosis can be distinguished from necrosis by staining the cells with two different DNA-specific fluorochromes (43). The two fluorochromes, propidium iodide and Hoechst 33342, produce different colored emissions, so that both can be detected simultaneously (43). The cells are first exposed to propidium iodide. This dye is excluded by the cells that have their plasma membrane integrity preserved, but it stains the DNA in cells that have damaged membranes. The cells are then washed and permeabilized with a low concentration of ethanol before they are stained with Hoechst 33342. This dye enters the live cells that have their membranes permeabilized and stains the DNA. Although the Hoechst dye can also enter the necrotic cells, the effect of this dye will be quenched by the propidium iodide that the cells were first stained with (131). A major advantage of this technique is that very few cells are needed for the analysis as compared to an analysis that involves examining the genome by gel electrophoresis. This is critical, as most colonies with substantial cell death never accumulate large numbers of cells. In certain cases, such as the DIF-induced cell death in *Dictyostelium*, it has been reported that apoptosis can occur without the process of genome fragmentation (26). In these cases, extensive vacuolization has been observed, which is supported by cells undergoing differentiation to form stalk structures. This apoptotic trait of extensive vacuolization has also been observed in *C. elegans* and in some cell types of higher eukaryotes (22).

In order to determine whether developmental signals can enhance the cell death process with a mutated allele, regrowth experiments can be performed as described (26). In *Dictyostelium*, one of the signals that initiates development is deprivation of nutrients.

Most wild-type cells can go through development while retaining the ability to resume vegetative growth in the presence of nutrients. Thus, in the regrowth experiments, we can look for the ability of the mutants to resume vegetative growth after nutrient deprivation by replacing the rich vegetative growth medium.

G proteins can be studied with respect to development and differentiation in a simple eukaryote, *Dictyostelium discoideum*, where Gα subunits seem to be required for proper cell migration and cellular differentiation during fruiting body development (58, 62, 86).

Dictyostelium discoideum as a Model System to Study Development:

During *Dictyostelium* development, a several other processes like chemotaxis during aggregation, morphogenesis during multicellular differentiation and gene regulation during the life cycle are mediated through signal transduction pathways that initiate at G protein coupled cAMP receptors. Delineation of the signaling pathways and determining how extracellular signals act to induce cellular differentiation may significantly aid understanding of morphogenesis.

Developmental Life Cycle: The cellular slime mold *Dictyostelium discoideum* provides a convenient eukaryotic model system for study of intercellular communication and signal transduction. This organism has a relatively simple and rapid developmental life cycle (Figure 5). It grows as a single celled vegetative amoebae in the presence of a food source. At this phase of its life cycle, it is attracted by a sensory system that involves chemotaxis to folic acid and other pteridines liberated by bacteria (125). The individual cells ingest the bacteria by phagocytosis or, in the case of axenic derivates, take up nutrients by pinocytosis.

Deprivation of certain amino acids and the action of several diffusible and environmental factors may play a decisive role in signaling the onset of development (55, 107). Starvation induces the amoebae to develop the components of an oscillatory

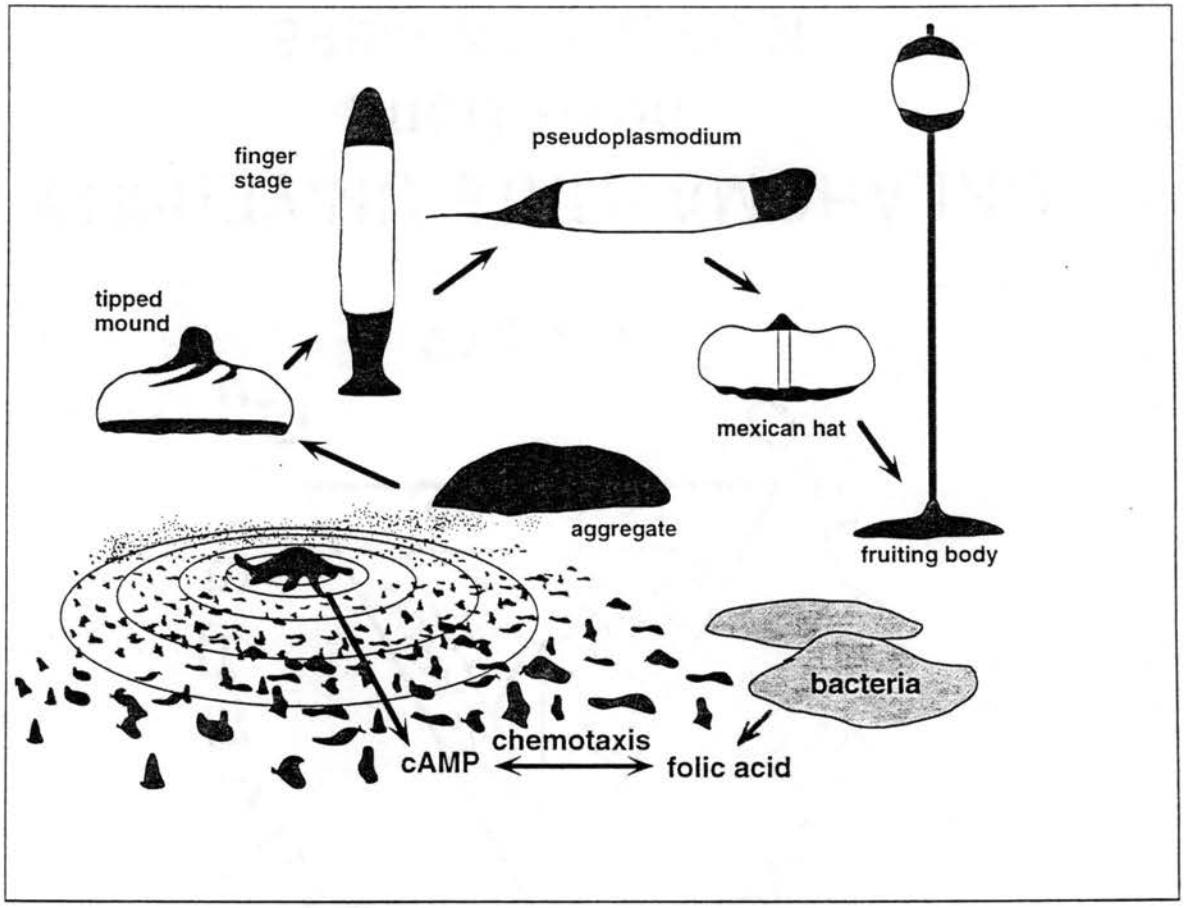

Figure 5. Developmental life cycle of *Dictyostelium discoideum*. The vegetative single celled amoebae, upon starvation undergo aggregation to form undifferentiated muticellular mounds, which ultimately differentiate to form a fruiting body at 24 hours post-starvation.

intercellular signaling process that is based on the synthesis, release and detection of extracellular adenosine 3', 5' monophosphate (cAMP). cAMP is secreted by a starved group of cells at six minute intervals. The production of cAMP by the cells helps in creating a cAMP gradient. This gradient in turn helps orient the adjacent cells which, in turn, start secreting out cAMP. cAMP thus induces the transient synthesis and secretion of more cAMP. A relay of cAMP signaling is thus initiated by a group of cells at a point, which serves as a focal center or a pacemaker for aggregation, and then radiates outward. The wave of cAMP is then propagated by a relay of the chemotactic signal. An external phosphodiesterase (PDE) then degrades the signal and resensitizes the system, at which point it is ready to respond to the next wave.

Subsequent to aggregation, differentiation is initiated where about 10^5 individual cells chemotax to a central collection point (aggregation or signaling center), and stick together by means of cell adhesion molecules located on the cell surface, to form a multicellular aggregate (10). Differentiation continues at about 12 hours post-starvation by the formation of a small tip at the apex of each undifferentiated aggregate. This tip then elongates to produce an upright finger-like structure. At about 16 hours into development, the tipped aggregate forms a migratory slug (pseudoplasmodium), which crawls towards light and warmth, leaving behind the secreted slime sheath along its trail. A mature fully differentiated fruiting body, that contains spore and stalk cells, is formed at about 24 hours after starvation.

In the undifferentiated state, two major cell types are observed, that are sorted out to different regions in the multicellular organism at the tipped mound stage. The prestalk cells (precursors to the stalk cells), occupy the anterior end of the tipped mound and slug, while the prespore cells (precursors to the spore cells), form a band at the center of the multicellular mound and are found in the posterior portion of the slug. There is evidence that suggests that sorting out involves differential chemotaxis to cAMP (156). Studies of developmental gene regulation have shown successive expression of sets of

developmentally controlled genes. In addition, bifurcation has been observed along two pathways that correspond to prestalk and prespore cell types. These phenomena seem to be dependent on ongoing intracellular signaling via cAMP.

At the beginning of culmination, the posterior end of the slug continues to move while the tip of the pseudoplasmodium stops forward movement. Consequently, the undifferentiated cells gather directly under the tip. At the upper end of the prestalk zone near the tip, the stalk cells now begin to form by progressive vacuolization. These cells also deposit cellulose on the cell walls and when differentiation is complete, the cells die.

The apical stalk is pushed down though the prespore zone by a reverse fountain movement. When the stalk reaches the base, cells that occupied the posteior region in the slug stage, differentiate and form vacuolate stalk like cells to give rise to the basal disc. The prespore cell mass then rises as the stalk elongates, and the structure is referred to as a sorogen. On its upward journey, each prespore cell starts to differentiate into spores by encapsulating itself in a complex cellulose coat. A mature fully differentiated fruiting body or sorocarp, that contains spore and stalk cells is formed at about 24 hours after starvation.

Phylogenetically, *Dictyostelium* can be considered as one of the evolutionary attempts at multicellularity. Selected cell death is an important part of multicellular development (26), and *Dictyostelium* seems to exhibit this phenomenon. The sorocarp contains two main cell types: the viable spores and the stalk cells. Differentiation of cells to stalk cells seems to be a result of both cAMP promoting and DIF promoting factors. Stalk cells are vacuolated and are considered non-viable as they cannot be regrown in a culture medium. This indicates a type of programmed cell death leading to the formation of stalk cells.

Studies have shown that various intracellular events such as an early irreversible step, subsequent massive vacuolization, cytoplasmic condensation, focal chromatin condensation and late membrane lesions are developmentally induced and lead to the programmed cell death of the stalk cells. However, no early DNA fragmentation could be

observed (26). Also in *Dictyostelium*, apoptosis is characterized by spherical surface protruberances and focal condensation of nuclei. Thus *Dictyostelium* seems to exhibit apoptosis, but has morphological traits of is own that characterize the phenomenon.

Growth and Culture Conditions: *D. discoideum* has two alternate life cycles: sexual and asexual. The asexual life cycle is easy to produce in the lab and is most often used for experimental studies. Free living *Dictyostelium* amoebae can easily be grown and maintained in the laboratory. The wild-type strains have doubling times of three hours and can be grown to very high densities, either on bacterial lawns and suspensions, or in axenic media that contain particulate nutrients. Axenic cultures that can be maintained in simple media and which have a generation time of 8 hours are also available (152). Cell lines can also be stored for long periods of time as spores or frozen amoebae.

Environmental parameters that affect the rate of development include cell density, light conditions, temperature and moisture present on the surface. The timing and stages of the 24-hour life cycle are reproducible within one hour limits, provided the parameters are held constant. The ability to initiate large numbers of cell into synchronous development allows biochemical analysis of cellular processes throughout development.

In *Dictyostelium*, the vegetative growth and the multicellular developmental phases of the life cycle are completely separated and can switch from one mode to the other. When cells are depleted of nutrients, growth ceases and development starts. But, the differentiation is completely reversible until the late stages of the program. Developing cells can "erase" their developmental markers and resume growth if nutrients are reintroduced (146). Thus developmental mutants, which are not lethal to the cells, can be propagated and maintained for further analyses.

Genetic and Biochemical Analyses: *Dictyostelium* has seven chromosomes and its genome is made up of 40,000kb (83). It has a relatively small DNA content as compared to that of

animal cells. Most biological phenomena in *Dictyostelium* are studied in haploid cells, although the cells do possess a true diploid phase. Diploids can be formed from opposite mating types as in yeast in the sexual life cycle. Parasexual diploids can also be formed by spontaneous cell fusion in a population of identical cells at a low frequency (99). Most whole cell genetic studies have involved the parasexual cycle, where the stable or metastable diploids that are formed can be destabilized, resulting in the random segregation of chromosomes which in turn lead to the production of haploids. Since the diploids that are formed are stable for a number of generations, the recessive and dominant characteristics of mutations can be examined.

There are a large number of tools available to study signal transduction in *Dictyostelium discoideum.* Cells can be easily transformed with exogenous DNA using either the calcium phosphate precipitation or electroporation (118). Transformants can be positively selected with several antibiotic resistance genes such as the neomycin phosphotransferase gene (*G418*^{r}) and the blasticidin deaminase (*Bsr*^{r}) gene. Auxotrophic markers such as *THY1* (encoding the thymidylate synthetase gene) and *PYR5-6* (encoding the gene necesssary to make uracil) are also available for selection (126). Integrating and nonintegrating vectors have been developed for use in incorporating exogenous DNA into the genome. Integrating vectors usually insert into the genome in tandem repeats of 10-300 copies. Endogenous plasmids also exist in *Dictyostelium*, which may aid in the construction of new vectors (31).

Homologous recombination can be used to alter or disrupt genes in order to create, isolate and analyze pertinent mutations. This is the consequence of its microbial style of growth and its capacity to keep the processes of growth and development independent of each other. Complementation studies can be used to shed considerable light on developmental genes (99). The antisense approach can be applied to *Dictyostelium* to mimic a mutation. Cells expressing the antisense construct can display phenotypes that are

correlated with the specific loss of the targeted gene product and can help identify the roles that a particular protein plays in development (137).

Dictyostelium has been used to express a number of different gene products from other cells with retention of functional properties. Mammalian gene products may be produced in large scale quantities because the *Dictyostelium* cells seem to have a mammalian–like glycosylation system. Membrane proteins that are difficult to express in bacteria and are not functional in yeast may be expressed in these cells. A wide array of reporter genes can also be used to tag developmentally regulated genes to study their spatial and temporal expression patterns (31). Various dyes that selectively stain particular cell-types are also available to look at spatial distribution of those cells in the multicellular aggregate during development.

Repressible promoters such as the *discoidin-1* promoter can be employed to study the effects of mutations on genes. The *Dictyostelium discoidin* genes are induced in bacteria-grown cells shortly before the onset of development, but are also expressed in high levels in the axenic media. Axenically grown cells strongly respond to the extracellular signal folate by suppressing discoidin gene synthesis. However, cell growth and development is not substantially affected. Removal of these signal molecules or setting up cells for development results in the rapid reactivation of the promoter. This provides a convenient system for the controlled expression of a gene of interest in growing cells and early development.

Temperature-sensitive *Dictyostelium* mutants can be generated and analyzed on *Klebsiella aerogenes* bacterial lawns at 22°C and 27°C. *Dictyostelium* is compatible to "random" insertional mutagenesis by restriction enzyme mediated integration (REMI), in which mutated genes can be tagged and retrieved (90). Because *Dictyostelum discoideum* wild-type cells are haploid, this feature can be used to examine recessive mutants that are perturbed in developmental functions using both biochemical and genetic analysis (58).

Cell Biology: The mechanisms of signal transduction in eukaryotic microorganisms appear to be similar to that in mammalian cells. *Dictyostelium*, due to its relatively short and simple life cycle, is especially well suited for studies of signal transduction, motility and chemotaxis, cell-cell communication, gene expression and pattern formation, all of which are processes involved in signal transduction pathways. Chimeric organisms created from mixed populations of cells are particularly helpful in characterizing mutants with deficiencies in intercellular signaling.

Chemotactic phenomena have important functions in inflammation, wound healing and metastasis in mammalian cells. *Dictyostelium* provides an excellent system to study this process because the chemotactic behaviour displayed by these amoeboid cells is very similar to that observed in mammalian phagocytic cells such as neutrophils and macrophages (33). The amoebae respond to folic acid, platelet activating factor (PAF), lysophosphatidic acid (LPA) and cAMP (73, 148). PAF, LPA and many chemokines elicit responses in leukocytes also (114). The spectra of biochemical reactions and cellular responses initiated by these attractants are very similar in both mammalian phagocytic cells and in *Dictyostelium*, although the two cell types are evolutionarily quite distant.

The ability to dissect pathways using a combination of biochemical, molecular and genetic approaches in *Dictyostelium* helps in coupling a wide range of mutants into defined biochemical pathways that regulate development in this system. The history of the analyses in yeast, *Drosophila* and *C. elegans* indicates that basic cellular decisions can be studied and understood by examining lower and less complex systems that can provide powerful tools. The relation of the signaling properties of individual cells to the behavior of aggregates of cells is more clearly understood in *Dictyostelium* than in other organisms. *Dictyostelium* is thus an ideal organism for studying how signaling pathways are integrated to control morphogenesis and gene regulation in development. Hence, *Dictyostelium* can be used as a model organism to study various processes in development.

Signal Transduction in *Dictyostelium*:

During *Dictyostelium* development, a large number of developmental processes like chemotaxis during aggregation, morphogenesis during multicellular differentiation and gene regulation during the life cycle are mediated through signal transduction pathways that initiate at G protein coupled cAMP receptors. Identifying the specificity of these signaling pathways and determining how extracellular signals act to induce cellular differentiation may significantly aid in the understanding of morphogenesis.

Extracellular cAMP is an important biomolecule in both the early and late stages of development. Its function in aggregation has been characterized extensively. The components of the signaling pathways during aggregation and also during later development include cell surface cAMP receptors, adenylyl cyclases, cAMP phosphodiesterases and a number of other proteins (55).

In *Dictyostelium*, many of the events which occur in development are controlled by seven-span serpentine receptors that help transduce external signals like cAMP through G protein dependent or independent pathways. Genes encoding four cAMP receptors (cARs) have been identified in *Dictyostelium*. The cAR1 and three other related cAMP receptors of *Dictyostelium* (cAR2, cAR3, cAR4) have a heptahelical structure that is characteristic of receptors coupling to G proteins (139). Each of the receptors seems to be coupled to similar signal transduction pathways, and can activate and adapt to adenylyl cyclase and chemotaxis. However, each gene is expressed at a different time in development and they also differ in their affinities to bind cAMP, in order to function appropriately in the environment in which they are expressed (82). Thus switching of receptor subtypes and retaining similar mechanisms of signal transduction and regulation, seems to be the method used by the cells to appropriately respond to different changes in the cellular environment (81). Recent published data indicates functional promiscuity of gene regulation by the different cAR receptors. The data suggests that in the absence of a particular *cAR* gene,

other cARs, which are expressed at the same stage or cell-type as the deleted *cAR* gene, are capable of taking over the function of the deleted *cAR* (167).

cAR1 mRNA is present mainly during aggregation, although two additional transcripts are induced at lower levels later on in development. The next *cAR* gene to be expressed is *cAR3*, which is maximally expressed at the mound stage and continues to be present at lower levels in the later stages. The *cAR2* transcript is found mainly in prestalk cells and is also present at the mound stage but is predominantly expressed at the slug stage. The last *cAR* to appear is *cAR4* , which is expressed during culmination (139, 140, 151). The temporal expression patterns of the *cAR*'s have been pictorally represented in Figure 6.

Vegetative *Dictyostelium* amoebae are chemotactically attracted by pteridine compounds such as folic acid (125). Upon starvation, the cells lose their sensitivity to folic acid and gain receptors to cAMP (29). There is evidence that suggests folates, other pteridines, and cAMP are detected by different types of cell surface receptors. There are two classes of folic acid receptors which can be activated differentially by different folic acid analogs. The formation of a gradient by the folic acid degrading enzyme, folate deaminase, is also responsible for chemotaxis of cells towards folic acid (5).

Like the mammalian and yeast receptors, cAR1 and the other cARs as well as folic acid receptors are possibly linked to heterotrimeric G proteins (47). At least eight *Gα* genes, each of which is expressed at a different time in development, and one gene that encodes the Gβ subunit have been identified in *Dictyostelium discoideum*. Recently, a Gγ subunit has also been identified. The amino acid sequences of the Gα subunits maintain the conserved GTP-binding and hydrolysis motifs and share a 30-50% identity with each other and with mammalian G protein α subunits (Appendix). However, the eight subunits do not fall into any obvious subtypes and do not bear a direct relation to the G protein classes found in mammalian cells (126).

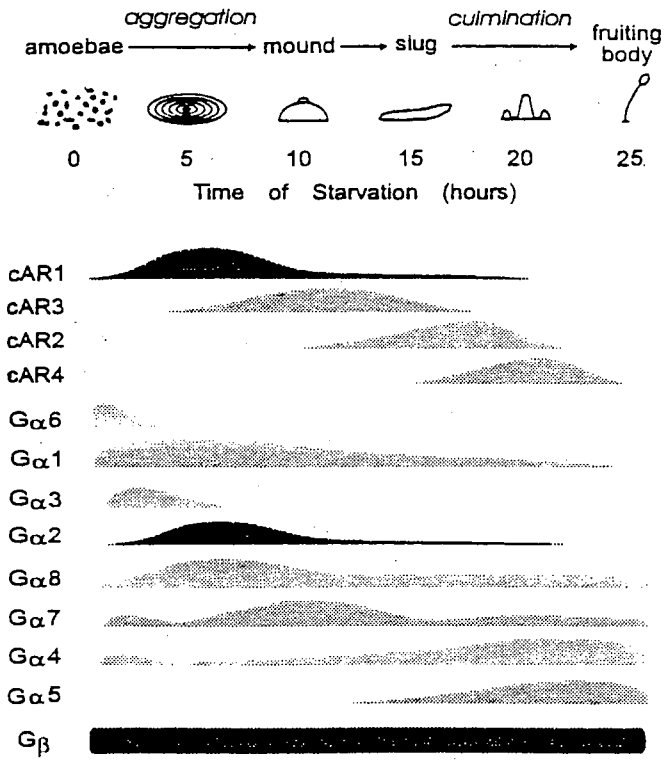

Figure 6. Temporal expression patterns of the cAMP receptors and Gα subunits identified in *Dictyostelium* with respect to developmental stages (top panel) (126)

Northern blot analysis indicates that each of these *Gα* genes is expressed at distinct times during the developmental life cycle (Figure 6). *Gα6* is mostly expressed in vegetative cells and upon starvation, i.e., at the onset of development, the levels of *Gα6* mRNA drops rapidly. *Gα8* is expressed similar to *Gα2* and this parallels the time course expression of *cAR1* during early development. The level of *Gα7* peaks in late aggregation and early mound stages and declines after that. *Gα1* is expressed throughout development with the transcript levels decreasing from the vegetative phase till culmination. A *gα1* null mutant resulted in no visible growth or developmental phenotypes. Similarly, *gα7* and *gα8* null mutants did not exhibit any defects in their growth rate or timing, nor did the mutants show any obvious defects in their morphological phenotypes during development as compared to wild-type cells. This suggested that some Gα subunits are functionally redundant or have very subtle effects. The question of redundancy between the subunits has not been addressed and it is necessary to construct cells that are deficient in multiple G protein α subunits to examine this possibility.

Analyses of null mutants created by gene disruptions suggest that four Gα subunits (Gα2, Gα3, Gα4 and Gα5) play an important role in development. *Gα3* mRNA is detected primarily in the growing and early aggregation stages. Recent reports indicate that Gα3 is required for proper development and that *gα3* null mutants are arrested at the mound stage after forming the tip (15). *Gα2* is expressed at very low levels in vegetative cells. At the onset of development, the levels of *Gα2* mRNA rise reaching a maximum at aggregation, followed by a steady decline. A second transcript of *Gα2* is preferentially expressed late in development in the anterior prestalk region. Previous analysis of the *Dictyostelium gα2* deletion mutants, including those resulting from gene disruptions, show that mutants that lack *gα2* do not aggregate and lack all known cyclic AMP receptor mediated responses such as chemotaxis and accumulation of secondary messengers (cAMP and cGMP) in response to extracellular cAMP(79, 86). This suggests that the Gα2 subunit is directly coupled to a cAMP receptor. In support of this hypothesis, disruption of the

cAMP receptor 1 (*cAR1*) gene has been observed to result in a phenotype almost identical to that of the *gα2* null mutant (151). The cAR1 and three other related cAMP receptors of *Dictyostelium* have a heptahelical structure that is characteristic of receptors coupling to G proteins (139). Gα2 seems to be important for many key transmembrane signaling processes. However, it is not required for responses to folic acid, and several cAMP stimulated responses do occur in its absence (123). This suggests that either other G proteins are mediating these functions or that the cAMP responses are due to G protein independent pathways via Ca^+ influx (112). The discovery of at least eight Gα subunits provides candidates that might fulfill these roles.

The functions of Gα2 were further characterized by analyzing the expression of Gα2 containing amino acid substitutions in the highly conserved G-3 region of the GTP-binding regions. The two mutations studied were Q208L and G40V. Analogous mutations in *ras* and mammalian $G\alpha_s$ substantially reduced the intrinsic GTPase activity of these proteins. Expression of Gα2 proteins carrying these mutations in wild-type cells resulted in an aggregation deficient phenotype, and the activation of downstream effectors such as phopholipase C and guanylyl cyclase was almost completely blocked. The activation of adenylyl cyclase was also substantially inhibited. The overexpression of an unmutated allele of *Gα2* results in a cAMP dependent activation of guanylyl cyclase and an inhibition of adenylyl cyclase activity (123). These results suggested a dominant negative phenotype associated with these mutations *in vivo*.

Gα4 and Gα5 are the closest related Gα subunits in *Dictyostelium* and have 51% sequence identity with each other. Both of these genes are primarily expressed during the multicellular stages of development and to a lesser degree during vegetative growth (61) (Figure 6). At any stage of development, each of these genes is expressed in an apparently undifferentiated subset (10-20%) of cells scattered throughout the multicellular organism (58). Whether each of these subsets of cells is identical, overlap or are distinct from one another has not yet been determined. They could therefore play a part in signal

transduction pathways associated with cell type differentiation or a number of morphological changes in the multicellular organism (61). Nevertheless, with the high degree of identity in amino acid sequence and similarity in temporal expression patterns, the phenotypes of the *gα4* and *gα5* null mutants implicate them in different developmental processes (58).

The developmental phenotypes of *gα4* null and *Gα4* overexpression (*Gα4*^{HC}) mutants suggest that Gα4 function is required for proper spore production (Figure 7). When starved, *gα4* null mutant cells aggregate to form multicellular mounds but then terminate in development as mounds with extremely extended rounded tips referred to as the extended finger morphology. This phenotype seems to result from a deficiency in the production of prespore cells and hence the *gα4* null cells do not produce any spores. In contrast to *gα4* null mutants, cells that contain a high copy number of the *Gα4* gene, *Gα4*^{HC}, are deficient in prestalk cell production as suggested by deficiencies in forming proper tips (prestalk cell zone) in aggregates (58). The terminal phenotype is an abnormal fruiting body-like structure, which shows a 25% reduction in the total number of spores produced. The *gα4* null cells can be complemented with a low copy number of the*Gα4* expression vector, and this restores the wild-type phenotype with normal spore production.

In addition to early spore development, the Gα4 subunit is absolutely essential for chemotaxis to folic acid, a mechanism by which the *Dictyostelium* cells seek out nearby bacterial food sources (125). Folic acid binds cell surface receptors and activates chemotactic movement as well as a very rapid accumulation of cAMP (~12 secs to reach a maximum level) and a slower accumulation of cAMP (~8 minutes to reach a maximum level) (29, 30). *gα4* null mutants are completely non-responsive to folic acid. In contrast, *Gα4*^{HC} mutants are supersensitive to folic acid (58). The coupling of folic acid receptors to G proteins had been predicted several years ago from studies that showed a decrease in receptor affinity by the presence of guanine nucleotides- a characteristic response of G

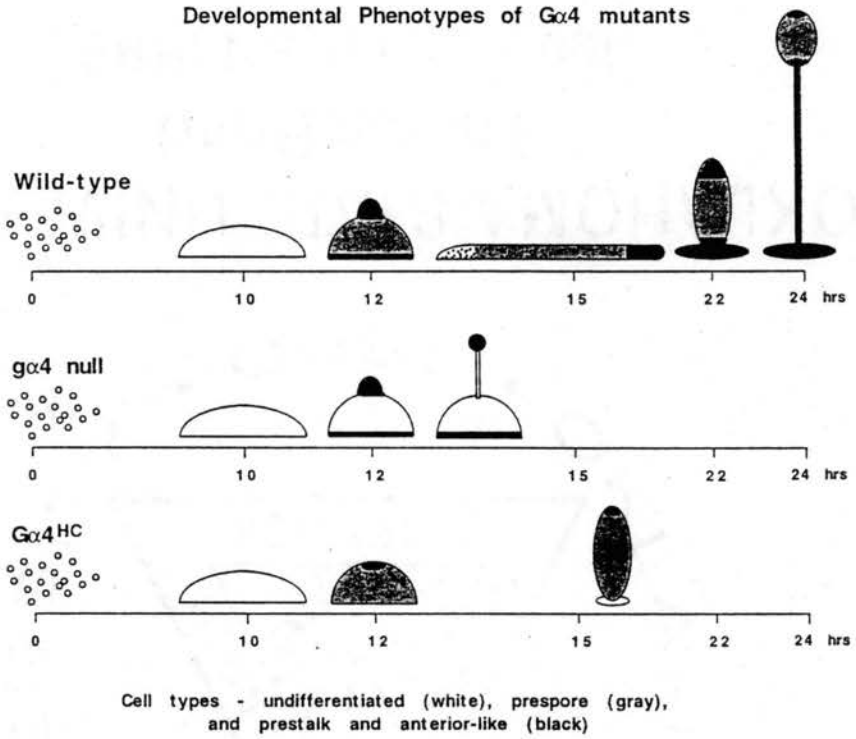

Figure 7. Developmental phenotypes associated with the Gα4 mutants and with respect to wild-type cells over time. The *gα4* null cells aggregate to form multicellular mounds but then terminate in the extended finger morphology. The $G\alpha4^{HC}$ cells form large mounds and have an abnormal terminal structure.

protein coupled receptors (29, 30). Gα4 function is required for prespore development and for all known responses to folic acid in *Dictyostelium discoideum* suggesting a possible role for folic acid as a developmental morphogen that activates the Gα4 mediated signal transduction pathway.

In contrast to the requirement of Gα4 function for prespore differentiation, the subunit encoded by the *Gα5* gene appears to play a pivotal role in the differentiation and/ or migration of prestalk cells during tip-formation (62)(Figure 8). *gα5* null mutants show a three hour delay in the formation of a tip on the multicellular mound which results in a delay of all subsequent developmental stages. They also form larger morphogenetic structures than the wild-type cells. The *Gα5* overexpression mutant forms a precocious tip approximately three hours earlier than the wild-type cells and their aggregates, slugs and spore masses tend to be smaller than the corresponding morphogenic structures in wild type cells. The correlation of Gα5 function and the temporal regulation of tip morphogenesis was also studied with respect to developmental gene expression. RNA blots indicated that the expression of the prespore specific gene *SP60*, and two distinct prestalk specific genes, *ecmA* and *ecmB* were delayed in the null mutant and precocious in the $G\alpha5^{HC}$ mutant compared to the wild type cells. Therefore, the Gα5 mediated signal transduction pathway may be affecting both tip morphogenesis and developmental gene expression by either promoting cellular differentiation or by indirectly promoting spatial segregation of prespore and prestalk cells (i.e., cell-type specific genes).

The Gα5 G protein also appears to be important for intercellular signaling. This conclusion has been drawn from chimera studies in which equal numbers of each of the Gα5 mutants and wild-type cells were mixed to form chimeras and were analyzed with respect to regulation of rate of tip morphogenesis. Aggregates containing equal portions of *gα5* null cells and wild-type cells formed tips at the rate expected for wild-type cells, which indicates that the cells expressing the *Gα5* gene exert a dominant role in determining the rate of tip

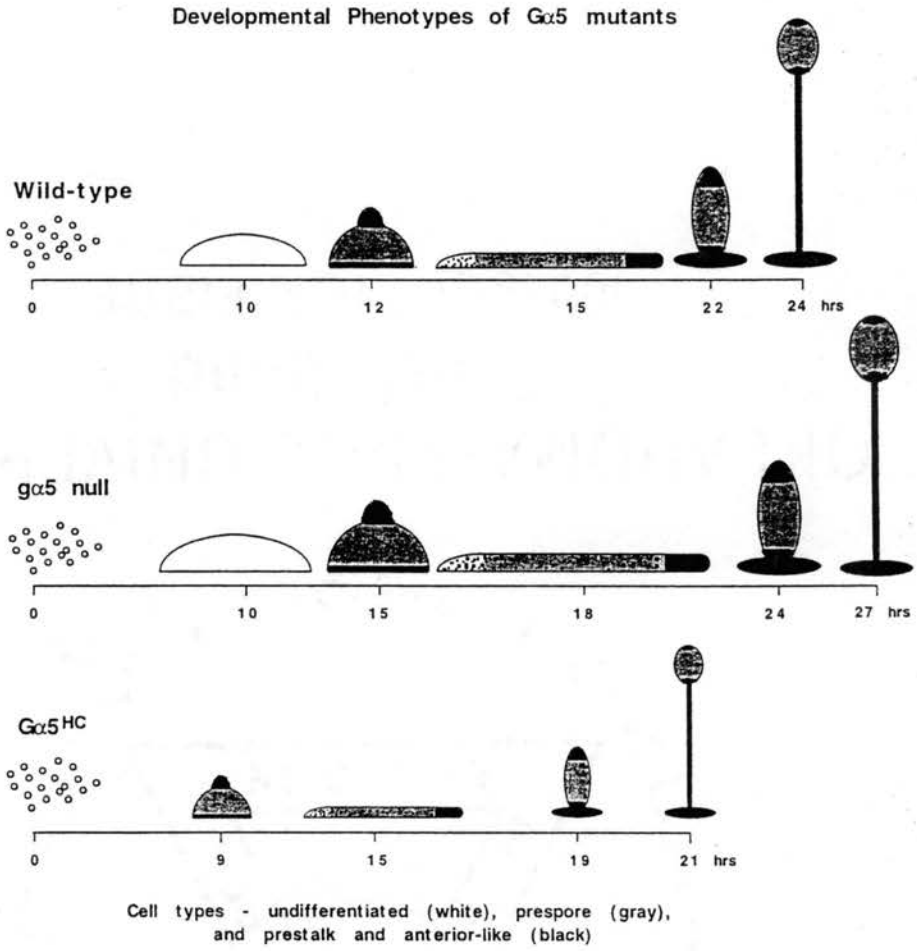

Figure 8. Developmental morphology of the Gα5 mutants as compared to wild-type cells with respect to time in development. The *gα5* null cells form larger mounds and are slower by about three hours in the process of tip formation than wild type cells, while the $G\alpha5^{HC}$ cells form smaller structures and precocious tips about three hours earlier than wild-type cells. Both the mutants can form fruiting bodies.

formation. Chimeric organisms formed using the $G\alpha5^{HC}$ cells indicated that the rate of tip formation affected by the *Gα5* gene is dependent on gene-dosage(62).

These studies indicated some sort of signaling between the two cell types in the multicellular organism that leads to the dominance of the cells expressing *Gα5*. However, studies have not been carried out to characterize the nature of this signaling and its effects on controlling the timing of cell-type specific gene expression. Another approach to analyzing Gα5 function would be to study specific mutations in conserved regions of the Gα protein that have been well characterized in other systems. Two such mutations are the substitution mutations of a conserved glutamine (Q) and a conserved glycine (G) residue in the G-3 region of Gα subunits. The substitution of glutamine with leucine has been associated with inhibition of GTPase activity and the glycine to threonine mutation has been associated with decreased G protein signaling due to a reduction in adenylyl cyclase activity in the systems analyzed.

Extracellular cAMP regulates tip formation (150). Tip-formation has been shown to be the result of prestalk cells moving up a cAMP gradient to reach the top of the mound (150). The loss of a cyclic AMP receptor cAR2 results in a severe delay in tip formation (140). Since mutational analysis indicates that the Gα5 subunit is required for tip formation as well (59, 150), this suggests that the Gα5 G protein might be coupled to a cAMP receptor and thus cAMP could provide the external signal that triggers the Gα5 mediated signal transduction pathway. Initial studies using cAMP as an external signal have been performed to determine whether the response of cells is dependent on or associated in any way with the Gα5 protein. Although the null mutants did chemotax towards cAMP, their response was slightly delayed relative to both the wild-type cells and the overexpression mutants, suggesting a possible association of the Gα5 protein with chemotaxis to cAMP. This phenotype has not been characterized and an activator of the Gα5 pathway has not yet been determined.

The *Dictyostelium* Gβ subunit has a high degree of homology to the β subunits found in humans, *Drosophila* and *C. elegans* (48, 51). With the exception of the first fifty amino acids in the very divergent amino terminus, the identity between the various β subunits is 70%. The β subunit is expressed at very high levels in growth and development (Figure 6). It has been proposed that it partakes in heterotrimer formation with each of the eight Gα subunits. The null mutants created by disrupting the *Gβ* gene indicate that the absence of the subunit does not have dramatic effects on growth of the *Dictyostelium* cells, although the cells do produce a smaller plaque on a lawn of *Klebsiella aerogenes*.

However, the subunit does play an important role in development. The Gβ null mutants, like the *gα2* null mutants, fail to aggregate under various conditions. This is because the null mutant fails to respond to cAMP and hence has been classified under the Frigid class of mutants (27). The absence of the Gβ subunit prevents adenylyl cyclase (AC) activation by GTPγS. This suggests that the βγ complex activates adenylyl cyclase (169).

As in mammalian cells, adenylyl cyclase activity is regulated by G proteins in *Dictyostelium*. Two adenylyl cyclase genes, ACA and ACG, which are topologically distinct cyclases, have been isolated in *Dictyostelium*. The two genes are expressed at specific stages in development and are regulated differently. ACA, which resembles the mammalian adenylyl cyclase, is expressed maximally during aggregation and is activated in response to a stimulated cAR1. Experiments have shown that ACA may not be a direct effector of the Gα2 G protein and also that activation of ACA requires a cytosolic protein (80, 155). *aca* null cells fail to aggregate, thus demonstrating that ACA is the adenylyl cyclase that coordinates aggregation. However, surprisingly, these cells indicate that ACA is not necessary for cell motility, chemotaxis, cell division or viability. ACG on the other hand, is expressed only during germination and is insensitive to guanine nucleotides.

The mechanism by which adenylyl cyclase is activated through cAMP binding to cell surface receptors at the aggregation stage is not well understood. However, it has been

shown that ACA activity is sensitive to extracellular cAMP levels (130). AC activity in wild-type cells, as measured by the concentration of intracellular cAMP in the cells, increases within the first two minutes and decreases to the pre-stimulus levels within ten minutes. Thus, ACA is both positively and negatively regulated by extracellular cAMP (130).

Of the G protein subunits that have been identified in *Dictyostelium*, at least two of the Gα subunits and the Gβ subunit are required for chemotaxis to cAMP and folic acid. Gα4 is needed for the cells to move towards folic acid, while Gα2 is essential for chemotactic responsiveness to cAMP. However, the Gβ subunit seems to be required for both these responses as well, which suggests that the Gβ subunit is capable of associating with either Gα subunit (169).

The Gα4 and Gα2 G proteins share a 46% identity to each other in terms of amino acid sequence, but Gα4 is even more closely related to Gα5. In addition to the a 51% identity in amino acid sequence between the Gα4 and Gα5 subunits, the genes encoding the two subunits have introns placed in identical positions, which suggests an evolutionary relationship between the two. Both genes are expressed throughout the vegetative phase and development, but are induced dramatically upon aggregation. The expression patterns of both the genes are very similar to each other. Their expression is most prominent in subpopulations of cells distributed throughout the multicellular organism, and is not similar to the spatial expression of prespore or prestalk cells. This suggests that both the genes are not cell-type specific in terms of spatial expression patterns and positional characteristics.

The similarity in the structure and expression of the *Gα4* and *Gα5* genes suggests that the corresponding subunits may play similar roles in development and possible interact with common regulatory components of signal transduction pathways. The difference in developmental roles of these subunits can possibly be attributed to differences in Gα subunit structure or to segregated expression of the Gα subunits. If these subunits are co-expressed in the same cell, the Gα subunits must not be fully functionally redundant or

interchangeable, based on the different phenotypes of the *gα4* and *gα5* null mutants. However, if the expression of the subunits is segregated, the Gα subunits could be functionally redundant. If the subunits interact with identical components, there might be significant cross-talk between the pathways. These issues on Gα subunit specificity have not yet been addressed.

Over the past several years, the number of identified G protein genes had increased steadily, but the number of each type of subunit remains unequal with the number of the Gα subunits being far greater than the number of Gβ or Gγ subunits. This disparity in gene number could be due to one of many factors. It could be a result of the degeneracy of Gβγ subunit interactions with the Gα subunit. There might be substantial diversity in Gβγ subunit structure such that unknown subunits might have gone unrecognized. Another possibility is that some Gα subunits may act independently of Gβγ subunits. Furthermore, the specificity of other G protein subunit interactions such as with receptors and downstream effectors remains to be fully characterized. A number of effector enzymes such as adenylyl and guanylyl cyclases, phosphodiesterases, phospholipases, protein kinases, ion channels etc., have been implicated in these pathways. Even for pathways that modulate the same second messenger, such as cGMP in photoreception and chemotactic movement, the directions of concentration change and kinetics can be quite different. It has been suggested that all three subunits stimulate adenylyl cyclases, but these cyclases appear to be different from each other. Hence one of the important parameters in establishing G protein functions is to define the specificity of the G protein associations.

When the stoichiometry of G protein subunits is altered due to the loss or over-expression of a particular subunit, an altered signal transduction pathway is observed. In *Dictyostelium*, the loss of a Gα or Gβ subunit involved in chemotactic pathways, results in a loss of signaling, while the overproduction of α subunits leads to increased signaling. However, in other systems such as yeast, the overexpression of the Gα subunit or the loss

of the Gβ or Gγ subunits, results in decreased signaling. This emphasizes the importance of G protein specificity.

The focus of the research outlined in this thesis is the Gα5 subunit and G protein and its similarity to the Gα4 subunit. All the intermediary players, (such as downstream effector molecules), inclusive of the initial signal stimulating a Gα5 associated signal transduction pathway have not been clearly defined. The experiments presented here aim at elucidating the role of the Gα5 G protein subunit, the external signal triggering the Gα5-signal transduction pathway and the functional specificity of the Gα5 subunit.

CHAPTER 3

ANALYSIS OF POTENTIAL ACTIVATORS OF THE Gα5 SIGNAL TRANSDUCTION PATHWAY

Introduction

To fully understand the regulation and function of the Gα5 signal transduction pathway, it is important to identify the external signals that activate this pathway. Both extracellular cAMP and the Gα5 G protein subunit seem to be important for the early developmental morphogenetic process of tip formation in the multicellular aggregate (62). Hence, cAMP was the most likely candidate to be tested as the external signal that activates the Gα5-mediated signal transduction pathway.

Extracellular cAMP has been shown to be important in regulating tip morphogenesis in studies where a directed migration of prestalk cells was observed in response to exogenously added cAMP (150). Consistent with this result, cells overexpressing phosphodiesterase do not form tips, probably due to the hydrolysis of extracellular cAMP. Marked prestalk cells also sort to the base of the aggregate when mounds are placed on agar containing high levels of cAMP (156). In addition to regulating tip formation, extracellular cAMP is the primary signal for aggregation during early stages of development and an important inductive signal for developmental gene expression in the multicellular mound (32, 141). These results imply that cAMP is an integral component of the signaling mechanisms required for this morphogenetic process.

Gα5 function is also required for proper tip formation (62). The delay in tip formation phenotype associated with the *gα5* null mutation is very similar to the cyclic

AMP receptor *cAR2* null mutant phenotype, in which cells lacking *cAR2* are developmentally blocked at the stage of tip formation (140). The experiments described in the first part of this section were designed to test the hypothesis that cAMP is the signal that triggers the Gα5-mediated signal transduction pathway.

Another Gα subunit in *Dictyostelium*, Gα2, is required for extracellular cAMP mediated aggregation in early development (86). The absence of the *Gα2* gene results in an aggregation-deficient phenotype and a loss of several cAMP receptor mediated functions such as activation of adenylyl and guanylyl cyclases (which leads to accumulation of secondary messengers cAMP and cGMP), chemotaxis and gene expression. Thus, cAMP might be an external signal that triggers more than one Gα mediated signal transduction pathway in *Dictyostelium*. To better define the role of Gα5 function in responses to cAMP, it is important to determine whether Gα5 is required for Gα2-independent responses to cAMP.

Chemotaxis and accumulation of secondary messengers (cAMP and cGMP) are two of the responses triggered by folic acid as an external signal in the Gα4-mediated signal transduction pathway (29, 30). As Gα4 and Gα5 are structurally related, it is likely that Gα5 when coupled to a cAMP receptor, might trigger similar cellular responses to the external signal such as second messenger (cAMP and cGMP) accumulation or chemotaxis.

The specific objectives of testing for cAMP as an external signal were as follows:

i) Perform chemotaxis assays and radioimmunoassays and compare the responses of *gα5* null cells, $G\alpha5^{HC}$ cells and wild-type cells.

ii) Create both a *gα2* null and a *gα2/gα5* double null *Dictyostelium* mutant to test whether Gα5 is required for Gα2-independent responses to cAMP.

ii) Perform chemotaxis assays with *gα2* null and *gα2 /gα5* double null mutants and compare their chemotactic response to cAMP.

iv) Perform radioimmunoassays with wild-type, *gα2* null and *gα2/gα5* double null strains and examine accumulation of secondary messengers in response to cAMP as an external signal.

Although cAMP is a likely external signal, other biomolecules like folic acid, lysophosphatidic acid (LPA) and platelet activating factor (PAF) have also been reported as external signals, some of which, activate G protein coupled receptors in *Dictyostelium* (73, 125, 148). The Gα5 mutants were also tested for responses to these biomolecules as potential activators of the Gα5 signal transduction pathway. The same assays described above were done using *gα5* null mutant cells (JH257), *Gα5* overexpression cells (JH258) and wild-type (KAx-3) cells using these biomolecules. In this section are described some of the experiments that were done to narrow the potential external signal activating the Gα5 signal transduction pathway.

Materials and Methods

Strains and Media

The following axenic haploid *Dictyostelium* strains were used: KAx-3 (wild-type) (161), the *gα5* null mutant strain JH257 (*gα5::THY1*) described in (62), the *Gα5* overexpression strain JH258 (contains a high copy number of the *Gα5* expression vector pJH206 ($G\alpha5^{HC}$) described in (62)). The auxotrophic strain JH8, constructed from the KAx-3 strain by site specific disruption of the *PYR5-6* gene has been described earlier (58). The JH10 strain which is a thymidine (*THY1*) auxotroph was also used (58). The *THY1* gene that disrupted the *Gα5* locus in JH257 was disrupted by a defective *PYR5-6* gene to create strain JH333. All strains that are used in the study are isogenic to the wild-type strain KAx-3, except at loci noted and all strains were grown at room temperature either in HL-5 medium or with *Klebsiella aerogenes* on SM nutrient agar plates, unless otherwise noted. All the cloning steps were carried out in *E.coli* strains using standard

recombinant DNA techniques and the DNA constructs thus obtained were transformed into *Dictyostelium* by electroporation as described by Dynes and Firtel (37). Clonal isolates were obtained by plating transformed cell lines in 24 or 96 well microtiter plates.

DNA Constructs and Mutant Production

Three versions of the disrupted *Gα2* gene were used. A disrupted *Gα2* gene was created by insertional mutagenesis of the *Gα2* cDNA (86) at the internal *BclI* site using a 1.5kb *Bam*HI fragment of the drug resistance marker, the Blasticidin gene (*Bsr*) (153) to give pKN11. Another version of the disrupted *Gα2* gene, pJH110, created by insertional mutagenesis of the *Gα2* cDNA by the *THY1* gene (37) at the *BclI* site was available in the lab (86). A third construct of the mutagenized *Gα2* gene was created using the *PYR5-6* gene (13) as a 3.7kb *Bam*HI fragment from pJH58 to disrupt the *Gα2* cDNA at the *BclI* locus to give pKN8. In pKN8 and pJH110, the disrupted *Gα2* cDNA was cut out of the cloning vector as an *Eco*RI fragment before being electroporated into the *Dictyostelium* cells. The disrupted *Gα2* gene was excised from the vector as a *KpnI/ Eco*RI fragment from pKN11 before being electroporated into *Dictyostelium* cells.

Wild-type KAx-3 and the *gα5* null mutant strain JH257 were grown in shaking cultures of HL-5 to mid-log phase ($2x10^6$ cells /ml). The two cell types were independently transformed with the *Bsr* disrupted *Gα2* construct pKN11, such that the disrupted *Gα2* gene inserted at the *Gα2* locus in the genome by homologous recombination. Potential transformants were selected based on their resistance to 10μg/ml of blasticidin and were plated in 24-well culture plates as clonal populations for further analysis. Similarly, pKN8 was digested with *Eco*RI and the fragment was electroporated into the uracil auxotrophic strains, JH8 and JH333. The *gα2/gα5* double null transformant selected from these *ura+* cells in the JH333 background was designated JH428. Also, the *Eco*RI digested fragment of pJH110 was transformed into the thymidine auxotroph JH10 to get the *gα2* null mutant JH425. Potential prototroph transformants in the latter two cases

were selected based on their ability to grow in the absence of uracil and thymidine respectively. The *Gα5* overexpression strain in the *gα2* null background, JH437, was created by introducing the *Gα5* expression vector pJH206 (62) in a high copy into the *gα2* null cells (JH110). Potential transformants were selected based on resistance to 10μg/ml of G418.

Potential transformants were washed in 12mM Na^+/K^+ phosphate buffer (pH 6.1) to remove nutrient medium. The washed cells were pelleted down and resuspended to a concentration of $2x10^7$ cell/ml in phosphate buffer. Each clone was then plated on non-nutrient Na^+/K^+ phosphate plates as well as on a lawn of *K. aerogenes* on SM plates. Solitary *Dictyostelium* amoebae feed on bacteria resulting in a clonal clearing or plaque on a bacterial lawn. As the plaque gets larger, the cells in the middle of the clone starve and undergo multicellular development. Developmental morphogenesis can then be viewed with the oldest aggregate closest to the middle of the plaque (46). A *gα2* null mutant was characterized by an aggregation deficient phenotype based on earlier studies (86). We thus looked for an aggregation deficient phenotype in the KAx-3, JH8 and JH10 transformants, which served as a control. The aggregation deficient phenotype was the most easily detectable phenotype in the double mutants as well and thus aggregation deficient transformants were selected in the JH257 and JH333 backgrounds also.

DNA and RNA Blots

Correct incorporation of the mutant alleles into the genome of the potential transformants in KAx-3, JH10, JH8, JH333 and JH257 was determined and verified by Southern blot analysis as described by Southern (149). Genomic DNA was isolated from cells as described by Nellen *et al* (118), digested with appropriate restriction enzymes and blotted to nitrocellulose membrane. The 1.2kb *Gα2* cDNA *Eco*RI fragment from JH105 (86) was radiolabeled and used for hybridization. A 1.5kb *Eco*RV fragment of the *Gα5* open reading frame from pJH214 was used for radiolabeling and the blots were probed to

verify incorporation of a high copy number of the *Gα5* gene in the *gα2* null background. DNA probes were generated by random primer probe synthesis using the protocol as described by Vogelstein (44) according to the manufacturer's instructions (Boehreninger Manheim). The clones whose DNA showed predicted altered hybridization patterns (that have a disrupted *Gα2* locus) were then picked and additional analyses was done with them.

Chemotaxis Assays

Cells that had grown to a mid-log phase in a shaking culture were washed and resuspended in phosphate buffer to a concentration of $2x10^7$ cells/ml. Cells were then starved on non-nutrient agar plates and induced to undergo development. Chemotactic responsiveness of the cells to cAMP was monitored at different stages of multicellular development (from six to fourteen hours after the onset of starvation). For analyzing chemotactic responses at different times in development, cells were plated at a density of $2x10^7$ cells/ml, harvested at specific times, washed in buffer, and resuspended at a concentration of $1x10^8$ cells/ml. Varying concentrations of cAMP (10μM-1mM), were spotted onto non-nutrient agar plates and the cells, at a concentration of $1x10^8$ cells/ml, were spotted ~ 2mm away from the chemoattractant droplet. The plates were incubated at room temperature and observed after 3 hours. Chemotaxis assays with folic acid as the chemoattractant were done in exactly the same way using 1mM folic acid droplets. Folic acid assays were done with vegetatively growing cells (0 hours post-starvation). The plates were incubated in the dark at room temperature and observed after three hours.

Although the Gα2 subunit is required for aggregation, under normal conditions, this requirement can be bypassed by pulsing starved cells (growing in Na^+/K^+ phosphate buffer in slow shaking cultures) with cAMP and then plating for development. For looking at responses of the *gα2 and gα2/gα5* double null mutants later on in development, cells were grown to mid-log phase in shaking cultures of HL-5 harvested and diluted out to concentrations of $1x10^6$ cells/ml in non-nutrient phosphate buffer. The cells were then

shaken at 90-100rpm at room temperature (~25°C) with plenty of aeration for six hours and then for an additional two-six hours with addition of 100μM cAMP every two hours. Presumably, the adhesion of cells in the slow shaking culture will allow aggregates to form independent of the normal aggregation process (141).

Radioimmunoassays (RIA) and Protein Assays

Cells were grown vegetatively in a shaking culture to a density of $3x10^6$ cells /ml. Cells were then harvested, washed and resuspended to a density of $1x10^8$ cells/ml in phosphate buffer. The cells were plated on non-nutrient plates until the desired time in development. The assays were done between six and fourteen hours of development when the expression of *Gα5* is the highest in wild-type cells. Cells were harvested and resuspended in phosphate buffer at the appropriate time at a concentration of $5x10^7$ cells/ml. Suspensions of intact multicellular structures were aerated for ten minutes and were then broken up by pipeting such that all cells in the multicellular aggregate received equal exposure to the external signal.

For responses to cAMP as an external signal, the cell suspension was stimulated with a 100μM concentration of cAMP at time zero. The cAMP analog, 2'-deoxy cAMP, was used in place of cAMP in the cAMP RIA, to help distinguish between exogenous and endogenous (secondary messenger) cAMP. However, cAMP was used to trigger the accumulation of cGMP as a secondary messenger in the cGMP RIA. At desired times following the stimulation (ranging from zero to eight minutes for a cAMP RIA and zero to forty five seconds for a cGMP RIA), constant volumes of aliquots from the mixture, were withdrawn and treated immediately with 3.5% perchloric acid (PCA) on ice. This extinguished activity at that point in the stimulated cells. The samples were then neutralized with 50% saturated $KHCO_3$ and the precipitate was discarded. The amount of acid soluble cAMP and cGMP was determined by means of cAMP and cGMP RIA kits (Amersham Corp.). All the assays were performed on duplicate samples. An aliquot that was first

treated with PCA and then had known amounts of either cAMP or 2' cAMP added served as a control to account for any background levels of cAMP and cGMP in the cell suspensions. To test other biomolecules as activators of the Gα5 signal transduction pathway, the following concentrations were used as mentioned in literature: PAF 10μM (148), LPA 100μM (73). Lysophosphatidic acid (LPA) is a hydrophobic molecule and 50mM BSA was used to solubilize the biomolecule before it was used as an external signal at the given concentration. Previous studies had indicated that Platelet Activating Factor (PAF) acts along with cAMP to enhance cAMP responses in *Dictyostelium* cells. Hence the stimulant PAF solution contained a mixture of 10μM PAF with 50nM cAMP. For the folic acid assays, a final concentration of 30μM folic acid with 0.33mM 5-azaguanidine solution was used as a stimulant in the radioimmunoassays. Cell extracts that were used for stimulation with the external signal were also assayed for their protein concentration to determine the concentration of the secondary messenger accumulated per mg protein. The standard dye-binding assay was used as described (14).

Results

Analysis of cAMP Responses

Analysis of cAMP Responses of Gα5 Mutants with Respect to Wild-Type Cells

The chemotactic ability of wild-type cells, *gα5* null and $G\alpha5^{HC}$ cells to varying concentrations of cAMP was studied. The results have been tabulated in Table 2. Gradient assays were performed by spotting cells about 2mm away from the chemoattractant droplet on non-nutrient phosphate plates. At cAMP concentrations of 10μM-100μM, all three cell types chemotaxed towards the chemoattractant within three hours at all the stages of development tested (6, 9 and 12 hours of development). The *gα5* cells seemed to chemotax a bit slower than wild-type cells, although the difference was marginal. The

TABLE 2

Chemotaxis of Gα5 mutants to cAMP

Cell Type	6hrs			9hrs			12hrs		
	10μM	100μM	1mM	10μM	100μM	1mM	10μM	100μM	1mM
KAx3 (Wild-Type)	++	+++	-	+	++	-	+	+	-
JH257 (*gα5* cells)	++	+++	-	+	++	-	+	+	-
JH258 (*Gα5^{HC}* cells)	++	+++	-	+	++	-	+	+	-

+, ++, and +++ indicate increasing positive chemotaxis towards the chemoattractant
- indicates negative chemotaxis away from the chemoattractant

Chemotactic ability of wild-type (WT) cells, and the Gα5 mutants to varying concentrations of cAMP at various times after starvation. Cells were grown in shaking culture to a density of $2x10^6$ cells/ml, washed twice in 12mM phosphate buffer, resuspended at a concentration of $1x10^8$ cells/ml and plated on non-nutrient phosphate plates for development. At the desired time in development, cells were washed off the plate and resuspended thoroughly in phosphate buffer, so as to break apart as many multicellular structures as possible, at a cell density of $5x10^7$ cells/ml. A 1μl droplet of cells was spotted about 2mm way from 1μl of varying concentrations of cAMP. Chemotaxis was monitored for about three hours and was measured qualitatively after three hours.

Gα5^{HC} cells seemed to chemotax at the same rate as the wild-type cells. The higher concentrations of cAMP seemed to disrupt the chemotactic ability of all three cell types. Cells seemed to be moving away from the cAMP droplet rather than toward it at cAMP concentrations of 1mM.

The three cell types were then tested for accumulation of secondary messengers, cAMP and cGMP, in response to cAMP as the external signal. As 10-100μM seemed to be the optimum concentration of cAMP that cells chemotactically responded to, the radioimmunoassays were done using a concentration of a 100μM cAMP. Cells were tested at ten hours of development when *Gα5* expression is at its highest. Wild-type cells had the best response to cAMP as the external signal as these cells had the highest accumulation of both intracellular secondary messengers, cAMP and cGMP. Although *Gα5*^{HC} cells had a lower response to extracellular cAMP than wild-type cells, they had a higher accumulation of intracellular cAMP and cGMP than *gα5* null cells (Figures 9 and 10). These results suggested that altering the stoichiometry of the Gα5 subunit altered intracellular secondary messenger accumulation in the Gα5 mutant cells. This indicated that although Gα5 may play a role in cAMP responses in a cell, the results may be obscured due to the presence of other Gα subunits that also play a role in cAMP responses in the cells. One other Gα subunit that is known to be essential for cAMP responses is the Gα2 subunit. Hence, to determine the role of Gα5 in cAMP response, it was essential to look at Gα2-independent responses of Gα5 to cAMP.

Analysis of cAMP Responses in *gα2* Null Versus *gα2 /gα5* Double Null Mutants Versus the *gα2 null/Gα5*^{HC} Cells

The ability to create specific mutations using homologous recombination allows for the analysis of mutant protein function in the absence of the wild-type protein in *Dictyostelium discoideum*. Three versions of the *gα2* null mutants were created as described in materials and methods (Figure 11). The *gα2* null mutant JH425, the *gα2 /gα5*

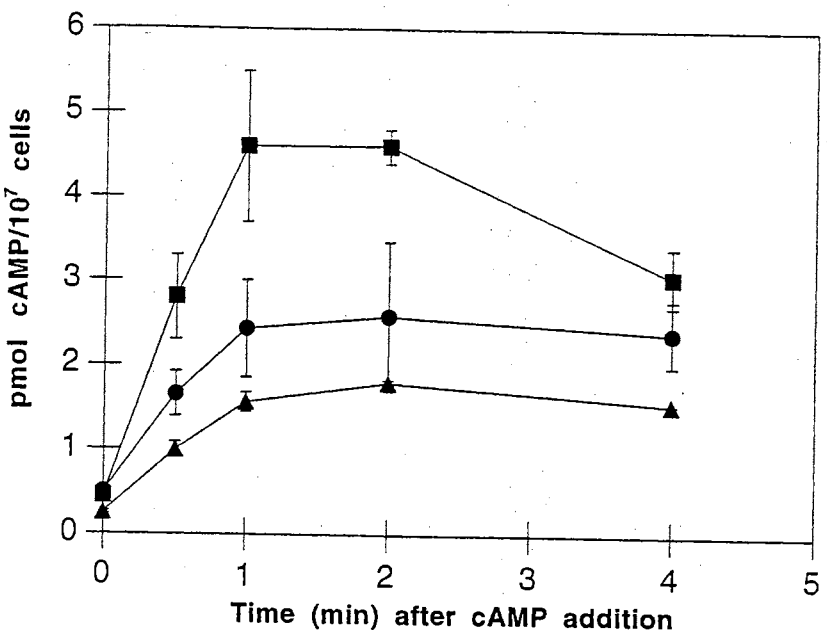

Figure 9. Accumulation of secondary messenger cAMP per 10^7 cells in wild-type cells (closed squares), *gα5* cells (closed triangles) and $G\alpha5^{HC}$ cells (closed circles) in response to dcAMP. Cells were grown in shaking culture to a density of $3x10^6$ cells/ml, harvested and washed in 12mM phosphate buffer. They were then resuspended in buffer and plated on non-nutrient phosphate agar plates at a density of $1x10^8$ cells/ml. At 10 hours post-starvation, the cells were washed off the plate, resuspended in buffer at a concentration of $5x10^7$ cells/ml, and kept aerated by bubbling to break apart the structures. The cells were stimulated with 100μM dcAMP. Extracts of the stimulated suspension were harvested at different times and enzyme reactions were stopped by the addition of perchloric acid, which was subsequently neutralized. The precipitate was discarded and the supernatant was used to determine levels of cAMP at that time point. Each sample was tested in duplicate. Radioimmunoassays were done using the cAMP kit (Amersham) as described in materials and methods. The vertical error bars represent deviations from the mean.

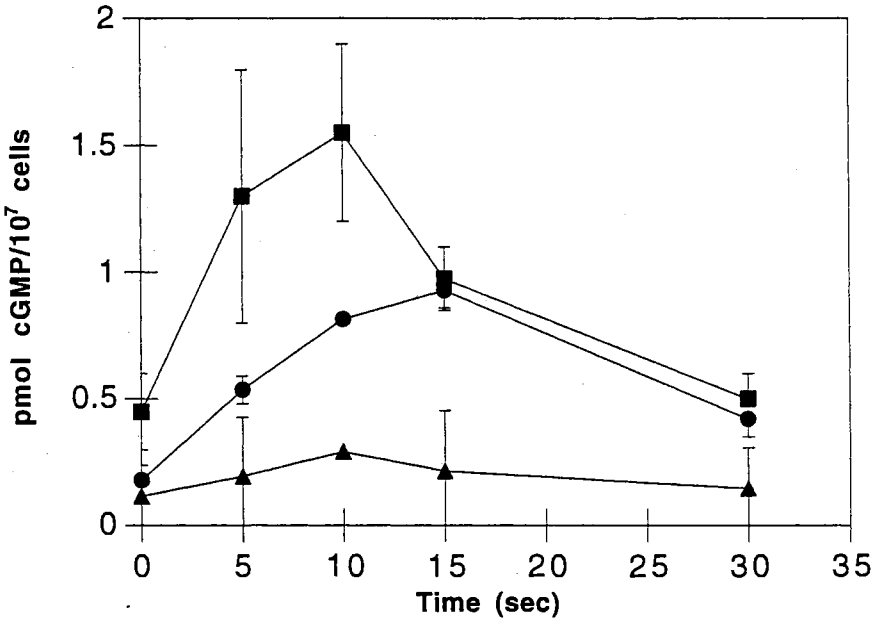

Figure 10. Accumulation of secondary messenger cGMP per 10^7 cells in wild-type cells (closed squares), *gα5* null cells (closed triangles) and $G\alpha5^{HC}$ cells (closed circles) in response to cAMP. The cells were stimulated with 100μM cAMP. Cell extracts were collected as decribed in figure 9 and used to determine levels of cGMP at each time point. Each sample was tested in duplicate. Radioimmunoassays were done using the cGMP kit (Amersham) as described in materials and methods. The vertical error bars represent deviations from the mean.

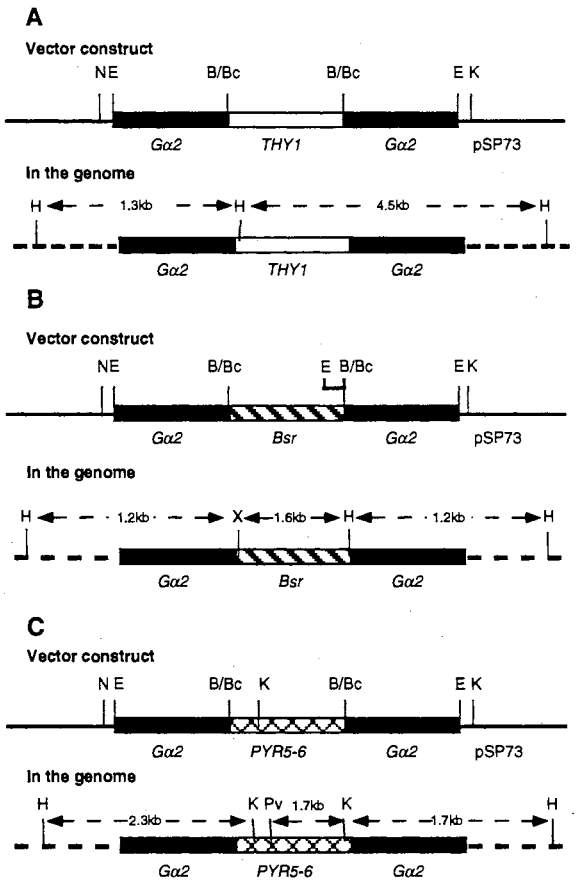

Figure 11. Constructs carrying the disrupted of the *Gα2* gene that were used in the analyses. The creation of these constructs is described in detail in the Materials and Methods section. A unique *BclI* site internal to the *Gα2* cDNA on the pSP73 vector was used in all the constructs to disrupt the *Gα2* gene. (A) *gα2::THY1* (B) *gα2::Bsr*^{r} (C) *gα2::PYR5-6*. Restriction enzyme sites indicated are *Bam*HI (B), *Bcl*I (Bc), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Nde*I (N), *Pvu*II (Pv) and *Xba*I (X). The first part of panels A, B and C indicate the plasmid vectors constructed bearing the *Gα2* gene disruptions. The second part of each of the panels indicates the restriction sites used and sizes of fragments expected for a southern blot analysis of genomic DNA of *Dictyostelium* cells carrying these disrupted *Gα2* genes.

double mutant strains JH428 and JH444, and the $G\alpha5^{HC}$ in the *gα2* null strain JH437 were used for the analyses.

A previous strain of a *gα2* null mutant was available in the lab as a frozen amoeba stock. However, this culture could not be revived and thus we had to create another *gα2* null mutant strain. As pJH110, the construct carrying the *THY1* disrupted version of the *Gα2* was readily available, it was used to recreate the *gα2* null mutant strain, JH425 (*gα2::THY1*). However, the *THY1* gene had been already used to disrupt the *Gα5* locus in the *gα5* null mutant strain JH257. Hence, pJH110 could not be used to create the *gα2/ gα5* double null strain in the JH257 background as the *THY1* could not be as a selectable marker in a strain that is already a thymidine prototroph. Hence, the construct carrying the *Gα2* gene disrupted by the *PYR5-6* gene (*gα2::PYR5-6*) was used to create a *gα2/ gα5* double null strain(JH428) in a *gα5* null mutant uracil auxotroph (JH333), so that it could be selected for with an auxotrophic marker. The construct carrying the *Gα2* gene disrupted by the *Bsr* gene (pKN11) was also used to create the *gα2/ gα5* double null strain in JH257 (*gα5* null background), due to an ease in selection for a low copy drug resistance gene. Attempts to create a *gα2* null strain using pKN8 and pKN11 in a wild-type background were unsuccessful.

The incorporation of the disrupted *Gα2* gene in a number of aggregation deficient transformants in the various background strains was confirmed by southern blot analysis (Figure 12). Genomic DNA from the various strains was digested using restriction enzymes. A *Hin*dIII digest of genomic DNA from JH10 cells on the southern blot indicates a single 2.6kb band corresponding to the intact *Gα2* gene as compared to two bands, 1.3kb and 4.5kb in size in JH425. This indicates the presence of a single 3kb *THY1* gene inserted at the *Gα2* locus in JH425. The *Gα2* cDNA probe recognized two bands (2.3kb and 1.7kb) in the DNA of JH428 that had been digested with *Hin*dIII and *Kpn*I as compared to just one 2.6kb band in the *gα5* null strain JH333. Also, the *Hin*dIII/*Pvu*II digest of the same DNA yielded two bands of 3kb and 3.4kb as compared to one 2.6kb

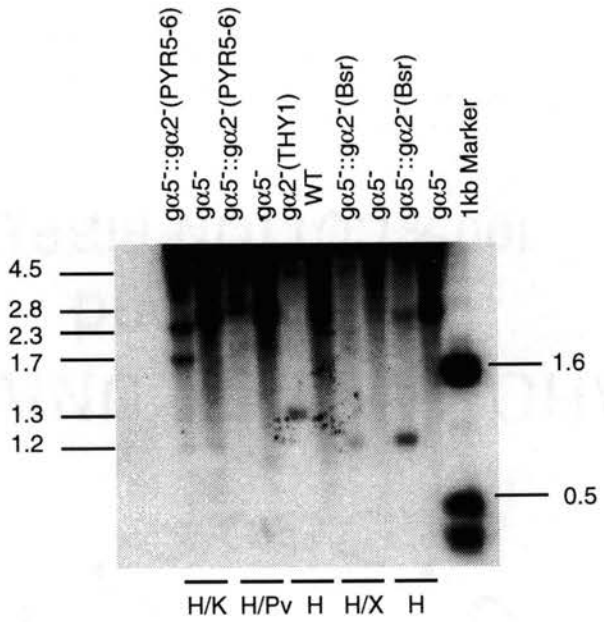

Figure 12. Southern blot of the *gα2* null and the *gα2/gα5* double null mutants. Restriction enzyme digests were done on genomic DNA isolated from the cells as specified and a 1.2kb *Eco*RI *Gα2* cDNA fragment was used as the probe. Details of the southern blot analysis are described in the Materials and Methods section. Restriction enzymes indicated are *Hin*dIII (H), *Kpn*I (K), *Pvu*II (Pv) and *Xba*I (X). The *Gα2* probe hybridizes a 2.6kb *Hin*dIII band in wild-type cells and *gα5* null cells. The sizes associated for the various enzyme digests with the disrupted versions of *Gα2* are shown in Figure 11.

band in JH333, indicating the presence of a single 3.7kb *PYR5-6* insert disrupting the *Gα2* locus. In the case of JH444, the genomic DNA was digested independently with *Hin*dIII/ *Xba*I and *Hin*dIII and probed with the *Gα2* cDNA probe. These digests yielded two 1.2kb bands in the earlier case, and a 2.8kb and 1.2kb bands in the latter, as compared to JH257 which showed a 2.6kb band with each of the two digests. This indicated the presence of a 1.5kb *Bsr* gene disrupting the *Gα2* locus in the genome.

The presence of multiple copies of the *Gα5* gene in JH425 was also confirmed by doing a southern blot (Figure 13). In this case, several transformants that were resistant to 10μg/ml of G418 were picked and a southern blot analysis was done with them. Genomic DNA from wild-type cells served as a control. The genomic DNA was digested with *Eco*RV and a Gα5 probe was used to probe the DNA. Transformants that showed the highest copies of an 8kb fragment as compared to a single 2.5kb fragment were picked for further analyses.

Phenotypically, the *gα2/gα5* double null mutant was the same as the *gα2* null mutant – aggregation deficient. Also, the overexpression of the *Gα5* gene in the *gα2* null cells could not compensate for the aggregation deficient phenotype of these cells. The *gα2* null, the *gα2/gα5* double null, the *gα2* null/$G\alpha5^{HC}$ and wild-type cells were compared for chemotaxis under gradient conditions both at 0 hours and after different lengths of starvation. To simulate development in the aggregation deficient cell types, cells were allowed to randomly aggregate and form clumps in slow shaking culture with exogenous addition of cAMP as described in the Materials and Methods section. Wild-type cells treated in exactly the same manner, served as a control. The clumps of cells were then washed multiple times to remove exogenous cAMP and then plated as described earlier for chemotaxis assays. All three cell types formed aggregates which when plated on non-nutrient plates developed into fruiting bodies. If cAMP triggers the Gα5 signal transduction pathway and also triggers cellular responses like chemotaxis, we should

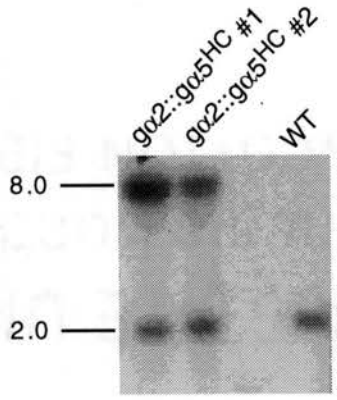

Figure 13. Southern blot of the *gα2 null::Gα5^{HC}* strain to show insertion of a high copy number of the *Gα5* gene. Genomic DNA was digested with *Eco*RV and was picked and a southern blot analysis was done with them. Genomic DNA from wild-type cells served as southern blot analysis was done as described earlier. A 1.5kb *Eco*RI/*Eco*RV fragment carrying the *Gα5* open reading frame was used to make the *Gα5* probe. The *gα2* null cells show a wild-type *Gα5* locus as seen by the 2kb *Eco*RV band that hybridizes to the *Gα5* probe. The 8kb hybridized band seen in the *gα2/ Gα5^{HC}* strains is due to the insertion of the entire *Gα5* expression vector pJH206 in tandem in multiple copies.

observe decreased or no chemotaxis in the *gα2/gα5* double null mutant as compared to the *gα2* null mutant alone. The wild-type cells, KAx-3, served as a positive control for maximum chemotactic responsiveness. Wild-type cells showed the same chemotaxis, as observed earlier, at three hours after spotting the cells next to the chemoattractant. *gα2* null cells were devoid of any chemotactic response to 100μM cAMP at all stages observed. The *gα2/gα5* double null cells and the *gα2* null/$G\alpha5^{HC}$ cells also did not show any chemotaxis to cAMP, indicating that alteration of Gα5 in *gα2* null cells did not in any way affect the chemotactic ability of these cells.

Due to the difficulty in quantifying chemotaxis, radioimmunoassays were done with these cell types as a quantitative assay. When an extracellular signal initiates a signal transduction pathway transduced via a G protein, one of the first intracellular responses is an increase in the level of secondary messengers like cAMP and cGMP over the activation cycle period. Radioimmunoassays were used to monitor the accumulation of these intracellular secondary messengers. The increase in the level of intracellular cAMP in the cells was monitored over a period of eight minutes, while the intracellular cGMP accumulation in response to cAMP was monitored over 45 seconds as described earlier (86).

The levels of cAMP and cGMP were assayed in both *gα2* null and *gα2/gα5* double null mutants and compared to levels induced by cAMP or 2' cAMP in wild-type cells. Initially, the assays were done using *gα2* null cells and *gα2/gα5* null cells to determine if there was any difference in accumulation of secondary messengers between the two strains. However the levels of intracellular cAMP and cGMP were very low and at basal levels for both these strains at six and ten hours post-starvation. Hence, we decided to look at differences in secondary messenger accumulation in the *gα2* null strain as compared to the *gα2* null/$G\alpha5^{HC}$ strain to see if an overexpression of *Gα5* could rescue the ability of the *gα2* null strain to respond to cAMP. The response of the wild-type cells to cAMP was much higher than either of the two mutants. The accumulation of intracellular cAMP in

gα2 null/$G\alpha5^{HC}$ strain was comparable to that in the gα2 null strain, although the concentration of cAMP values were still at basal levels in both mutant strains. The cGMP accumulation in both the mutant strains in response to external cAMP at the different stages in development was negligible also as compared to the wild-type responses.

Analysis of Responses to Lysophosphatidic Acid, Platelet Activating Factor and Folic Acid

Although cAMP is a likely external signal, other biomolecules like lysophosphatidic acid (LPA) and platelet activating factor (PAF) have also been reported as possible external signals activating G protein coupled receptors in *Dictyostelium* (73, 125, 148). However, the reported responses to these biomolecules in *Dictyostelium* have been subtle responses such as changes in cell shape. We tried to monitor this response in wild-type cells with LPA and PAF using light microscopy, but were unable to observe the slight changes that had been reported earlier. As both these biomolecules did not seem to evoke distinct cellular responses such as chemotaxis, we decided to do radioimmunoassays to determine if there was any effect on accumulation of secondary messenger concentrations in the cells. This assay was done with the wild-type cells, *gα5* null cells and $G\alpha5^{HC}$ cells at six hours post-starvation for PAF responses and at ten hours in development for LPA responses. PAF has been shown to increase cAMP responses by increasing the activation of guanylate cyclase in *Dictyostelium* as well as in other sytems (148). Both the *gα5* null cells and the $G\alpha5^{HC}$ cells seemed to have no accumulation of cGMP in response to the PAF stimulant mixture as compared to the response seen in the wild-type cells (Figure 14). In the case of the lysophosphatidic acid, however, there seemed to be a higher accumulation of intracellular cAMP in the $G\alpha5^{HC}$ cells as compared to the wild-type and *gα5* null cells (Figure 15). In terms of cGMP accumulation, there was no response in any of the cell types in response to LPA (Figure 16). This may be expected as there was no distinct chemotaxis that had been observed with the wild-type cells in response to LPA. This

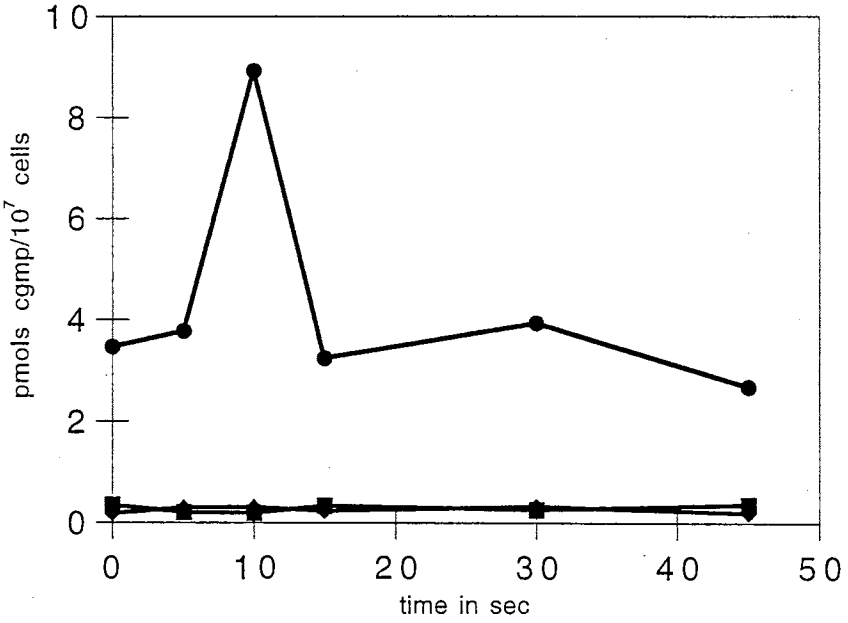

Figure 14. Accumulation of secondary messenger cGMP per 10^7 cells in wild-type cells (closed circles), *gα5* null cells (closed squares) and $G\alpha5^{HC}$(closed triangles) cells in response to 10μM PAF with 50nM cAMP. Cell extracts were collected as described in figure 9 at 6 hours post-starvation, and they were used to determine levels of cGMP at each time point. Each sample was tested in duplicate. Radioimmunoassays were done using the cAMP kit (Amersham) as described in materials and methods.

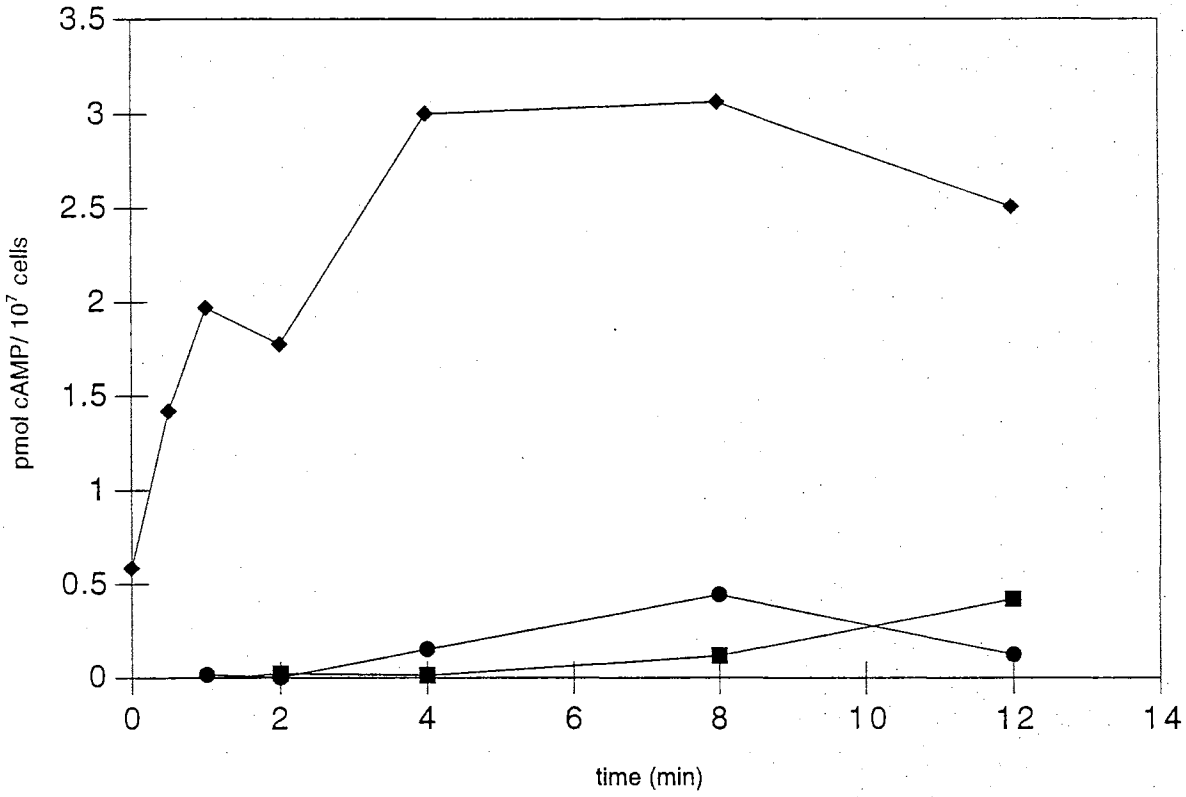

Figure 15. Accumulation of secondary messenger cAMP per 10^7 cells in wild-type cells (closed diamonds), *gα5* null cells (closed squares) and $G\alpha5^{HC}$ (closed circles) cells in response LPA. The cells were stimulated with 100μM LPA. Cell extracts were collected as described in figure 9 at 10 hours post-starvation and they were used to determine levels of cAMP. Each sample was tested in duplicate. Radioimmunoassays were done using the cAMP kit (Amersham) as described in materials and methods.

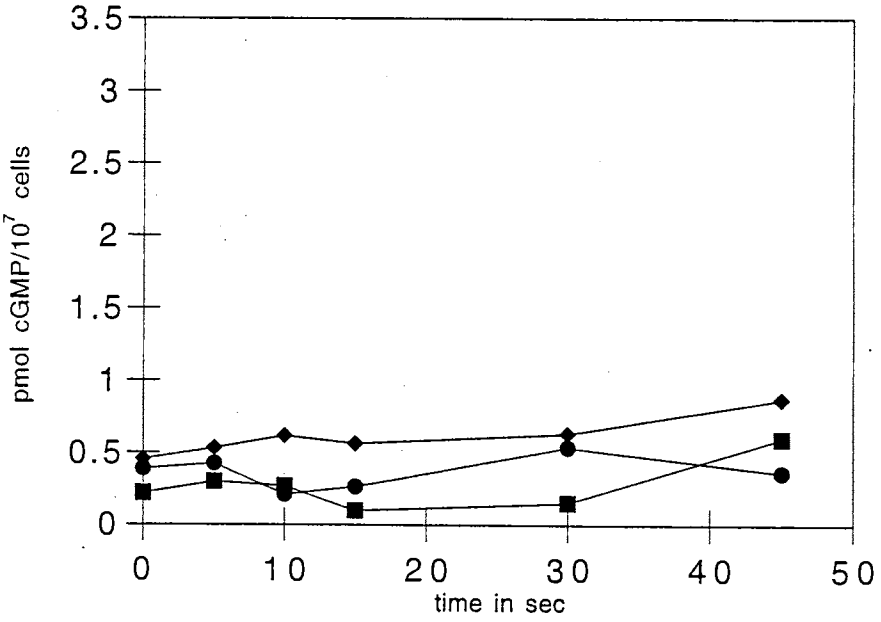

Figure 16. Accumulation of secondary messenger cGMP per 10^7 cells in wild-type cells (closed diamonds), *gα5* null cells (closed squares) and $G\alpha5^{HC}$ cells (closed circles) in response LPA. The cells were stimulated with 100μM LPA. Cell extracts were collected as described in figure 9 at 10 hours post-starvation and they were used to determine levels of cGMP. Each sample was tested in duplicate. Radioimmunoassays were done using the cGMP kit (Amersham) as described in materials and methods.

indicated that LPA or an LPA like molecule may be the external signal triggering the Gα5 signal transduction pathway, but may be doing so at the stage of tip formation in development.

Folic acid stimulates the Gα4 signal transduction pathway. Given that Gα4 and Gα5 are structurally similar, it is possible that folic acid may be the external signal triggering the Gα5 mediated signal transduction pathway (59). Chemotaxis assays done with the *gα5* null cells, wild-type cells and $G\alpha5^{HC}$ cells indicated that the $G\alpha5^{HC}$ cells were delayed in responding to the folic acid droplet, as compared to the wild-type cells and the *gα5* null cells during the first few hours of starvation (Figure 17). This suggested that the increased *Gα5* expression was interfering with the response to folic acid. After this initial delay in chemotactic responsiveness, $G\alpha5^{HC}$ cells display chemotactic movement, but the movement is very different from wild-type cells. The $G\alpha5^{HC}$ cells are more evenly distributed as opposed to the halo phenotype seen in the wild-type cells, where the cell concentration is higher in the leading edge of the chemotaxing cells. The three cell types were also tested for cGMP accumulation as cGMP is thought to be an important second messenger in *Dictyostelium* chemotactic responses (126). Consistent with the chemotaxis data for folic acid, the $G\alpha5^{HC}$ cells seemed to display low levels of cGMP that did not dramatically change upon folic acid stimulation. The *gα5* null cells seemed to have a higher basal level of cGMP and also seemed to display slightly higher levels of cGMP accumulation in response to folic acid as compared to wild-type cells (Figure 18).

Discussion

Interaction of the G protein with a receptor that has been activated with an external signal promotes the exchange of GDP that is bound to the α subunit, for GTP, and the subsequent disassociation of the α-GTP complex from the βγ heterodimer. The α-GTP complex and the free βγ heterodimer may interact with effector proteins such as adenylate and guanylate cyclases that further amplify the signal by generating secondary messengers

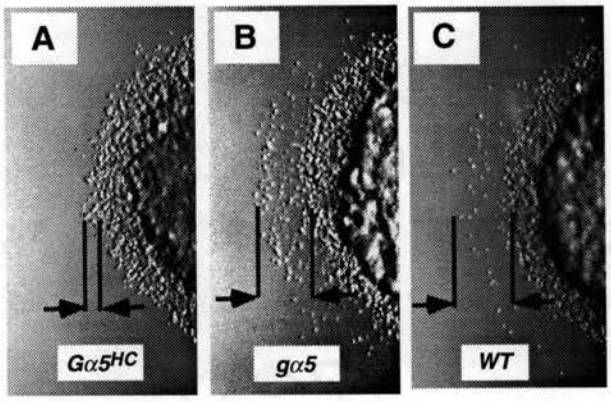

Figure 17. Chemotaxis of the $G\alpha5^{HC}$ (A), $g\alpha5$ null (B), and wild-type cells (C) to folic acid. Cells were grown in shaking culture mid-log phase (~$2x10^6$ cells/ml), harvested and washed in 12mM phosphate buffer. The cells were then resuspended to a density of $2x10^8$ and spotted for chemotaxis as described in materials and methods. This gradient assay was monitored over three hours and pictures were taken at this time. Folic acid source is to the left of all cell droplets. Bars represent the leading edge of chemotaxis and the perimeter of the cell droplets.

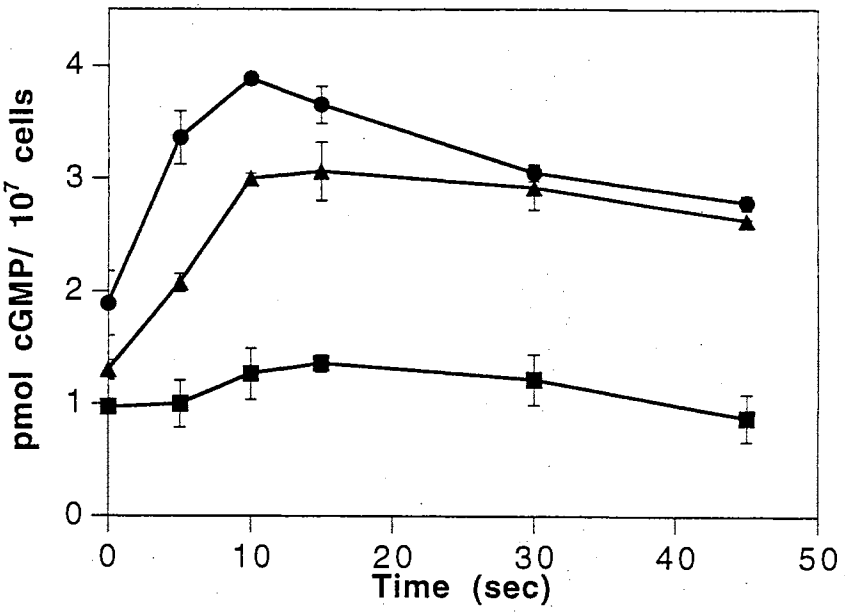

Figure 18. Accumulation of secondary messenger cGMP per 10^7 cells in wild-type cells (closed triangles), *gα5* null cells (closed circles) and $G\alpha5^{HC}$ cells (closed squares) in response to folic acid. Cells were stimulated with 30μM folic acid and 0.33mM 5-aza-guanidine. Cell extracts were collected as described in figure 9 at 10 hours post-starvation and they were used to determine levels of cGMP at that time point. Each sample was tested in duplicate. Radioimmunoassays were done using the cGMP kit (Amersham) as described in materials and methods.

such as cyclic AMP (cAMP) and cyclic GMP (cGMP). During effector stimulation, the intrinsic GTPase activity of the Gα subunit converts GTP to GDP allowing the α and βγ subunits of the G protein to reassociate, thereby completing the activation cycle (6). Many of the factors that participate in the Gα5 signal transduction pathway are unknown. Once the external signal triggering the pathway is identified, several experiments can be done to elucidate the other players in the pathway.

cAMP may be one of the external signals that triggers the Gα5-mediated signal transduction pathway. A decrease in the levels of cAMP and/or cGMP in the *gα5* null mutants as compared to the wild-type cells, and a decrease in the level of secondary messengers in wild-type cells as compared to the $G\alpha5^{HC}$ cells, would have suggested that cAMP is the extracellular signal triggering the Gα5 signal transduction pathway.

Gα2 is one of the G proteins that is known to be activated by cAMP (86). Hence, a decreased response to cAMP in the *gα2/gα5* double null mutant as compared to the *gα2* null mutant may suggest that cAMP is the external signal triggering the Gα5 pathway. Recently, Gα3 was reported as another G protein that was required for cAMP mediated responses(15). Hence it may be important to look at Gα5 response to cAMP in *gα2/gα3* null mutants to determine if cAMP is the external signal triggering the Gα5 signal transduction pathway.

Another possibility is that cAMP may not trigger cellular responses such as chemotaxis or accumulation of secondary messengers, which may be why we observe variable results with these assays. It might be a signal that triggers other phenomena such as protein phosphorylation or regulation of ion channels (Scott et al., 1987). If cAMP is the external signal that triggers the Gα5-mediated signal transduction pathway, but does not trigger responses such as chemotaxis or accumulation of secondary messengers, the intrinsic GTPase activity of the Gα5 subunit in response to cAMP can be analyzed by doing cAMP binding assays as described by Kesbeke *et al* (80).

Chemotaxis may be a morphological process affected by responses to cAMP through the Gα5 pathway, but it may be a subtle phenotype that was not observed with the chemotaxis assays performed. Light scattering techniques, using cell suspensions that have been treated with cAMP, can be used to reflect structural changes and indicate alterations of cell shape or the size of cell aggregates, or both, in the *gα2* and *gα2/gα5* double null mutants as compared to the wild-type cells as described earlier (148). These morphological changes are associated with chemotaxis to an external signal. The attraction of the light scattering technique is that if cAMP induces responses that result in structural changes of cells or cell aggregates, it will be immediately observed as a change in light wave oscillation or spike profile. These procedures may serve as a more sensitive means of measuring chemotactic responses to cAMP.

cAMP may be one of the external signals that triggers the Gα5-mediated signal transduction pathway. There may be multiple signals activating the pathway at different stages in the life cycle and development. This implies that the G protein can bind to multiple receptors and such cases have been reported before (69). Hence, cAMP may not serve as an activating signal between six and fourteen hours post-starvation, but might be the signal affecting downstream Gα5 effectors at a different time in development.

An overexpression of the Gα5 subunit inhibits responses to folic acid. Based on these results obtained from folic acid responses of the Gα5 mutants, it may be likely that the Gα5 signal transduction pathway has some effect on folic acid responses in cells. Downstream effectors of the Gα5 pathway may act as negative regulators of proteins activated by folic acid through another signal transduction pathway, such as the Gα4 pathway.

Changing the stoichiometry of Gα5 in *Dictyostelium* cells seems to affect the responses of the cells to either PAF or LPA. It is possible that the two biomolecules do not directly activate the Gα5 signal transduction pathway, but downstream effectors of the Gα5 signal transduction pathway may have an effect on other pathways activated by LPA and

PAF. Thus, both LPA and PAF seem to be potentially interesting candidates that affect the Gα5 pathway. LPA has been thought to act via G proteins in a number of systems (36). LPA is a normal precursor in the de novo lipid biosynthesis in all cells – both prokaryotic and eukaryotic. Thus there may be extracellular levels of LPA present in high enough concentrations to be detected by amoebae and may also be high enough to activate signal transduction pathways. PAF is produced by *Dictyostelium* cells during development and in response to cAMP. Thus if the Gα5 signal transduction pathway is affected by extracellular cAMP signaling, PAF may be a likely enhancer of the cAMP responses.

If the GTPase assays done with some of the biomolecules that gave any response do not give any precise data, combinations of the biomolecules using the same concentrations can be used subsequently, to do the assays. Hence, although it was not possible to define the external signal triggering the pathway, a number of potential candidates were preliminarily tested.

CHAPTER 4

ANALYSIS OF THE Gα5 G PROTEIN AND THE EFFECTS OF SITE SPECIFIC POINT MUTATIONS IN THE GTPase DOMAINS OF THE Gα5 SUBUNIT.

Introduction

The Gα5 G protein is important in tip morphogenesis as indicated by the *gα5* null mutant phenotype, where the rate of tip formation of the multicellular aggregate and developmental gene expression is delayed by about three hours as compared to the wild-type cells (62). This suggestion is further supported by the fact that the cells overexpressing *Gα5* form precocious tips about three hours earlier than the wild-type cells. The developmental gene expression in these overexpressor mutants was also advanced by three hours as compared to wild-type cells. Chimeric organisms created by mixing together equal proportions of Gα5 mutant cells and wild-type cells were used to determine whether the aberrant temporal regulation of tip morphogenesis could be affected by intercellular signaling. Aggregates containing equal proportions of *gα5* null and wild-type cells formed tips at the rate expected for wild-type cells, suggesting that the cells expressing the *Gα5* gene exert a dominant role in the rate of tip formation. Similarly, cells overexpressing the *Gα5* gene also exerted a dominant role on the chimeric organism with respect to tip formation, as aggregates containing *gα5* null/$G\alpha5^{HC}$ or wild-type/$G\alpha5^{HC}$ cells formed tips at the rate expected for aggregates of only $G\alpha5^{HC}$ cells (62).

Mutations that impair the GTPase activity of Gα subunits have been reported to enhance downstream signaling of their respective transduction pathways and in certain

cases cause cancerous growth (93, 102). Analysis of analogous GTPase mutations in the *Dictyostelium* Gα subunits may provide insight into the regulation of various parameters such as cell division, differentiation and cell migration, that are usually altered in cancer cells. The analysis of GTPase mutations in *Dictyostelium* Gα subunits also has an advantage in testing whether the mutated gene is dominant when expressed at normal levels. In most cases, the analysis of the GTPase mutations in mammalian Gα subunits have been with mutated genes at an undefined copy number and/or expressed from heterologous promoters. The overexpression of these mutant subunits might also contribute to increased downstream signaling as has been shown for some wild-type Gα subunits. Thus, in order to completely understand the regulation of GTPase-deficient Gα subunits, it is important to control the levels and expression of these Gα subunits, as can be done in *Dictyostelium.*

Certain mutations in the highly conserved G-3 region (DVGGQR- using nomenclature as described in Bourne *et al.*, 1991) have been extensively studied in mammalian and other systems, including *Dictyostelium* (12, 78, 93, 123). Some mutations like the Q227R of $G\alpha_s$ replace the conserved glutamine residue within the G-3 region of the Gα subunits with other amino acid residues (12). These changes in the conserved glutamine residue are of particular interest as analogous mutations, such as the substitution of the glutamine to leucine, in the mammalian *ras* genes has been associated with oncogenesis (4). In these, as well as numerous other cases, Gα subunits and Ras proteins which have this glutamine residue altered, have reduced GTPase activity. The inhibition of the GTPase activity is thought to prolong the activation states of these proteins, which results in increased signaling of downstream effectors.

In addition to the alteration of the glutamine residue in the conserved G-3 region, another substitution mutation (glycine to threonine) in the same region, has also been previously studied in the mammalian $G\alpha_s$ gene and the *Dictyostelium Gα1* and *Gα2* genes

(34, 123, 124). This mutation in the in the mammalian $G\alpha_s$ and *Dictyostelium Gα1* was associated with a decrease in adenylyl cyclase activity leading to a decreased response of these two subunits. This effect was termed as a dominant negative phenotype (34, 124). However, in the *Dictyostelium* Gα2 subunit, the substitution of the glutamine residue in the G-3 region had little or no effect on the cells (123).

This section aims at trying to elucidate some of the features of the Gα5 signal transduction pathway. Studies on the effects of two site-specific mutations, the Glutamine to leucine substitution at position 199 ($G\alpha5^{Q199L}$) and the Glycine to Threonine substitution at position 197 ($G\alpha5^{G197T}$), in *Gα5* gene are also described here.

Materials and Methods

Strains

The following axenic haploid *Dictyostelium* strains were used: KAx-3 (wild-type) (161), the *gα5* null mutant strain JH257 (*gα5::THY1*) described in (62), the Gα5 overexpression strain JH258 (contains a high copy number of the *Gα5* expression vector pJH206 ($G\alpha5^{HC}$) described in (62)). *gα5* null cells carrying either a prespore-specific (*pSP60-lacZ*) or a prestalk-specific (*pecmA-lacZ*) were also used (JH287 and JH289 respectively). All strains that are used in the study are isogenic to the wild-type strain KAx-3, except at loci noted and all strains were grown at room temperature either in HL-5 medium or with *Klebsiella aerogenes* on SM nutrient agar plates, unless otherwise noted. All the cloning steps were carried out in *E.coli* strains using standard recombinant DNA protocols (105) and the DNA constructs thus obtained were transformed into *Dictyostelium* by electroporation as described by Dynes and Firtel (37). Clonal isolates were obtained by plating transformed cell lines in 24 or 96 well microtiter plates.

For growth in the presence of folic acid, cells were cultured in plates as well as grown in shaking culture in the presence of 1mM folic acid. All plates and flasks were kept in the dark to prevent exposure to light since folic acid is light sensitive. Cells were grown

at a density below $1x10^6$ cells/ml for two days in shaking culture in standard HL-5 media and HL-5 supplemented with 1mM folate to be compared for studying the effects of induction of the *discoidin-1* promoter in the absence of folic acid. Cells were washed and resuspended in 12 mM phosphate buffer and then plated for development on non-nutrient phosphate plates and on a lawn of *Klebsiella aerogenes* at a concentration of $1x10^7$cells/ml to monitor the effects of the induction of the $G\alpha5^{Q199L}$ mutation on development. KAx-3 cells plated at the same density served as the wild-type control.

DNA Constructs

Gα5 point mutations were created by site directed in vitro mutagenesis as described by Kunkel (88). Oligonucleotides 5'-CTTCTTTCTGATCTTAGTCCACCAACATC-3' ($G\alpha5^{Q199L}$) or 5'-CTGATCTTTGTCCAGTAACATCTAACATTC-3' ($G\alpha5^{G197T}$) were used as primers to mutagenize a 0.3kb *SpeI/BclI* fragment of the *Gα5* gene. The mutagenized *Gα5* fragments were used to replace wild-type fragments in pJH230 (62). The 1kb *Hin*dIII/*Eco*RV fragments of the resulting plasmids were deleted to remove the *Gα5* promoter and the initial coding region. A 3.2kb *Bam*HI fragment containing the *Dictyostelium THY1* gene (58) was then inserted into the *Bam*HI site of these vectors to create vectors pJH250 ($G\alpha5^{Q199L}$) and pJH252 ($G\alpha5^{G197T}$). A wild type control vector pJH255 was also constructed and is identical to pJH250 and pJH252 except for the point mutations. The three constructs, pJH250, pJH252 and pJH255, were linearized at the *Spe*I site and electroporated into the thymidine auxotrophic strain JH10 (58) to allow for site specific recombination at the *Gα5* locus. Transformants were selected based on their ability to grow in the absence of thymidine i.e., prototrophs. Transformants with a single copy of the vector that had integrated precisely in the *Spe*I site of the *Gα5* locus genome were identified by Southern blot analysis (149).

To allow for high copy selections of the mutant alleles, the *Bam*HI/*Spe*I fragments of pJH250 and pJH252 were used to replace the *Bam*HI/*Spe*I fragment of pJH206 (62) to create vectors pKN1 and pKN2 respectively which carry the mutants $G\alpha5^{Q198L}$ and $G\alpha5^{G196T}$, along with the neomycin resistance gene that confers resistance to G418. For high copy integration of the mutant *Gα5* genes, the two vectors pKN1 and pKN2 along with the control vector pJH206, which is the construct containing the wild-type *Gα5* gene with the neomycin gene, were electroporated into wild-type KAx-3 cells. Potential transformants that were resistant to 10μg/ml of G418 were selected and were plated in a 24 well culture plate for further analysis. Southern blot analysis was done on these potential transformants to identify the correct integration and copy number of the vectors into the genome (149). Genomic DNA was isolated from cells as described by Nellen *et al* (118), digested with *Eco*RV and blotted to nitrocellulose membrane. A 1.5kb *Eco*RI/ *Eco*RV fragment of the Gα5 open reading frame from pJH214 was used for radiolabeling, and the blots were probed to verify incorporation of a high copy number of the *Gα5* mutant gene. DNA probes were generated by random primer probe synthesis using the protocol as described by Vogelstein (44) according to the manufacturer's instructions.

To construct an inducible $G\alpha5^{Q199L}$ gene, a promoterless version of the $G\alpha5^{Q199L}$ allele was made first, by introducing a 2kb *Hin*dIII/*Pst*I fragment containing the $G\alpha5^{Q199L}$ open reading frame and downstream sequences into the same sites of pJH235 (62) to give pKN38. The *discoidin-1* promoter, isolated from pVEII (9) as a 0.8kb *Pac*I/*Xho*I fragment was cloned into pT7T318U (Pharmacia) and a 0.8kb *Hin*dIII/*Eco*RI fragment from this construct, containing the *discoidin-1* promoter, was then introduced into the *Hin*dIII site of pKN38 to yield pKN39. A 2.2kb neomycin resistance gene, that confers resistance to the drug G418 for purposes of selection in *Dictyostelium*, was then introduced into pKN39 as a *Xba*I/*Sal*I fragment from pJH63 to give pKN41.

As a control for the repressible construct with the $G\alpha5^{Q199L}$ mutant allele, a construct carrying the unmutated *Gα5* gene driven by the repressible *discoidin* promoter

was made. To make the control construct, a 1.3kb *SpeI/BamHI* fragment of pJH198 was used to replace the *Spe*I/*Bam*HI fragment of pKN39 to give pKN37. The 2.2kb neomycin resistance gene as an *Eco*RI fragment of pJH64 was then cloned into the *Eco*RI site of pKN37 to give pKN44.

The two constructs, pKN41 and pKN44, were linearized at the *Sal*I and *Xho*I sites respectively and electroporated into the wild-type cells (KAx-3) as described earlier (37). Repression of the gene was achieved by adding folate to a final concentration of 1mM to the cells growing in HL-5. Colonies that are resistant to 10μg/ml of G418 were selected and grown vegetatively in 24 well culture dishes under drug selection and with a concentration of 1mM folate. Incorporation of a high copy number of the inducible constructs were verified by a Southern blot analysis genome (119). Genomic DNA was isolated from cells as described by Nellen *et al* (118), digested with *Eco*RI and blotted to nitrocellulose membrane. A 1.5kb *Eco*RI/ *Eco*RV fragment of the Gα5 open reading frame from pJH214 was used for radiolabeling and the blots were probed to verify incorporation of a high copy number of the inducible *Gα5* genes. DNA probes were generated by random primer probe synthesis using the protocol as described by Vogelstein (44).

Analysis of Morphology and β-Galactosidase Activity

Cells were grown in HL-5 as a shaking culture till mid-log phase (~$2x10^6$ cells/ml). The cells were then harvested and washed in 12 mM Na^+/ K^+ phosphate buffer (pH6.1) to remove nutrients. The washed cells were pelleted and resuspended at a concentration of $2x10^7$ cells/ml in phosphate buffer. Each clone was then plated for development on non-nutrient Na^+/K^+ phosphate plates as well as on lawns of *Klebsiella aerogenes* on SM plates as described earlier. Phenotypes of the various transformants were then analyzed at different times after the onset of starvation. The rate of tip formation and phenotypes associated with development in the transformant cells were compared to wild-

type KAx-3, *Gα5* overexpressing cells JH258 ($G\alpha5^{HC}$) and *gα5* null cells (JH257), which served as the controls.

For analysis of cell sorting using β-galactosidase activity, cells were grown to mid-log phase, washed in 12 mM phosphate buffer and resuspended as described earlier. Cell suspensions of the different strains were mixed in a ratio of 1:4 before plating the mixture on nitrocellulose membrane filters (Millipore) resting on top of the non-nutrient phosphate plates. At the desired time in development, the multicellular structures were stained for β-galactosidase activity as described earlier (57). The multicellular structures were fixed on the membrane in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$ pH 7.0) containing 2.5% gluteraldehyde and 0.1% Triton X-100. The fixing solution was then washed off with Z-buffer and the structures were stained in a solution that contained 1mM $K_3[Fe(CN)_6]$, 1 mM $K_4[Fe(CN)_6]$, 0.1% Triton X-100 and 1mg/ml of the substrate X-gal (Sigma) (from a stock of 20 mg/ml X-gal in N, N, dimethylformamide). For quantitative β-galactosidase enzymatic assays, the same cell suspensions of above were subjected to a freeze-thaw cycle and then the extracts were incubated with ONPG (o-nitro phenyl-b-D-galactosidase) as the substrate and the standard enzyme assay as described by Dingermann et al was performed (35). The cell extracts were also assayed for protein concentration using a dye-binding assay(14).

DNA and RNA Blots

Dictyostelium DNA blots were performed as described earlier. Genomic DNA was isolated from cells as described by Nellen *et al* (118) digested with *Eco*RV, electrophoresed on a 0.7% agarose gel and blotted on a nitrocellulose membrane. RNA blots were done as described by Mann and Firtel (106). Cells were grown to a concentration of $3x10^6$cells/ml, washed twice and RNA isolated as described (118) at the vegetative stage. RNA was then electrophoresed through a 1.2% agarose gel with 6% formaldehyde and blotted onto a nitrocellulose membrane. For later stages in development, cells at a concentration of $5x10^7$

cells/ml were developed on presoaked Whatman filters placed on non-nutrient agar plates. At the desired time in development, cells were washed off the filter and harvested for RNA. RNA isolated from wild-type cells from a developmental stage when *Gα5* is expressed was used as a positive control. The 1.5kb *Eco*RI/*Eco*RV fragment from pJH214, containing the *Gα5* open reading frame, was used to generate a probe. Some DNA probes were generated by random primer probe synthesis using a Genius kit (Boehringer Mannheim) according to the manufacturer's instructions. At other times, radiolabeled DNA probes were generated by random primer probe synthesis using the protocol as described by Vogelstein (44) according to the manufacturer's instructions. Genomic DNA from wild-type cells served as the control.

Results

Analysis of the Effect of Intercellular Signaling in the Chimeras on Developmental Gene Expression

gα5 null mutant cells carrying developmentally important cell-type specific reporter genes were made use of in chimeras to further examine the role of Gα5 function in possible intercellular signaling and temporal regulation of developmental gene expression. *gα5* null cells carrying the prespore-specific (*pSP60-lacZ* fusion) and the prestalk-specific (*pecmA-lacZ* fusion) reporter genes were mixed with wild-type, *gα5* null and $G\alpha5^{HC}$ cells to examine the ability of the wild-type and $G\alpha5^{HC}$ cells to accelerate these cell-type specific reporter genes, when these cells were developed as chimeras. Based on the quantitative β-galactosidase assay, prestalk-specific gene expression (*pecmA-lacZ* fusion) in the *gα5* null cells was precociously induced by the presence of wild-type or $G\alpha5^{HC}$ cells. The level of this induction was consistently greater in the *gα5* null/ $G\alpha5^{HC}$ chimeras as compared to *gα5* null/ wild-type chimeras, suggesting that prestalk gene expression is dependent on the level of Gα5 function in accompanying cells (Figure 19). The prespore-specific reporter gene expression (*pSP60-lacZ* fusion) was also consistently accelerated in the *gα5* null cells the

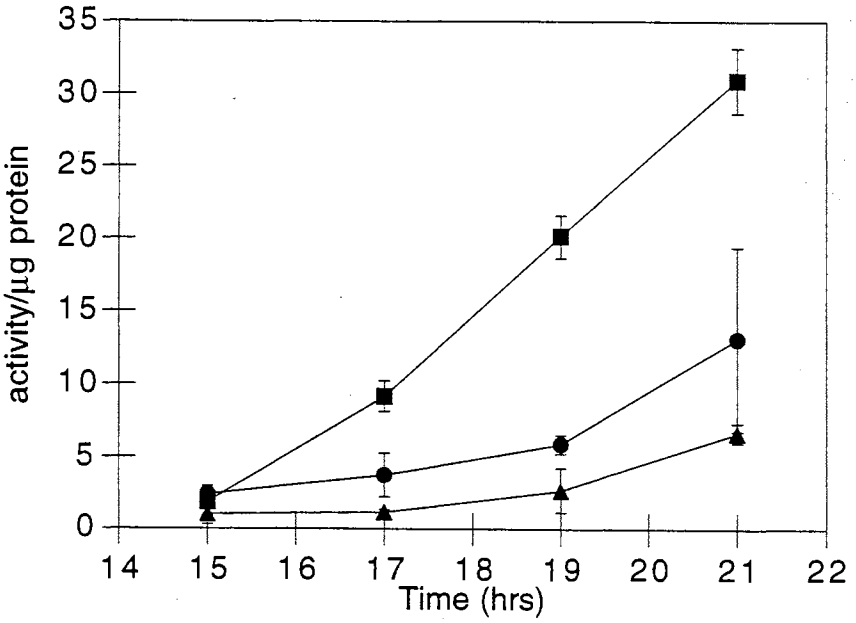

Figure 19. Expression of prestalk specific gene fusions in *gα5* null mutant cells developed in the presence *of gα5* null, wild-type and $G\alpha5^{HC}$ cells. *gα5* null cells carrying *pecmA-lacZ* gene fusions were mixed at a ratio of 1:4 with *gα5* null cells(triangles). Wild-type cells (circles) or $G\alpha5^{HC}$ cells (squares) and developed as chimeras. At the times indicated, the chimeras were assayed for β-galactosidase activity as described in Materials and Methods. The values represent the mean of the activities measured from two sets of chimeric organisms developed simultaneously and the error bars represent the standard deviation of these activities. β-galactosidase activities are given as pmoles of substrate hydrolyzed (µg protein/minute) at 22°C.

presence of $G\alpha5^{HC}$ or wild-type cells in the chimeric aggregates. However, the difference in the level of induction between the *gα5* null/wild-type and the *gα5* null/ $G\alpha5^{HC}$ chimeras was variable suggesting that parameters other than the level of Gα5 function may be important for levels of induction of the prespore-specific genes (Figure 20).

Cytological staining of the *gα5* null cells carrying the prespore- or prestalk- specific *lacZ* reporter gene fusions showed a spatial pattern of localization of prespore and prestalk cells that was similar to that of wild-type cells (Figure 21 A, D). However, in the chimeras made with the *gα5* null cells expressing *pSP60-lacZ* and wild-type cells, there is a greater concentration of the *gα5* null cells towards the anterior of the prespore region (Figure 21 B). In contrast, when *gα5* null cells expressing *pecmA-lacZ* were mixed with $G\alpha5^{HC}$ cells, the region of greatest staining, appears to be more extended (Figure 21 F) than when *gα5* null cells expressing *pecmA-lacZ* were mixed with the *gα5* null cells (Figure 21 D) or wild-type cells (Figure 21 E). Hence, the capacity of *gα5* null cells to move to either the prespore or prestalk region suggests that Gα5 function is not essential in a cell-autonomous manner for cell-type sorting. The variations in the distribution of the *gα5* null cells carrying the *pecmA::lacZ* within the prestalk region, when mixed with $G\alpha5^{HC}$ cells as compared to *gα5* null cells, suggests that the difference in *Gα5* gene dosage may affect other aspects of spatial patterning. One possibility is that Gα5 could be important in the rate of cell sorting.

Analysis of the Cells Carrying the $G\alpha5^{Q199L}$ and the $G\alpha5^{G197T}$ Mutant Genes

The effects of two site-specific mutations of the conserved G-3 region (glutamine to leucine substitution and glycine to threonine substitution) that alter G protein activity and have been well characterized in a number of systems were studied in the Gα5 subunit. The analogous site-specific point mutations were created in*Gα5* by site directed mutagenesis to give the alleles $G\alpha5^{Q199L}$and $G\alpha5^{G197T}$ and these were analyzed for their effects on Gα5 function and the development of *Dictyostelium.*

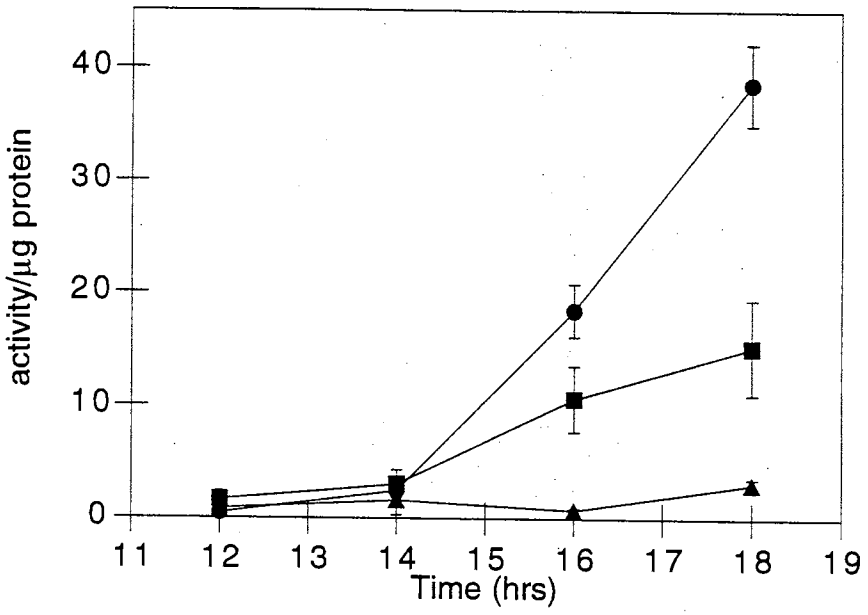

Figure 20. Expression of prespore specific gene fusions in *gα5* null mutant cells developed in the presence *of gα5* null, wild-type and $G\alpha5^{HC}$ cells. *gα5* null cells carrying *pSP60-lacZ* gene fusions were mixed at a ratio of 1:4 with *gα5* null cells(triangles), wild-type cells (circles) or $G\alpha5^{HC}$ cells (squares) and developed as chimeras. At the times indicated, the chimeras were assayed for β-galactosidase activity as described in Materials and Methods. The values represent the mean of the activities measured from two sets of chimeric organisms developed simultaneously and the error bars represent the standard deviation of these activities. β-galactosidase activities are given as pmoles of substrate hydrolyzed (µg protein/minute) at 22°C.

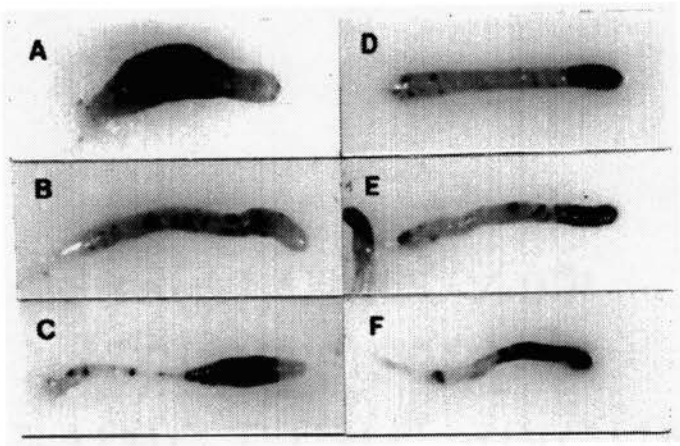

Figure 21. Cytological staining of *gα5* null mutant cells developed in the presence of *gα5* null, wild-type and $G\alpha5^{HC}$ cells. *gα5* null cells carrying the *pSP60-lacZ* (A-C) or the *pecmA-lacZ* (D-F) gene fusions were mixed at a ratio of 1:4 with *gα5* null cells(A, D), wild-type cells (B, E), or $G\alpha5^{HC}$ cells (C, F) and developed as chimeras. Chimeras were fixed and histochemically stained as described in Materials and Methods.

These alleles were then independently introduced in both a single copy and in multiple copies into wild-type KAx-3 cells. A plasmid containing the mutant $G\alpha5^{Q199L}$ with the G418 resistance marker gene (pKN1) was transformed into wild-type cells to determine effects of this allele on the cells. Transfomants that were selected with lower concentrations of G418 (2-5μg/ml) remained viable although transformants selected in growth medium containing 10μg/ml G418 formed colonies, but then gradually died. This suggested hat a high copy number of this plasmid was lethal to the cells. As a control, the *Gα5* expression vector (pJH206) (a plasmid identical to pKN1 except for the Q199L mutation), was simultaneously transformed into wild-type cells. This transformation yielded several high copy number transformants that remained viable in media containing ~15μg/ml of G418. Genomic lots of the viable $G\alpha5^{Q199L}$ transformants indicated a low copy number of the $G\alpha5^{Q199L}$ mutant plasmid (about 1-5 copies using the endogenous *Gα5* gene as the reference). The transformants studied had multiple copies of an 8kb *Eco*RV fragment as compared to single copy of a 2kb fragment in the genomic DNA from wild-type cells.

This data is consistent with the lower resistance to G418 in the media. This cell death phenotype appears to be present only in the cells with a high copy number of the $G\alpha5^{Q199L}$ allele as transformants with a lower copy number of this allele continued to grow on plates. The characterization of the viable transformants indicated that there were no obvious developmental abnormalities associated with the cells containing the low copy numbers of the mutant alleles. Also, in other studies done in the lab, the wild-type *Gα5* gene was replaced with a single copy of the $G\alpha5^{Q199L}$ allele. This was accomplished by transforming the mutated allele as a truncated gene on a plasmid, linearized within the Gα5 open reading frame, so as to direct the integration of the plasmid into the *Gα5* locus. About 40% of the transformants obtained with this method had a single full length copy of the $G\alpha5^{Q199L}$ gene and a truncated wild-type *Gα5* gene. These transformants also

displayed no gross vegetative or developmental phenotype, suggesting that the $G\alpha5^{Q199L}$ allele was capable of providing sufficient Gα5 function and complementing the loss of the wild-type *Gα5* gene.

In addition to the analysis of the $G\alpha5^{Q199L}$ allele, the effects of the$G\alpha5^{G197T}$ allele on the cells was also studied. This allele was expected to have a dominant negative effect on the wild-type cells resulting in decreased G protein function as was seen in cases studied earlier(123, 124). The $G\alpha5^{G197T}$ allele, like the $G\alpha5^{Q199L}$ allele, appeared to be lethal at a high copy number. Stable transformants contained less than ten copies of the plasmid carrying the $G\alpha5^{G197T}$ allele and were not resistant to as high a concentration of G418 as wild-type cells transformed with the wild-type *Gα5* gene. None of the viable transformants displayed any alteration of tip morphogenesis or any other obvious developmental phenotype. As done with the $G\alpha5^{Q199L}$ allele, a truncated copy of $G\alpha5^{G197T}$ mutant allele was also integrated in a single copy at the *Gα5* locus as described earlier to test the ability of the allele to complement the lack of a wild-type *Gα5* gene. All transformants with a single copy of the allele in the absence of a functional *Gα5* gene developed similar to wild-type cells. The ability of $G\alpha5^{G197T}$ allele to complement a wild-type allele suggests that this mutant gene can provide sufficient Gα5 function and may not behave as a dominant negative allele, as previously expected. The phenotypes associated with the mutant *Gα5* genes have been summarized in Table 3.

Analysis of a Repressible Mutant $G\alpha5^{Q199L}$ Allele

We considered the possibility of the rapid cell death in the mutants being a result of programmed cell death or apoptosis. Both oncogenesis and apoptosis are processes that involve cell growth and differentiation. As it has been observed that mutant G proteins are associated with oncogenesis, it might not be unexpected to have mutant G proteins associated with apoptosis as well. Apoptosis has been observed in a number of organisms

TABLE 3

Phenotypes associated with cells carrying the $G\alpha5^{Q199L}$ and $G\alpha5^{G197T}$ mutant genes.

Mutation	Wild-Type *Gα5*	Phenotype
$G\alpha5^{Q199L}$(singlecopy)	Absent	Wild-type phenotype
$G\alpha5^{Q199L}$ (low copy)	Present	Wild-type phenotype
$G\alpha5^{Q199L}$ (high copy)	Present	Cell-death (lower threshold)
$G\alpha5^{G197T}$(single copy)	Absent	Wild-type phenotype
$G\alpha5^{G197T}$(low copy)	Present	Wild-type phenotype
$G\alpha5^{G197T}$(high copy)	Present	Cell-death (higher threshold)
Gα5 (high copy)	Present	Rapid development
gα5	Absent	Delayed development

and seems to be an important process in regulation of cell growth and differentiation (163). Cell-death seems to be the phenotype observed in *Dictyostelium* when a very high copy of the $G\alpha4^{Q200L}$ gene is introduced into wild-type cells. Some experiments to assay for common characteristics associated with apoptosis, such as the early fragmentation of genomic DNA before the cell loses other cellular functions like membrane integrity, had been done with the $G\alpha4^{Q200L}$ mutants, using the DNA specific fluorochromes Propidium Iodide and Hoechst dye(43, 163). A qualitative examination of extent of vacuolization by light microscopy, as described in the literature, had also been done earlier with the $G\alpha4^{Q200L}$ mutants (26). No observable results could be obtained with these assays due to too few cells being available for the analysis. Hence we tried using another approach to study the $G\alpha5^{Q198L}$ mutants by overpassing the effect of the $G\alpha5^{Q199L}$ mutation in the vegetative phase and hence be able to study the effect of the $G\alpha5^{Q199L}$ mutation in the developmental stages with a larger number of cells. This was done by making use of a repressible promoter to drive the transcription of the $G\alpha5^{Q199L}$ gene.

One such repressible promoter that could be used was the promoter of the *discoidin-1* gene. As a control for the repressible construct with the $G\alpha5^{Q199L}$ mutant allele, a construct carrying the unmutated $G\alpha5$ gene driven by the repressible discoidin promoter was made (Figure 22). This was done to determine if the effects that were being observed, when the promoter was induced was only due to the mutation in the $G\alpha5$ gene and not due to any other discrepancy due to artifacts, or additional mutations that might have been picked up. It also helped to identify problems due to leaky activity of the promoter when it was uninduced.

The constructs were then electroporated into wild-type KAx-3 cells. Transformants were selected and grown in the presence of folic acid, such that expression of the genes could be repressed. The transformants whose genomes contained a high copy number of a 4kb *Eco*RI fragment were selected and grown in shaking culture (as determined by a

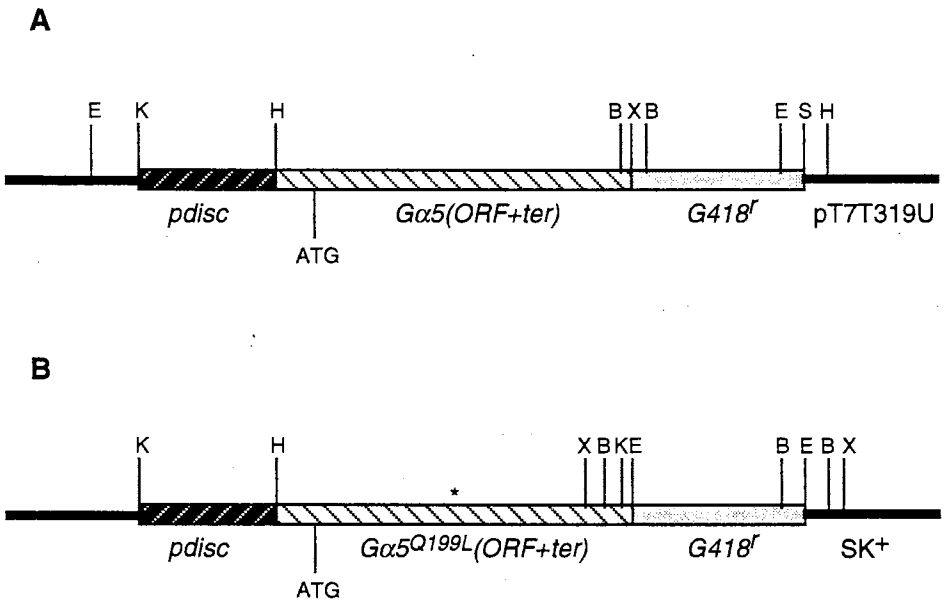

Figure 22. Inducible gene constructs with the *discoidin-1* promoter. (A) Map of the inducible $G\alpha5^{Q199L}$ gene (*pdisc-*$G\alpha5^{Q199L}$) and (B) Map of the inducible wild-type *Gα5* gene (*pdisc-Gα5*). A detailed description of the constructs is described in Materials and Methods. The initiation codon is represented by ATG. *G418*^{r} represents the *pACT6::Neo*^{r} gene that confers resistance to the drug G418. Restriction enzymes shown are *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K) and *Xba*I (X).

southern blot (Figure 23)) with and without folic acid. They were then plated for development on phosphate plates. Preliminary results indicated a difference in the phenotypes of the cells that carried the mutant allele constructs, grown in the presence and absence of folic acid. The cells grown in the absence of folic acid seemed to develop slower than the cells grown in the presence of folic acid. In the control cells, the cells carrying the *discoidin-1* promoter fused to the wild-type version of the Gα5 open reading frame, also showed a difference in their phenotypic morphology in the absence and presence of folic acid. In the control cells, the wild-type *Gα5* gene driven by the discoidin promoter resulted in a $G\alpha5^{HC}$ phenotype in the absence of folic acid, which was consistent with the phenotype associated with cells overexpressing *Gα5*. However, this phenotype was also observed in the cells that were grown in the presence of folic acid, although the effect was not as dramatic. This indicated that the promoter activity was not being tightly regulated with the folic acid.

These different strains were then grown in the presence and absence of folic acid and the vegetative cells were harvested for an RNA blot analysis. This analysis indicated that transcripts of the mutant alleles and the wild-type alleles were present in cells that were grown both with and without folic acid (Figure 24). This could possibly be due to the fact that the RNA was harvested from cells in which the folic acid was not at the optimum concentration to repress the *discoidin-1* promoter, or that the promoter may be leaky. If cells were grown vegetatively for far too long in the absence of folic acid, it is possible that the lethality of the Q-->L mutation would have killed the cells or allowed for rearrangement of the genes in the genome. The transcript levels of the repressible mutant *Gα5* gene was much lower than that of the wild-type transcripts, indicating that high expression of the mutant allele could not be tolerated by the cells.

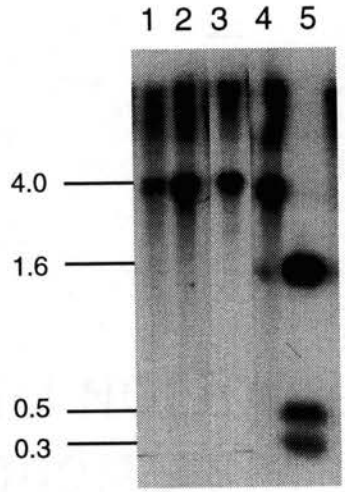

Figure 23. Southern blot of the cells transformed with the repressible *Gα5* constructs. Cells were grown to mid-log phase and genomic DNA was isolated as described in Materials and Methods. The DNA was then digested with *Eco*RI and run on a 0.7% agarose gel and blotted onto a nitrocellulose membrane. The blot was hybridized with a 1.5kb *Eco*RV/ *Eco*RI fragment containing the Gα5 open reading frame from pJH214 as described earlier. Lanes 1, 2 – Genomic DNA from transformants carrying the repressible mutated *Gα5* gene (*pdisc-Gα5*^{Q199L}); Lanes 3, 4 - Genomic DNA from transformants carrying the repressible unmutated Gα5 gene (*pdisc-Gα5*); Lane 5 –1kb molecular weight standard. The 1.6kb, 0.5kb and 0.3kb bands on the molecular weight marker cross-hybridize with the *Gα5* probe.

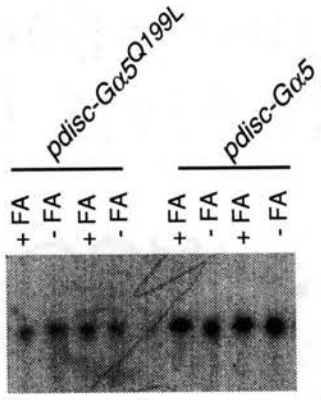

Figure 24. Expression of the repressible *Gα5* gene constructs in the cells. Cells were grown with and without folic acid (FA) and were harvested for isolation of RNA as described in Materials and Methods. Total RNA was isolated from the vegetative cells and 4μg of RNA was run per lane on formaldehyde gels as described in Materials and Methods. Lanes 1-4 represent RNA from cells carrying the mutant *pdisc-Gα5*^{Q199L} allele and Lanes 5-8 represent RNA from cells carrying the inducible *pdisc-Gα5* gene. The blots were hybridized with a 1.5kb *Eco*RV/ *Eco*RI fragment from pJH214 as described earlier.

Discussion

The delay in tip morphogenesis and gene expression in *gα5* null cells and the precocious tip development and gene expression in the $G\alpha5^{HC}$ cells suggest that Gα5 plays and important role in temporally regulating this process. The *Gα5* expressing cells (wild-type cells and$G\alpha5^{HC}$) have the ability to accelerate the expression of prestalk or prespore gene expression in *gα5* null cells when developed as chimeras, as was observed in the experiments done with the *gα5* null cells expressing either *pSP60-lacZ* or *pecmA-lacZ*. This suggests that the Gα5 signal transduction pathway is important for the production of an intercellular signal rather than a reception of one, which regulates late developmental gene expression.

Tip formation involves accumulation of prestalk specific cells on the top of the cellular aggregate and hence, the dependence of the intercellular signal on *Gα5* gene dosage for the induction of prestalk-, but not prespore-specific gene expression is consistent with the role of Gα5 in tip morphogenesis. However, it is not known whether this induction of prestalk-gene expression is due to increased cell differentiation and/or migration although previous studies have indicated that the expression of many cell-type-specific genes (e.g. *SP60*, *ecmA* and *ecmB*) can be initiated before cell sorting. This suggests that cell-type-specific gene expression does not require cell sorting (42, 57, 75). Whether the *gα5* null dependent intercellular signal directly affects cell differentiation or migration, the cellular aggregate must have alternative, but less effective mechanisms to carry out these processes and Gα5 appears to play an important role in them. The results presented in this section also showed that the *Gα5* dosage did not have a significant effect on the spatial patterning of cell types within the chimeric organisms.

Based on earlier observations (78, 93, 102), the transformants with the $G\alpha5^{Q199L}$ mutation, i.e.pJH250 and pKN1, would have been expected to have an inhibition of GTPase function. This would indicate a constitutively turned on Gα subunit. Hence, in the single copy mutants we would expect a *Gα5* overexpression phenotype with an

increase in rates of tip morphogenesis as compared to wild-type cells and/or multiple tip formation, and a very small spore mass at the fruiting body stage, presumably due to increased prestalk cell development. However, the $G\alpha5^{Q199L}$ mutation did not appear to affect tip morphogenesis, as suggested by the replacement of the wild-type allele with a single copy of the mutant allele. The inability to obtain viable transformants carrying high copy numbers of these alleles suggests that overproduction of the mutant proteins affect processes for vegetative growth. The *Gα5* gene is expressed at low levels before multicellular development and the Gα5 function does not seem to be essential during vegetative growth, but the Gα5 signal transduction pathway might be involved in pre-aggregative functions. Many of the known mammalian subunits function in fully differentiated cells while the Gα5 subunit in *Dictyostelium* appears to function primarily in undifferentiated cells. The rapid cell death associated with the "activated allele" might possibly result from inappropriate or precocious stimulation of developmental processes that are incompatible with vegetative growth. Such a hypothesis might explain the lack of extremely aberrant phenotypes during the development of cells that carry low copy numbers of these mutant *Gα* alleles. Whether the mutant Gα5 subunits affect only the signal transduction pathway mediated by Gα5 or other pathways remains to be determined.

The transformants with the high copy of the $G\alpha5^{G197T}$ mutation, i.e. pJH252 and pKN2, were expected to have a decrease in adenylyl cyclase activity based on previous studies which associated this mutation with a dominant negative phenotype (34, 124). This led us to look for a loss in Gα5 function phenotype in these mutants. We expected rates of development in these mutants to be slower than the wild type cells, similar to a *gα5* null mutant and a large spore mass at the fruiting body stage due to a potential decrease in prestalk cell development and production. The cell death phenotype associated with the overexpression of the mutant alleles does not appear to be due to increased subunit levels since wild-type alleles can be introduced at very high copy numbers without the cell-death phenotype. The phenotype is also not likely to be due to a dominant negative Gα function,

since the Gα5 mediated signal transduction pathway is not needed for vegetative growth. It is possible that these mutant Gα subunits interfere with other G protein mediated signal transduction pathways needed for growth, but this seems unlikely as most *Dictyostelium Gα* genes have been shown not to be essential for vegetative growth. Very few of the glycine to threonine mutations have been studied in other systems and although the G-3 region of all known Gα subunits is highly conserved, it is not clear whether all such mutations would function as dominant negative mutations in all Gα subunits. As the expected dominant negative phenotype was not observed in strains in which the wild-type gene was replaced with the G197T allele, it was difficult to evaluate the effect of the $G\alpha5^{G197T}$ mutation in the cells. As both these mutations ($G\alpha5^{Q199L}$ and $G\alpha5^{G197T}$) had a similar effect on the cells in high copy number, we decided to further analyze the effect of the$G\alpha5^{Q199L}$ mutation first, before trying similar experiments on the cells carrying the $G\alpha5^{G197T}$ subunit.

The possibility to down-regulate the activity of the discoidin promoter during growth is a useful tool for expression of sequences that may be lethal to the cells. Considering the possibility that $G\alpha5^{Q199L}$ mutation is lethal to cells at the vegetative phase, use of a *discoidin-1* promoter and folate to regulate the $G\alpha5^{Q199L}$ activity enabled us to look at the effects of the subunit only during development, by repressing the promoter and hence inhibiting transcription of the mutant gene during vegetative growth. The ability to get some difference in phenotype between the cells carrying the repressible mutant $G\alpha5^{Q199L}$ allele initially indicated that the inducible promoter may have been effective. However, the comparable levels of *Gα5* transcripts seen in RNA isolated from both induced and non-induced cells indicated either a leaky promoter due to high basal levels of transcription or non-optimal repression of the promoter by the exogenously supplied folate. Differences in levels of transcript between the mutant *Gα5* and wild-type *Gα5* indicated that the mutant gene was in some way down-regulated, supporting the hypothesis that the

mutant *Gα5* gene was lethal to the cell when expression levels of this gene are high. This prevented further characterization of the effect of the mutant allele.

CHAPTER 5

SPECIFICITY OF Gα SUBUNIT FUNCTION IN *DICTYOSTELIUM*

Introduction

Heterotrimeric G proteins mediate the transduction of a wide range of extracellular signals in eukaryotic organisms, implying that the versatility of this form of signal transduction is quite extensive. While multitudes of G protein subunits, G protein-coupled receptors, downstream effectors, and other associated proteins have been identified, the networking between these components in different transduction pathways remains to be fully understood. Even with respect to the interactions between G protein subunits, the relationship between subunit associations and G protein specificity is not well characterized. Genetic and biochemical studies have provided evidence supporting the association of different Gα subunits with common Gβγ dimers and the association of different Gβγ dimers with common Gα subunits (69). In most eukaryotes, including those with sequenced genomes, the number of identified Gα subunits greatly overshadows that of the other subunit types, Gβ and Gγ, suggesting that different Gα subunits interact with common Gβγ dimers and that the Gα subunits are likely to be the primary determinants of signaling specificity (17, 20, 53, 160). The stoichiometry of each subunit type is also important for signal transduction since the loss or overproduction of any one of these three subunits can result in altered stimulation of downstream effectors (24, 58, 62, 86, 89, 166). Therefore, the interactions between different G protein subunits and other G protein-associated proteins within a given cell may have considerable impact on the transduction of external signals.

In *Dictyostelium discoideum*, several G protein subunits have been identified, including at least eight Gα subunits and one Gβ subunit (95, 126). The analyses of G protein mutants has revealed that several of the Gα subunits and the Gβ subunit are important for the chemotactic responses and the transduction of signals during the developmental life cycle of this organism (15, 59, 87, 159, 169). The Gα2, Gα3, and Gβ G protein subunits are required for starved *Dictyostelium* cells to form a multicellular mound (consisting of ~ 10^5 cells) through an aggregation process that involves the relay of an extracellular cAMP signal between cells (15, 80, 86, 169). Subsequent morphogenesis requires the Gα3, Gα4, Gα5 and likely the Gβ subunits so that the aggregate can differentiate into a migratory slug and eventually culminate into a fruiting body, consisting of a mass of spores on top of a stalk (15, 58, 62).

Chemotactic responses to cAMP and folic acid prior to and during multicellular development are specifically mediated by the Gα2 and Gα4 subunits, respectively, together with the Gβ subunit (169). Although the function of Gα subunits in these chemotactic responses is very specific, some of the downstream responses such as the accumulation of cAMP and cGMP appear to be a convergence of the two signal transduction pathways. While G proteins have been identified for different external signals, G protein-coupled receptors have only been reported for pathways that respond to external cAMP signals (140) (77, 85, 100). There are four cAMP receptors (cAR1, cAR2, cAR3, and cAR4) that have been identified and three of these receptors share some functional redundancy with respect to cAMP responses when they are expressed from heterologous promoters (167). However, differences in cAMP affinity and expression patterns suggest that most of these receptors are important for different developmental processes (82).

The *Dictyostelium* Gα subunits, Gα4 and Gα5, share some sequence identities that are not found in any other subunits of this organism and the overall sequence identity between these subunits is 51% (61). The location of introns within the *Gα4* and *Gα5* genes are identical and both genes are expressed with similar temporal and spatial patterns

throughout developmental life cycle. However, the developmental phenotypes associated with the loss or overexpression of these genes is quite distinct indicating that they promote different developmental processes (58, 62). Deficiencies in spore development and morphogenesis are observed in *gα4* null mutants and cells that overexpress the *Gα4* gene, due to high copy number (*Gα4* ^{HC}cells), are blocked in tip morphogenesis (formation of the anterior prestalk cell region) after aggregate formation (58). Gα4 function is also required for the inhibition of tip morphogenesis by folic acid (60). The role of Gα4 function in promoting spore development and inhibiting prestalk development is further supported by the localization and development of these cells in chimeric organisms. When mixed with wild type cells, *gα4* null cells localize primarily to the posterior end of the chimeric organism whereas *Gα4* ^{HC}cells localize to the central region of chimeric aggregates where prespore cells reside (60).

In contrast, *gα5* null cells form large aggregates that are delayed in tip formation and *Gα5*^{HC}cells (overexpress the *Gα5* gene due to high copy number) form small aggregates that develop a precocious tip, suggesting a role for Gα5 functions in prestalk cell development (62). Given the similarities of *Gα4* and *Gα5* gene structure and expression, it seems possible that these subunits might interact with similar or identical signaling components to coordinate the development of prespore and prestalk cells. In this study, we investigated the relationship of Gα4 and Gα5 subunit function during the developmental life cycle to determine if these subunits and their respective pathways interact with each other. We found that these subunits promote complementary developmental processes that function antagonistically with respect to cell differentiation and developmental morphogenesis. We also provide evidence that these subunits are expressed in different subsets of cells and that these functions cannot fully compensate for the loss of each other when expressed from endogenous or heterologous promoters.

Materials and Methods

Phylogenetic Analysis

The phylogenetic analysis of the *Dictyostelium* Gα subunits was conducted using the PHYLIP phylogeny inference program package (45). The predicted amino acid sequences of the *Dictyostelium* Gα subunits were aligned with minimal gapping between most subunits by using the internal conserved regions as guides. Two large regions of the Gα3 subunit that were not present in any of the other Gα subunit sequences were omitted from the phylogenetic analysis. The PROTDIST program from the PHYLIP package was used to calculate distance units and these units were processed by the Fitch program to produce a single best unrooted tree. The order of input data into the Fitch program was jumbled 200 times allowing 20 trees to be analyzed with only the best tree being selected. The Treeplot program of the PHYLIP package was used to plot tree branches with lengths proportional to the distance units.

Strains and Media

The following axenic haploid *Dictyostelium* strains were used: KAx-3 (wild-type) (161), the *gα5* null mutant strain JH257 (*(gα5::THY1)* described in (62)), the *Gα5* overexpression strain JH258 (contains a high copy number of the *Gα5* expression vector pJH206 ($G\alpha5^{HC}$) as described (62)). In addition the following strains were also used: the *gα4* null mutant strain JH142 (*gα4::PYR5-6* described in Hadwiger *et al.* (58) and the *Gα4* overexpression strain (that contains a high copy number of the *Gα4* expression vector pJH154, $G\alpha4^{HC}$) JH202 (58).

All strains that were used in the study were isogenic to the wild-type strain KAx-3, except at loci noted and all strains were grown at room temperature either in HL-5 medium or with *Klebsiella aerogenes* on SM nutrient agar plates, unless otherwise noted. All strains were grown in axenic HL-5 medium unless otherwise noted. All strains were isogenic to the axenic strain KAx-3 except at the loci noted. To create the double *gα4/gα5* null mutant,

the *PYR5-6* allele of the *gα5* null mutant strain was replaced with a mutated *pyr5-6* allele (carrying a missense mutation) by homologous recombination in a strategy previously described for other strains. The selection for a mutant *PYR5-6* gene was based on resistance to the drug 5-F0A to get strain JH333. A wild type *PYR5-6* allele was then used as a selectable marker to disrupt the *Gα4* locus as previously described in the construction of other *gα4* null strains (58). In brief, the wild-type *Gα4* gene in pJH56 (58) was disrupted at the *Bgl*II site with a functional *PYR5-6* gene (as a *BamH*I fragment from pJH58). This construct was linearized at the *Eco*RI site and then electroporated into JH333 cells as described earlier (37) to allow for homologous recombination at the *Gα4* locus and thus yield a *gα4/gα5* double null strain (JH334). Transformants were selected based on their ability to grow in the absence of uracil. Minimal medium (GIBCO/BRL) was used to select against uracil auxotrophs. Clonal isolates were obtained by plating transformed cell lines in 24 or 96 well microtiter plates.

Transformants with *gα4* null characteristics were isolated and analyzed by genomic DNA blots to confirm the disruption of the *Gα4* locus as previously described for other strains. *Dictyostelium* strains were electroporated using methods previously described by Dynes and Firtel (37) and, in each case, several independent transformants were collected and examined for developmental phenotypes. Developmental gene expression was examined by RNA blot analysis as previously described (106). All DNA probes used for hybridization were created by random primer probe synthesis as described by Feinberg and Vogelstein (44). All strains were grown in axenic HL-5 medium unless otherwise noted. Minimal medium (GIBCO/BRL) was used to select against uracil auxotrophs.

DNA Constructs

Molecular cloning techniques such as restriction enzyme digestion, ligation, bacterial transformation and screening of bacterial transformants were carried out in *E. coli* strains using standard recombinant DNA techniques as described in Maniatis *et al.* (105).

Gα4 and *Gα5* expression vectors (pJH154 and pJH206, respectively) that express the *Gα* genes from their respective promoters have been previously described (58, 62). Vectors expressing the *Gα4* gene from the *Gα5* promoter were created by fusing the *Gα5* promoter sequence to a *Gα4* gene that had been truncated 56bp upstream of the *Gα4* open reading frame. The truncated *Gα4* gene was constructed by inserting a *SalI/Nco*I fragment from a previously described PCR product of the Gα4 amino terminus (primers JH19 and JH20) (61) into the same sites of the *Gα4* gene vector pJH56, to create the vector pCA264. A *PstI/Eco*RV fragment containing the *Gα5* promoter from pJH198 was inserted into the same sites of pBluescript IISK$^+$ (Stratagene) and then the amino terminus coding region and 16 bp of upstream sequence of this *Gα5* sequence was removed by deleting a *Hin*dIII/*Ase*I fragment (regenerated the *Hin*dIII site). This *Gα5* promoter sequence was inserted as a *PstI/SalI* fragment into the same sites of pCA264 to create a *Gα5* promoter/*Gα4* open reading frame fusion vector pKN5 and then an *Eco*RI fragment of pJH64 containing the *pAct6::Neo^r* gene was inserted at the *Eco*RI site of pKN5 to create the *pGα5::Gα4ORF-ter4* expression vector (pKN7). The plasmid pJH64 had been previously created by adding a *Eco*RI linker to the *Sal*I site of pB10SX and then transferring the *pAct6::Neo^r* gene as a *Eco*RI fragment into pBluescriptIISK+. To construct the *pGα5::Gα4orf-5ter* gene fusion that contained *Gα5* rather than *Gα4* termination sequences, the open reading frame of the *Gα4* gene from pJH56 was amplified by PCR using the oligonucleotides JH2079 5'-GCCGGATCCGAATGAGATTCAAGTGTTTTGG-3' and JH2080 5'-GCCCGAATTCTAGAAGTGTTCTAAAGCTTGAG-3'and inserted as a *Bam*HI/*Eco*RI fragment into the same sites of pBluescriptIISK+, creating plasmid pKN31. A *Hinc*II/*Kpn*I fragment containing downstream *BclI/Hinc*II(both sites lost) fragment of pJH198 and the *pAct6::Neo^r*gene from pJH64 was inserted into the same sites of pKN31. The *BglII/Kpn*I fragment of this vector which contained the *Gα4* carboxyl terminus region,

the *Gα5* termination sequence, and the *pAct6::Neo*^{r} gene was inserted into pKN5 to give the *pGα5::Gα4ORF-ter5* expression vector (pKN33).

The *Gα5* gene driven by the *discoidin-1* promoter was constructed by isolating the *discoidin-1* promoter as a *Pac*I/ *Xho*I fragment from pVEII (9). This fragment was then inserted it into the same sites of the pBluescriptIISK+ which had a *Pac*I site created at the *Sal*I locus (pJS12) by using a linker, to give pJS13. A *Hin*dIII/*Eco*RI fragment from pJH268, containing the*Gα5* open reading frame and 17bp upstream of the start codon, was isolated from pJH268 and then inserted downstream of the *discoidin-1* promoter in pJS13 to give pKN37. The vector pJH268 was created by a *Hin*dIII/*Ase*I deletion (*Hin*dIII site regenerated) of pJH198 (62). The *Eco*RI fragment from pJH64 containing the *pAct6::Neo*^{r} gene was then introduced into pKN37 to give pKN44.

A total of seven different reporter constructs were made using the *Gα4* and *Gα5* promoters. The promoter of *Gα5* fused to the *lacZ* gene with the Blasticidin (*Bsr*^{r}) resistance cassette, *pGα5::lacZ-Bsr*^{r}, was created by inserting a 1.5kb *Eco*RI fragment of pKN14 carrying the *Bsr* ^{r}gene into the same site of pJH209(62). pKN14 was created by isolating the *Bsr* ^{r}gene as a *Bam*HI fragment from pJH280 (which contains the *Bsr* ^{r}gene from pUCBsrδBam (153) as a *Hin*dIII/*Kpn*I in the same sites of SK$^-$) and inserting it into pT7T318U. A Green Fluorescent Protein (18) reporter construct, *pGα5::GFP-G418*^{r}, carrying the promoter of *Gα5* and the *G418*^{r} gene was created by inserting an *Nhe*I site at the *Eco*RV site about 50bp downstream of the ATG in *Gα5* using linker JH2313 (5'-ATCGCTAGCGAT-3') to give pKN9. The GFP open reading frame was isolated from JH297 as a 0.7kb *Nhe*I/ *Bam*HI fragment and inserted into the *Nhe*I/*Bcl*I sites of pKN9 to give pKN12 which contained the GFP open reading frame downstream of the *Gα5* promoter with *Gα5* termination sequences. The *G418*^{r} gene was isolated as a *Bam*HI fragment from pJH63 and inserted into the same site of pKN12 to give pKN21. A GFP reporter construct (*pGα5::GFP*-G418*^{r}), carrying a mutated version of the *GFP* allele which allows for higher fluoresence (28), was constructed by replacing the *Nco*I/ *Bam*HI

piece of pKN12 with a 0.5kb PCR fragment cut with the same restriction enzymes (pKN23). The 0.5kb mutated *GFP* allele was amplified from a *KpnI*/ ScaI fragment of pJH322 by PCR using oligonucleotides JH2363 (5'-CCTGTTCCATGGCCAACACTTGTCACTACTTTCACTTATGGTGTTC-3') and the universal reverse primer. A fragment carrying the promoter of *Gα5* with the mutated *GFP* was isolated as a *NheI*/ *Bam*HI fragment from pKN23 and inserted into the *NheI*/*BclI* sites of pKN9 to give pKN25. The *G418*^{r} gene was isolated as a *Bam*HI fragment from pJH63 and inserted into the same site of pKN25 to give pKN27.

A set of four reporter vectors carrying the gene encoding two different versions of the β-glucouronidase enzyme (*gusA*) (74) were created. One of vectors used, pHUgus, is one in which the Gus protein is post-translationally cleaved to yield a Gus enzyme that is always the same and hence independent of the N-terminal sequences brought in by the promoter (175). These vectors carry the hygromycin resistance gene for purposes of selection in *Dictyostelium* (39). A *Gα5* reporter construct using this version of *gusA* (HUgus) was created by isolating the *Gα5* promoter from pKN46 as a 1kb *Bam*HI fragment and inserting it into the same site of pHUgus3. This put the HUgus open reading frame in frame with the amino terminus sequences of Gα5 to give pKN047 (*pGα5::HUgusA-Hyg*^{r}). pKN46 was created by inserting a *Bam*HI linker (oligonucleotide JH4056 (5'-GGGATCCCTGCA-3')) at the *Pst*I locus in pJH217, thereby duplicating the *Pst*I site. The second version of the *gusA* gene, pSAgusA, has a nuclear localization signal upstream of its open reading frame (38). The reporter construct pKN49, carrying the *Gα5* promoter with pSAgusA (*pGα5::SAgusA-G418*^{r}), was created by isolating the *Gα5* promoter as a 1kb *XbaI*/ *BglII* fragment from pKN47 and insert it into the same sites of pSAgusA. The vector pSAgusA carried the *G418*^{r} gene for purposes of selection in *Dictyostelium.*

Similar constructs using the two versions of the *gusA* gene were also created using the *Gα4* promoter. The *Gα4* promoter (with its own ATG) was isolated as a 2.1kb *Bam*H1 fragment from pJH152 and inserted into the *Bam*HI site of pSP73 to give pKN50.

The reporter construct carrying the promoter of *Gα4* fused to the *SAgusA* gene (*pGα4::SAgusA-G418*^{r}) was created by isolating the *Gα4* promoter as a 2.1kb *Xba*I/ *Bgl*II fragment from pKN50 and inserting it into the same sites of pSAgusA to give pKN52. The reporter construct carrying the *HUgus* fused to the *Gα4* promoter, pKN53, was created by inserting the same 2.1kb *Xba*I/ *Bgl*II fragment containing the *Gα4* promoter from pKN50 into the same sites of pHUgus3 (*pGα4::HUgus-Hyg*^{r}).

Histochemical Staining

Cells were grown to mid-log phase as shaking cultures in HL-5 medium. The cells were then washed free of medium and resuspended in 12mM phosphate buffer (pH 6.1). They were subsequently spread on nitrocellulose filters resting on top of non-nutrient agar plates for development. At various time points through development as well as during vegetative growth, the expression of the *Gα* promoter/reporter gene fusions were monitored by histochemical staining. The glucouronidase staining was done first, using X-Gluc (Sigma) as the substrate (74). The β-galactosidase staining was done second as described, using Magenta-Gal (Sigma) as the substrate (57). X-Gluc, when metabolized by the Gus enzyme, would produce a blue color while the magenta-gal would be metabolized by β-galactosidase to form a red colored product.

Development and Chemotaxis Assays.

Cells were grown to mid log phase (2 x 10^6 cells/ml) and washed in phosphate buffer (12 mM NaH_2PO_4, adjusted to pH 6.1 with KOH), and then resuspended at 2 x 10^7 cells/ml in the phosphate buffer unless otherwise noted before plating on nonnutrient plates (1.5% granulated agar in phosphate buffer) for development or chemotaxis assays. Development for RNA isolation was conducted in a similar manner except that cells were plated at a density of 1x10^8 cell/ml on Whatmann 50 filters saturated in phosphate buffer. Chemotaxis to folic acid was assayed as previously described by placing droplets of cells

and 1mM folic acid within 2 mm of each other on nonnutrient plates and then monitoring for directed cell movement over three hours. Development of cells in the presence of folic acid was conducted as previously described by Hadwiger and Srinivasan (60). Briefly, cells were grown in the presence of 1mM folic acid and then developed on nonutrient agar containing 100μM folic acid.

Cells were fluorescently labeled with CMFDA Celltracker Green (Molecular Probes) as described by Hadwiger and Srinivasan(60). Briefly, cells were grown to mid-log phase, washed in 12mM phosphate buffer and resuspended to a density of $2x10^7$ cells/ml. Cells were incubated for 30 minutes in the dark with 1mM cell-tracker dye CMFDA. Chimeric organisms were created by mixing the labeled cells with unlabeled wild-type cells in a ratio of 1:9. The cell mixture was washed twice in phosphate buffer and then spotted on nonnutrient plates at a density of $2x10^7$ cells/ml for development.

cGMP Assays

Cells grown in shaking culture to a concentration of $3x10^6$ cells/ml were harvested and washed twice in 12mM phosphate buffer solution. The cells were then resuspended in phosphate buffer at a concentration of $5x10^7$ cells/ml and stimulated with a 10^{-4} M concentration of folic acid as described earlier (59). Aliquots were then taken at the times indicated and analyzed for accumulation of cGMP by using a cGMP radioimmunoassay kit (Amersham). The amount of cGMP was normalized with respect to protein concentrations of the stimulated cell culture. Protein concentrations were determined using a dye binding assay (14).

Results

Phylogeny of *Dictyostelium* Gα Subunits

An unrooted phylogenetic tree of seven *Dictyostelium discoideum* Gα subunits (Gα1 - Gα8, except for Gα6) was created by using the PHYLIP phylogeny inference program (Figure 25). The primary sequence of these Gα subunits were obtained from previous reports or from GenBank records and the alignments included full length sequences from all subunits except for two large regions that are unique to the Gα3 subunit (15). The branch lengths of the tree represent the calculated distance units between the different Gα subunit sequences. The relatively short branch lengths between the Gα4 and Gα5 subunits were consistent with these subunits sharing the greatest percentage of identity (51%) between any pair of subunits. The branch lengths of the Gα1 and Gα2 subunit pairing were also relatively short as these subunits share 48% identity. The branch lengths were much greater for all other combinations of Gα subunits, consistent with the high degree of diversity among most *Dictyostelium* subunits, and none of the other pairs of Gα subunit exceeded 45% identity, with most of the identity being restricted to the guanine nucleotide binding regions conserved among most Gα subunits. The *Dictyostelium* Gα6 subunit sequence was not included in this phylogenetic analysis because of incomplete sequence data but the known sequence of this Gα subunit shares only 42% and 40% identity to the Gα4 and Gα5 subunits, respectively, in a region 51% identical between Gα4 and Gα5. The relatedness of the Gα4 and Gα5 subunit sequences was also supported by unrooted phylogeny trees determined by the Parsimony method included in PHYLIP package (data not shown).

Gα4 and *Gα5* genes Function in Complementary Developmental Processes

While the *Gα4* and *Gα5* genes share sequence similarity, the developmental phenotypes associated with loss or overexpression of these genes are quite different and these difference are exemplified by the morphogenesis of tip formation in aggregates of *Gα*

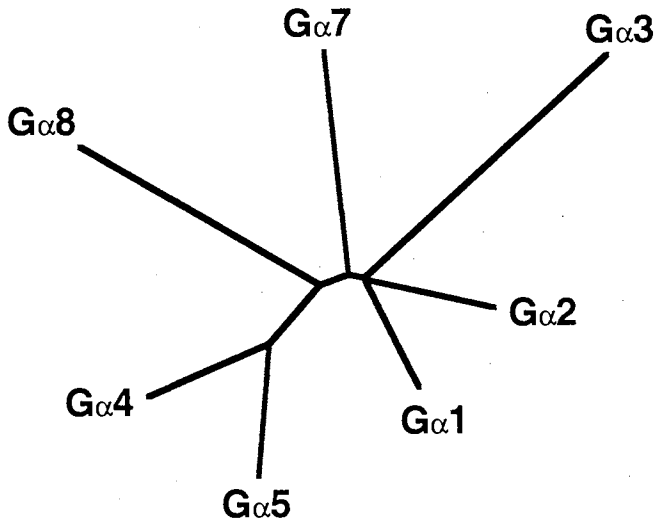

Figure 25. Unrooted phylogenic tree showing degree of identity in amino acid sequence between seven different Gα subunits in *Dictyostelium discoideum*, as determined by the PROTDIST protein sequence program in the PHYLIP phylogeny inference package. The branch lengths are representative of the distance maps as determined by the Fitch program of the PHYLIP package.

mutants (Figure 26). Aggregates of *gα4* null cells form a tip, composed of prestalk cells, that extends away from the mound, leaving behind the majority of prespore cells. The remaining mound will often produce a new tip as the previous tip moves away, resulting in a multiple tip phenotype. In contrast, *gα5* null aggregates typically form large aggregates that are delayed in tip formation, but once the tip is established the entire mound will participate in the formation of a migratory slug. Tip formation can be completely blocked in aggregates of $G\alpha4^{HC}$ cells and, like the *gα5* null cells, aggregate size is typically much larger than that of wild type cells. Aggregates of $G\alpha5^{HC}$ cells are typically small and form precocious tips compared to wild type aggregates. RNA blot analysis indicated that the level of *Gα4* gene expression in $G\alpha5^{HC}$ and *gα5* null cells was similar to that of wild type cells during vegetative growth and throughout development, suggesting that the altered tip development of *Gα5* mutants was not due to changes in *Gα4* expression (Figure 27). Likewise, *Gα5* expression was not altered in *Gα4* mutants except for a slightly lower level of *Gα5* expression in $G\alpha4^{HC}$ cells after developmental block at the mound stage, indicating that *Gα5* gene expression is not greatly influenced by *Gα4* function (data not shown). The expression of the *Gα5* gene also did not appear to be altered significantly by the presence of folic acid at concentrations that delay tip morphogenesis (data not shown).

Previous studies have indicated that chemotactic responses to folic acid require Gα4 function and that overexpression of the Gα4 subunit increases the responsiveness of cells to folic acid (59). To determine if Gα5 function is also important in this response, we tested the ability of *Gα5* mutants to chemotax to folic acid. The chemotactic responsiveness of *gα5* null mutants to folic acid appeared to be similar to that of wild type cells, indicating that Gα5 function is not essential for this response, but the overexpression of the Gα5 subunit significantly delayed chemotactic movement to folic acid during the first few hours of starvation, suggesting that overexpression of the *Gα5* gene interferes with this response (Figure 28A-E). After this initial delay in chemotactic responsiveness, $G\alpha5^{HC}$ populations display chemotactic movement but this movement differs from that of

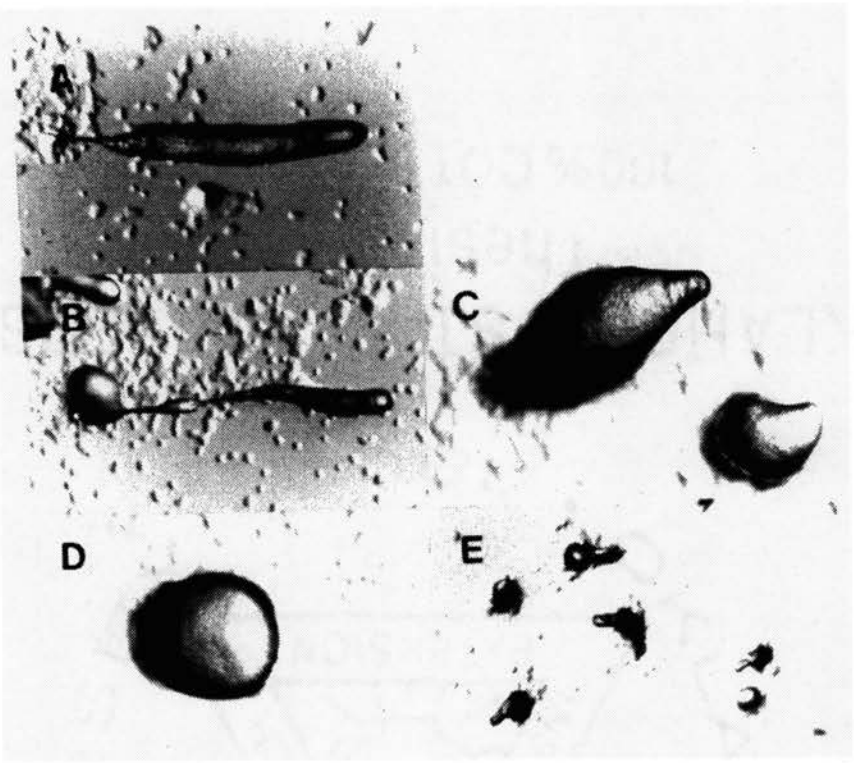

Figure 26. Morphological phenotypes associated with *Gα4* and *Gα5* mutants. Cells were grown to mid-log phase in shaking culture, washed in phosphate buffer, and then plated for development on nonnutrient plates as described in the Materials and Methods section. Photographs were taken at 15 hours after starvation. Developmental morphology associated with (A) Wild-type cells (B) *gα4* null cells (C) *gα5* null cells (D) $G\alpha4^{HC}$ cells (E) $G\alpha5^{HC}$ cells

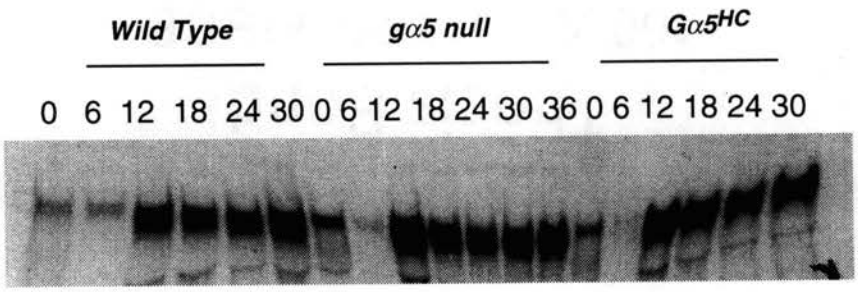

Figure 27. *Gα4* gene expression in the wild-type cells, *gα5* null mutants and $G\alpha5^{HC}$cells. Cells were grown in shaking culture to a density of $3x10^6$ cells/ml, washed with phosphate buffer, and plated on filters for development. Total RNA was isolated from cells at times indicated during development (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 blot was hybridized with a *Gα4* probe that covers the entire *Gα4* open reading frame.

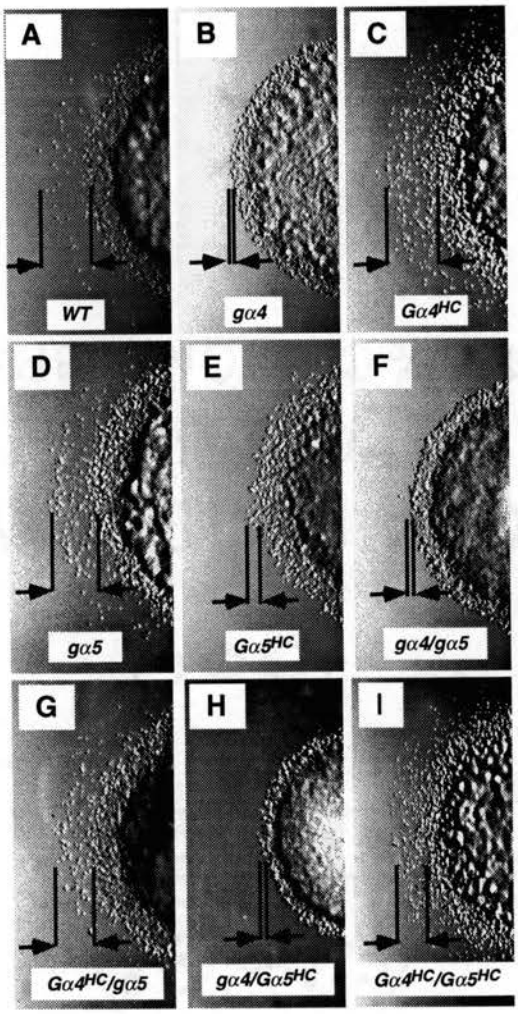

Figure 28. Chemotaxis of wild type, *Gα4,* and *Gα5* mutant cells to folic acid. Cells were prepared for development as described in figure 26 but then were plated at a concentration of $1x10^8$ cells/ml for chemotaxis assays as described in the Materials and Methods. Photographs were taken three hours after cells were plated. Panel A - Wild-type cells, B - *gα4* null cells, C - $G\alpha4^{HC}$ cells, D - *gα5* null cells, E - $G\alpha5^{HC}$ cells, F - *gα4/gα5* double null cells, G - *gα5* null overexpressing the *Gα4* gene, H - *gα4* null cells overexpressing the *Gα5* gene, and I - $G\alpha4^{HC}/G\alpha5^{HC}$ double overexpression mutant cells. Folic acid source is located to the left of all cell droplets. Bars represent the leading edge of chemotaxis and cell droplet.

wild type or *Gα4*^{HC} populations in that there are typically fewer cells on the leading edge of chemotaxing cells. *Gα5* overexpression and *gα5* null cells were also tested for the ability to accumulate cGMP in response to folic acid stimulation since cGMP is thought to be an important second messenger in *Dictyostelium* chemotactic responses (96, 120). Cells overexpressing the *Gα5* gene displayed low basal levels of cGMP, even after folic acid stimulation, that are consistent with inability of these cells to chemotax at this stage in development (Figure 18). Cells lacking the *Gα5* gene consistently displayed slightly higher levels of cGMP accumulation, before and during the response to folic acid, as compared to that of wild type cells. However, the relative increase of cGMP was less in *gα5* null cells than in wild type cells because *gα5* null cells have a higher basal level of cGMP before the stimulation. The reduced responsiveness of *Gα5*^{HC} cells to folic acid in early development did not allow these cells to escape the *Gα4*-mediated inhibitory effects of folic acid on the timing of tip morphogenesis described for wild type cells (Figure 29).

Previous studies of *Gα4*^{HC} and *gα4* null cells in chimeric organisms have indicated that Gα4 functions cell-autonomously to promote cell localization to the central prespore region of developing aggregates. To determine if *Gα5* mutants also have altered cell distribution within chimeric organisms, we labeled *Gα5* mutants with CMFDA (Cell Tracker Greeen) and monitored their distribution with respect to wild type cells in developing aggregates (Figure 30). Labeled *gα5* null cells were similar to *Gα4*^{HC} cells in that they localized to the central prespore region of the aggregates but the *gα5* null cells were not completely restricted from the anterior prestalk region. In contrast, *Gα5*^{HC} cells were found primarily near the posterior of the chimeric organism similar to the distribution observed for *gα4* null cells.

To determine whether the phenotypic differences between *Gα4* and *Gα5* mutants in aggregate size and tip morphogenesis are due to common or distinct developmental defects, chimeric organisms were constructed with equal amounts of *Gα4* and *Gα5* mutant cells and analyzed for tip morphogenesis and aggregate size (Figure 31). Four different

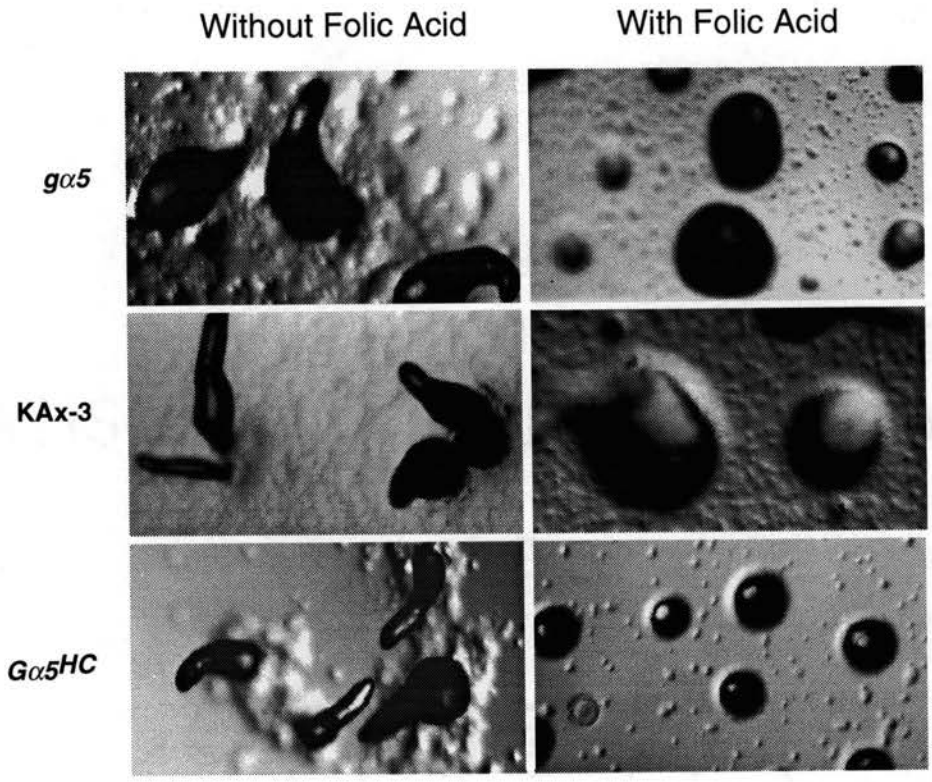

Figure 29. Development of wild-type, *gα5* null and $G\alpha5^{HC}$ cells with or without folic acid. Folic acid treated cells were grown in fresh media with 1mM Folic acid four hours before plating for development on non-nutrient gar containing 100μM folic acid, as described in the materials and methods. Control cells (without folic acid treatment) were grown and developed under identical conditions except for the absence of exogenous folic acid in the growth medium and non-nutrient agar. Developing structures were photographed at the same magnification when the control cells had reached the tip formation stage - Wild-type cells at 13 hours after the onset of starvation; *gα5* null cells at 16 hours post-starvation; $G\alpha5^{HC}$ cells at 10 hours of development.

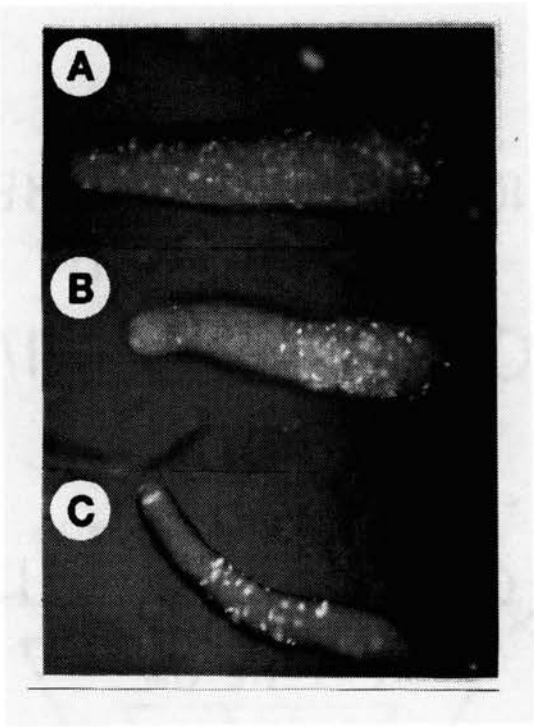

Figure 30. Localization of *Gα5* mutant cells in chimeric organisms. The *Gα5* mutant cells and wild type cells were prepared for development as described in figure 26. The *Gα5* mutants cells fluorescently labeled and mixed with unlabeled wild type cells as described in the materials and methods section. Photographs were taken after 18 hours of development on nonnutrient plates. Panel A - labeled wild-type cells, B - labeled $G\alpha5^{HC}$ cells, and C - labeled *gα5* null cells. The anterior of each organism is on the left of each panel.

TABLE 4

Combination of Gα4 and Gα5 mutant cells used in chimera analysis

	$G\alpha4^{HC}$	*gα4* null
$G\alpha5^{HC}$	Compensation	Exaggerated prestalk development
gα5 null	Deficient in prestalk development	Compensation

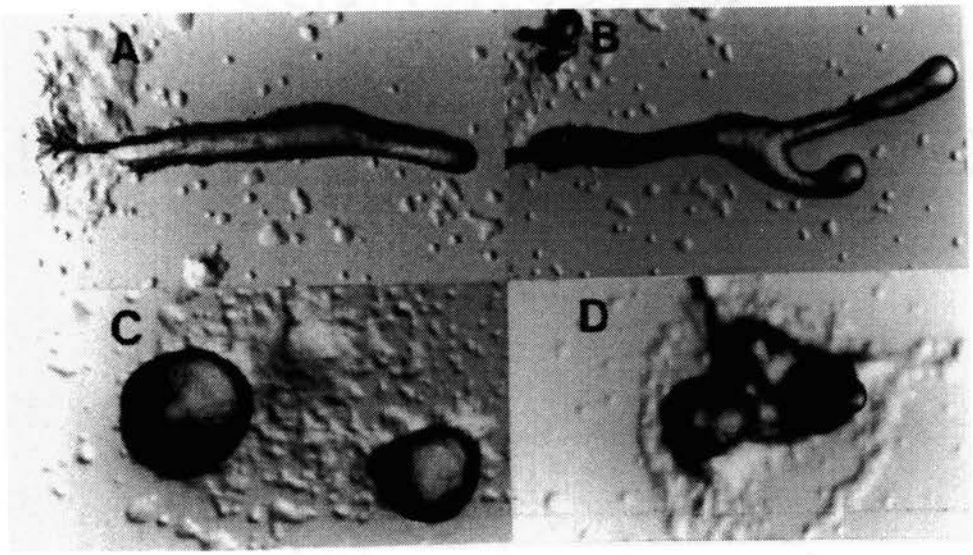

Figure 31. Chimeric organisms created with *Gα4* and *Gα5* mutant cells. Two different cell types were grown to mid-log phase in shaking culture, washed in phosphate buffer and mixed in a 1:1 ratio before being plated for development on non-nutrient plates. Photographs were taken after 15 of development. Panel A - *gα4* null/*gα5* null chimeras, B - *gα4* null cell/$G\alpha5^{HC}$ chimeras, C - $G\alpha4^{HC}$/*gα5* null chimeras, and D - $G\alpha4^{HC}$ /$G\alpha5^{HC}$ chimeras. Anterior of organisms in panels A and B are on the right.

combinations of mutant cells were used to create the chimeras and studied as indicated in Table 4. Mixtures of *gα4* null and *gα5* null cells resulted in aggregates with relatively normal tip morphogenesis and slug development, indicating that this combination of strains can compensate for the early developmental deficiencies specific to the individual strains. Chimeric organisms composed of $G\alpha4^{HC}$ and $G\alpha5^{HC}$ cells initially formed large loose aggregates but these aggregates separated into smaller aggregates that formed tip structures similar to those of wild type aggregates. Other combinations of *Gα* mutant mixtures, such as *gα4* null/$G\alpha5^{HC}$ or *gα5* null/$G\alpha4^{HC}$ chimeras, developed with much more distorted morphology compared to aggregates composed only of null mutants or overexpression mutants. Chimeras composed of *gα4* null and $G\alpha5^{HC}$ cells formed aggregates of different sizes and the larger of these aggregates formed slugs that aberrantly continued to produce new tips, even during slug migration. Chimeric mixtures of *gα5* null and $G\alpha4^{HC}$ cells produced aggregates that were delayed several hours, similar to that observed for *gα5* null mutants, and the aggregate size of these chimera was also much larger than that observed for wild type cells.

Chemotactic and Developmental Phenotypes of Gα4 and Gα5 Double Mutants

To investigate potential epistatic relationships between Gα4 and Gα5 function, we created strains containing null mutations in both *Gα* genes by disrupting the *Gα4* gene in a *gα5* null mutant (see Material and Methods for details). Transformants with *gα4* null DNA isolated from the JH333 cells served as a control. A 1.5kb *Bam*HI/*Eco*RI fragment from pJH317 (described in the DNA constructs section) containing the *Gα4* open reading frame was used to make the probe for the hybridization as previously described (44). Transformants that showed the predicted altered pattern were picked for the analysis. Clonal populations of this double null mutant aggregated to form relatively large mounds that were delayed in tip formation, typical of the phenotypes associated with *gα5* null

mutations. After this delay, tips formed and proceeded to extend away from the aggregate with a morphology very similar to that of *gα4* null mutants (Figure 32 A-B). Relatively few spores were detected in the double null mutants by visual examination and individual cells were completely nonresponsive to the chemoattractant folic acid, consistent with the dependency of these processes on Gα4 function (Figure 28 F). This also indicated that the loss of Gα5 function does not rescue *gα4* null defects.

Chemotactic and developmental phenotypes were also examined for cells that overexpress both the *Gα4* and *Gα5* genes. These $G\alpha4^{HC}/G\alpha5^{HC}$ strains were created by introducing a *Gα5* expression vector confering resistance to the drug G418 into a previously constructed G418-sensitive $G\alpha4^{HC}$ strain. Transformants with high levels of drug resistance (growth in the presence of > 10 μg/ml G418) were selected and analyzed for developmental morphology. To verify the incorporation of a high copy number of transformants, genomic DNA was isolated from these potential transformants and a southern blot hybridization was carried out as described earlier. A 1.5kb *Eco*RI/*Eco*RV fragment of pJH214 carrying the *Gα5* open reading frame was used to make a radiolabeled probe as described earlier for this analysis. Genomic DNA isolated from JH202 cells served as the control. Those transformants that showed a high incorporation of the *Gα5* gene into the genome of the JH202 ($G\alpha4^{HC}$) cells were then picked analyzed for developmental morphology, gene expression and chemotaxis.

The $G\alpha4^{HC}/G\alpha5^{HC}$ cells formed aggregates that differentiated into finger-like structures but this process occurred without the formation of a conical tip at the anterior (Figure 32 C-D). Developmental morphogenesis of the $G\alpha4^{HC}/G\alpha5^{HC}$ mutant terminated at this aberrant finger stage of development without detectable spore production, as determined by visual inspection of dissected aggregates. Cell-type specific gene expression was also analyzed by monitoring the expression of prespore (*cotC*) and prestalk (*ecmA*) specific genes over time. Total RNA was isolated from the cells at different times in development and RNA blots were done as described earlier. Fragments of cDNA of *ecmA*

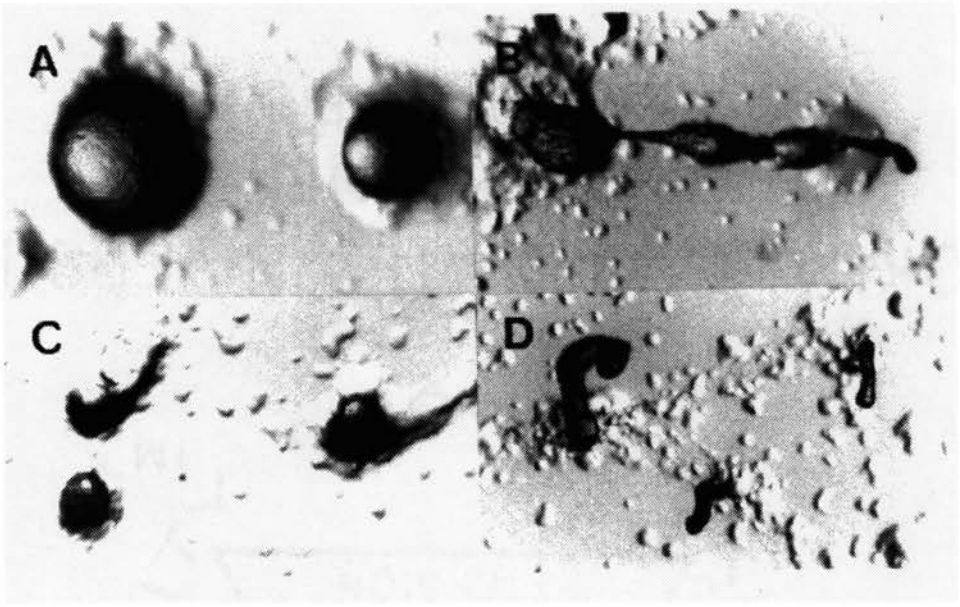

Figure 32. Morphological phenotypes of the double mutant strains. Cells were plated for development as described in Figure 26. Photographs were taken either 14 hours (A and C) or 30 hours (B and D) after starvation. Panels A - *gα4/gα5* double null mutant cells at 14 hrs, B - *gα4/gα5* double null mutant cells at 30 hrs, C - $G\alpha4^{HC}/\ G\alpha5^{HC}$ double overexpression cells at 14 hrs, and D - $G\alpha4^{HC}/\ G\alpha5^{HC}$ double overexpression cells at 30 hrs.

and *cotC* were radiolabeled and used as probed for the hybridizations. Prestalk and prespore specific gene expression of the $G\alpha4^{HC}/G\alpha5^{HC}$ cells resembled that of wild type cells suggesting that *Gα5* overexpression can at least partially rescue the loss of cell type specific gene expression associated with the overexpression of the *Gα4* gene (Figure 33). At the onset of development, the chemotactic responsiveness of the $G\alpha4^{HC}/G\alpha5^{HC}$ cells to folic acid was similar to that observed for wild type cells (Figure 28 I). The $G\alpha4^{HC}/G\alpha5^{HC}$ cells were also tested for cell localization in chimeric organisms by labeling the mutant cells with CMFDA (Cell tracker green) and mixing these cells in a ratio of 1:9 with wild type cells. The mutant cells were localized to the central and posterior regions of the aggregate throughout development of the chimeric organisms (Figure 34). This pattern of cell localization resembled a combination of patterns exhibited by the $G\alpha4^{HC}$ cells (localized to the central region) and the $G\alpha5^{HC}$ cells (localized to the posterior end). The absence of $G\alpha4^{HC}/G\alpha5^{HC}$ cells in the anterior region of chimeric organisms suggested that anterior prestalk cell development is aberrant even though prestalk gene expression in $G\alpha4^{HC}/G\alpha5^{HC}$ clonal aggregates is similar to that of wild-type aggregates.

Gα4 and *Gα5* Genes Fail to Compensate for the Loss of Each Other

While *Gα4* and *Gα5* genes encode Gα subunits with structural similarity, neither gene can fully compensate for the loss of the other as indicated by phenotypes associated with the *gα4* and *gα5* null mutants (58, 62). The lack of compensation might be due to differences in protein structure that affect interactions with other proteins in their pathways or differences in gene expression that affect subunit level and distribution. To determine if increased gene dosage allows one subunit gene to suppress the null mutant phenotype of the other subunit gene, we transformed *gα5* null mutants with the *Gα4* expression vector and transformed *gα4* null mutants with the *Gα5* expression vector. Overexpression of the *Gα5* gene due to increased gene dosage did not appear to alter any of the morphological

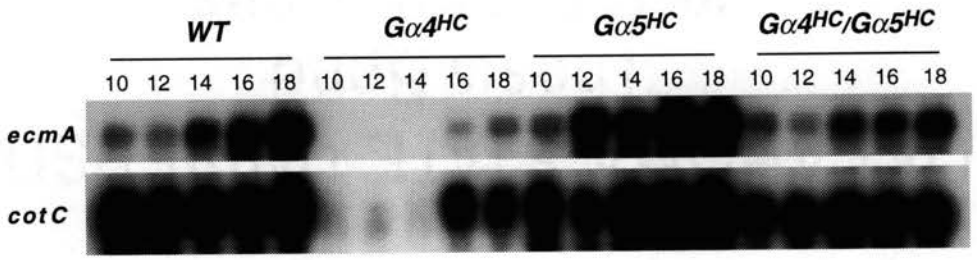

Figure 33. Expresssion of prestalk (*ecmA*) and prespore (*cot C*) genes in wild type, $G\alpha 4^{HC}$, $G\alpha 5^{HC}$ and $G\alpha 4^{HC}/G\alpha 5^{HC}$ double overexpression mutant cells. Cells were grown in shaking culture to a density of $3x10^6$ cells/ml, washed with phosphate buffer, and plated on filters for development. Total RNA was isolated from cells at times indicated during development (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 an *ecmA* probe or a *cotC* probe.

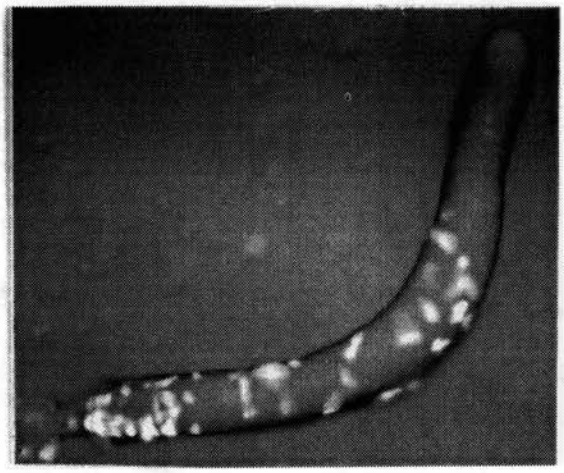

Figure 34. Localization of the double overexpression strain in a chimeric organism. The $G\alpha4^{HC}/G\alpha5^{HC}$ double overexpression cells were labeled with the cell tracker dye and mixed in a ratio of 1:9 with unlabeled wild-type cells to create a chimeric organism as described in Figure 30. The photograph was taken at 18 hours of development.

defects of developing *gα4* null cells or rescue the chemotactic responsiveness of these cells to folic acid (Figure 28 H). Likewise, the overexpression of the *Gα4* gene in *gα5* null cells did not rescue the timing of tip morphogenesis or reduce aggregate-size defects associated with the loss of the *Gα5* gene. Rather, the increased *Gα4* gene dosage completely prevented tip formation and subsequent developmental morphogenesis as it does in wild type cells. Overexpression of the *Gα4* gene did not appear to dramatically alter the chemotactic responsiveness of *gα5* null cells to folic acid (Figure 28 G).

The ability of *Gα4* or *Gα5* gene to compensate for the loss of each other was also tested by expressing these genes from heterologous promoters. A *Gα4* expression vector, *pGα5::Gα4(ORF)-5ter*, was constructed in which the *Gα4* open reading frame was fused between the *Gα5* promoter and downstream noncoding sequences of a *Gα5* expression vector (Figure 35). A DNA blot as described by Southern (149) was done to verify the incorporation of the chimeric genes into the genome. Genomic DNA was isolated from cells as described by Nellen *et al*(118), digested with the restriction enzyme *Hin*dIII, will be electrophoresed through a 0.7% agarose gel and blotted to nitrocellulose. Wild-type (KAx-3) genomic DNA served as the control. The *Gα4* open reading frame, isolated as a 1.5kb *Eco*RI-*Bam*HI fragment from pJH317, was radiolabeled and used for hybridization. Incorporation of the chimeric gene was confirmed by the hybridization of a 1.5kb *Hin*dIII fragment internal to the Gα4 open reading frame. In comparision, the wild-type *Gα4* hybridizes a 2kb *Hin*dIII fragment in the genome of wild-type cells. When present at a high copy number, the *pGα5::Gα4(ORF)-5ter* gene fusion was able to partially suppress the delayed tip morphogenesis in *gα5* null mutants but the gene fusion was not able to suppress the large aggregate phenotype associated with the loss of Gα5 function (Figure 36). The *pGα5::Gα4(ORF)-5ter* gene fusion was capable of rescuing folic acid chemotaxis and fruiting body development in *gα4* null cells indicating that there might be some overlap in the spatial expression patterns of the *Gα4* and *Gα5* genes (Figure 37 and Figure 38). Another *Gα4* expression vector with a gene fusion that contained *Gα4* downstream

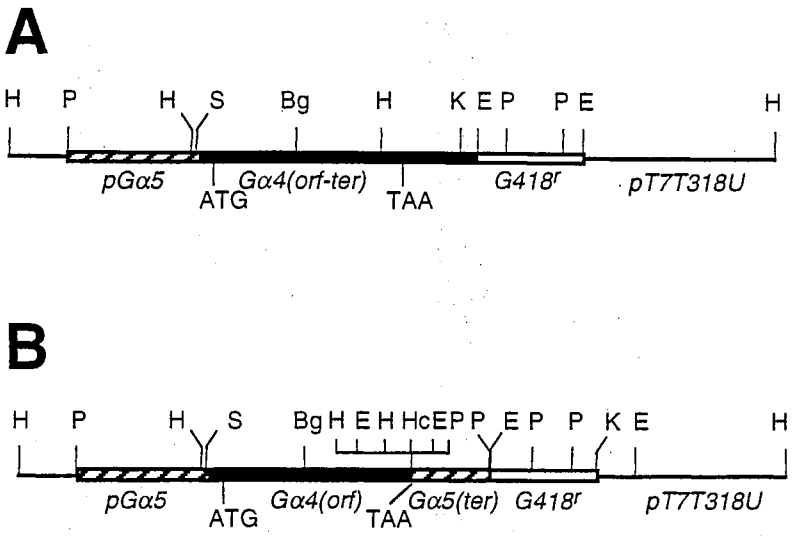

Figure 35. Vector constructs that contain *Gα4* open reading frame fused to the promoter of the *Gα5* gene. Restriction site maps of the *pGα5::Gα4orf-ter4* (A) and *pGα5::Gα4orf-ter5* (B) vectors that contain either the *Gα4* or *Gα5* downstream noncoding sequences, respectively. Vector construction is described in the materials and methods section. Solid segments represent *Gα4* sequences, striped segments represent *Gα5* sequences, open segments represent the *pAct6::Neo*^{r} (*G418*^{r}) gene, and the solid line represents vector backbone sequences. The initiation and termination codons are represented by ATG and TAA respectively. Restrictriction enzyme recognition sites shown are: *Bgl*II (Bg), *Eco*RI (E), *Hin*cII (Hc), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P) and *Sal*I (S).

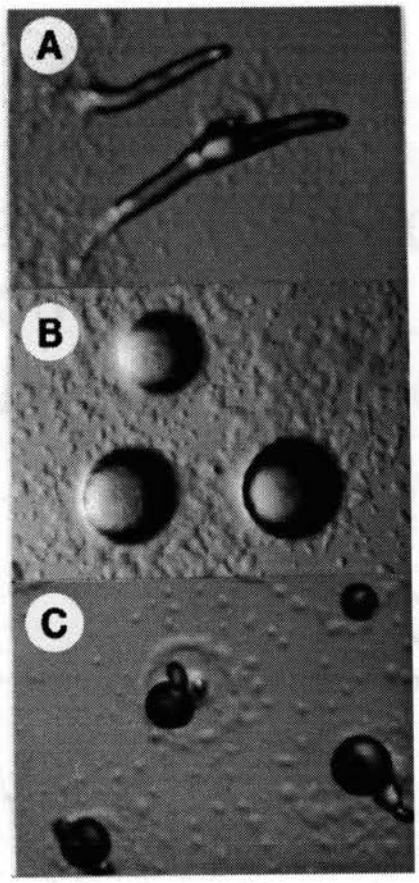

Figure 36. Morphological phenotypes of wild-type (A), *gα5* null cells (B), and *gα5* null cells that express the *pGα5::Gα4orf-ter5* gene fusion (C). Cells were prepared and plated for development as described in Figure 26. Photographs were taken after 13 hours of development.

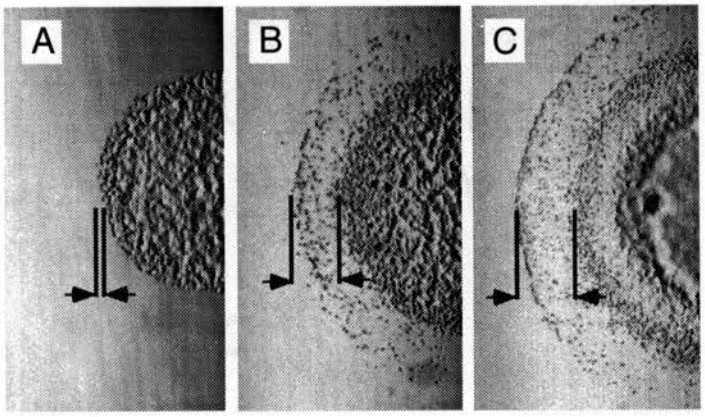

Figure 37. Chemotaxis of *gα4* null cells (A), *gα4* null cells expressing the *pGα5::Gα4orf-ter5* gene fusion (B), and wild-type cells (C) to folic acid. Chemotaxis assays were performed as described in Figure 28. Photographs were taken after three hours cells were plated. Folic acid source is located to the left of all cell droplets. Bars represent the leading edge of the chemotaxing cells and the cell droplet perimeter.

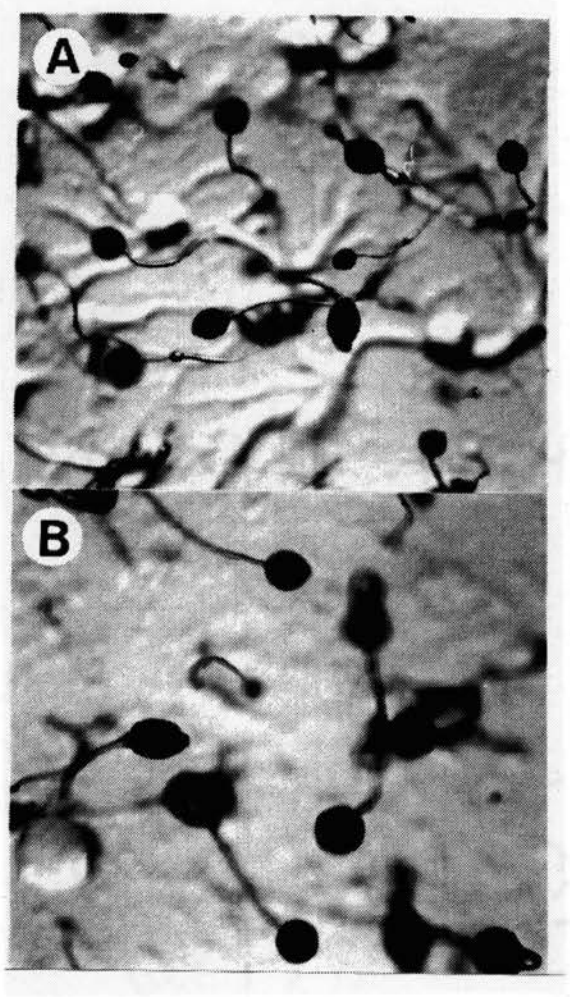

Figure 38. Fruiting body development of *gα4* null cells that have been transformed with *pGα5::Gα4orf-ter5* (A) or *pGα5::Gα4orf-ter4*. (B). Cells were prepared and plated for development as described in Figure 26. Photographs were taken after 30 hrs of development.

noncoding sequences instead of the *Gα5* downstream sequences, *pGα5::Gα4(ORF)-4ter* (Figure 35), gave phenotypes similar to those observed for cells carrying the *pGα5::Gα4(ORF)-5ter* gene fusion (Figure 38) except for a smaller transcript size (Figure 39). While present in high copy number, both *pGα5::Gα4(ORF)* gene fusions were only expressed at levels similar to the *Gα4* gene in wild type cells and neither gene fusion was capable of inhibiting tip morphogenesis in wild type, *gα4* null, or *gα5* null cells, suggesting that the *Gα5* promoter does not provide sufficient expression of the *Gα4* gene during later developmental stages (Figure 39).

When plated on bacterial lawns, *gα4* null cells with the *pGα5::Gα4(ORF)* vectors formed plaques with growth rates similar to that observed for wild type cells as opposed to plaques with slow growth rates that are typical of *gα4* null mutants. These plaques routinely became aggregation deficient after reaching diameters of 1-2 centimeters and the cells from these outer zones remained aggregation deficient when replated on bacterial lawns. This aggregation deficient phenotype was not observed in wild type or *gα5* null cell backgrounds implying that the phenotype is specific to the *gα4* null background. This phenotype was also not observed in populations of cells growing in axenic medium suggesting that growth on bacterial lawns provided a selection for this phenotype. Aggregates within the very center of these plaques often displayed *gα4* null developmental phenotypes suggesting that cells with higher expression levels of the *pGα5::Gα4(ORF)* gene preferentially migrate to the perimeter of the plaque.

Attempts to express the *Gα5* gene from the *Gα4* promoter were unsuccessful due the instability of the gene fusion vectors in several different bacterial strains, but vectors were constructed in which the *Gα5* gene is expressed from the *Dictyostelium discoidin-1* promoter. This promoter has been used to express the *Gα4* gene in *gα4* null cells, allowing for the complete rescue of folic acid chemotaxis and a partial rescue of developmental phenotypes (Srinivasan and Hadwiger, unpublished data). The *pdiscoidin-1::Gα5(orf)-5ter* gene fusion did not rescue folic acid chemotaxis or developmental

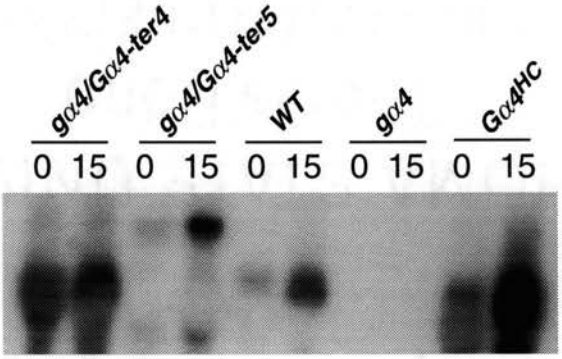

Figure 39. Expression of *Gα4* transcripts in *gα4* null cells expressing the *pGα5::Gα4orf-ter4* (*gα4/Gα4-ter4*), *gα4* null cells expressing the *pGα5::Gα4orf-ter5* (*gα4/Gα4-ter5*), wild-type cells (*WT*), *gα4* null cells (*gα4*), and *Gα4* overexpressors ($Gα4^{HC}$) cells. RNA was isolated from developing cells at times indicated (0 or 15 hrs) and then analyzed as described in Figure 33. The RNA blot was hybridized with a *Gα4* probe covers the entire *Gα4* open reading frame. Transcript size differences in the *gα4* null cells carrying the *pGα5::Gα4orf-ter5* construct are due to *Gα5* downstream sequences.

morphogenesis when present in *gα4* null cells indicating that the *Gα5* gene is not sufficient to compensate for the loss of Gα4 function. However, wild type and *gα4* null cells expressing this *pdiscoidin-1::Gα5(orf)-5ter* gene formed small aggregates with precocious tip morphogenesis, suggesting that the *Gα5* gene was being overexpressed.

Attempts were also made to determine if the *Gα4* and *Gα5* genes are expressed simultaneously in the same cells. This was done by trying to coexpress two different reporter genes, which were driven by the *Gα4* and *Gα5* promoters, in the same cells. Seven *Gα4* and *Gα5* reporter constructs were created for this purpose (Figure 40). Initially, cells transformed with the *pGα5::lacZ-Bsr*^{r} reporter gene, selected with >10μg/ml of blasticidin, and tested for gene expression by histochemical staining. No β-galactosidase staining was observed in these cells, suggesting that we had not been able to select for a high enough copy number of this vector in the cells. Similarly, the cells transformed with the reporter construct carrying the wild-type version of the GFP fused to the *Gα5* promoter did not show fluorescence above the background fluorescence of *Dictyostelium* cells, although these transformants had been selected with very high (>15μg/ml) G418 concentrations.

The transformants carrying the mutated GFP reporter constructs (*pGα5::GFP*-G418*^{r}) showed distinct fluorescence when selected for with very high concentrations of G418(>15μg/ml). These transformants were then retransformed with a reporter gene carrying the *Gα4* promoter fused to the *lacZ* gene on a vector conferring resistance to G418. The transformants were selected with concentrations of G418 that exceeded 30μg/ml. However, the GFP in the cells lost its ability to fluoresce when the cells were fixed for histochemical staining. Also, two different types of microscopy were needed to monitor each individual reporter gene expression. Hence it was not possible to monitor both *Gα* expression patterns simutaneously using the *GFP* and the *lacZ* reporter genes.

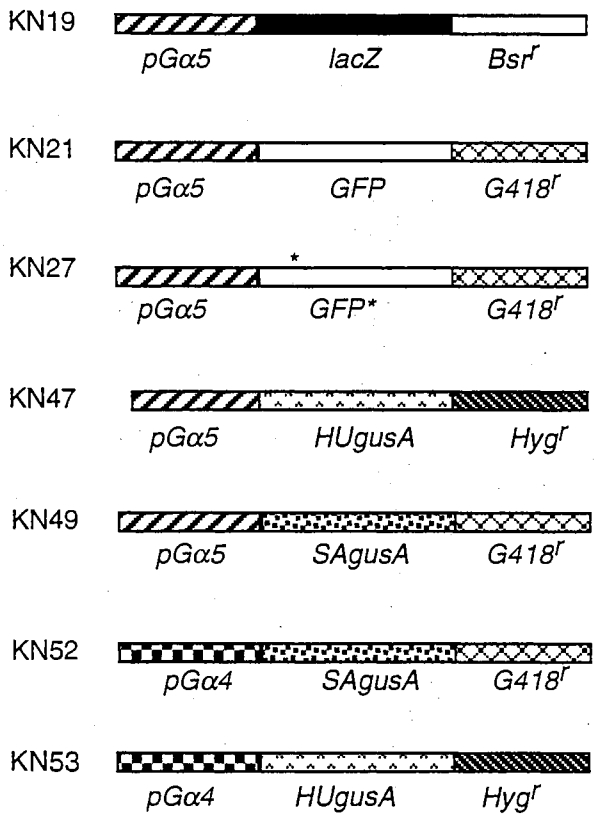

Figure 40. Maps of *Gα5* and *Gα4* reporter constructs created. Construction of these vectors has been described in detail in Materials and Methods. *G418*^{r} represents the *pACT6::Neo*^{r} gene fusion which confers resistance to G418. *Bsr*^{r} represents the blasticidin resistance gene and *Hyg*^{r} represents the gene that confers resistance to hygromycin. *gusA* represents the gene encoding the β-glucouronidase enzyme; *lacZ* represents the gene that codes for β-galactosidase; GFP and GFP* represent the wild-type green fluorescent protein and the mutated green fluorescent protein which has a higher fluorescence respectively; *HUgusA* is the version of the Gus enzyme which is post-translationally modified and *SAgusA* represents the gene encoding the nuclear localized version of the Gus protein.

The ability to co-express and monitor two different subunits using reporter genes which utilize similar protocols for histochemical staining made the β-glucouronidase expressing *gusA* gene a potentially interesting candidate for use in the spatial expression experiments. Cells transformed with the *Gα5* reporter gene fused to the wild-type, bacterial version of the *gusA* gene gave a diffused staining pattern as compared to Gα4-β-galactosidase expressing cells, making it difficult to observe distinct cells that expressed the gus reporter construct. Hence the two different versions of the *gusA* gene-pHugusA and pSAgusA- were used to make reporter constructs. The *HugusA* gene when driven by either the *Gα4* or *Gα5* promoter did not show any evidence of expression, although transformants carrying the constructs were resistant to >50μg/ml of hygromycin. Also, the fact that the hygromycin resistance gene confers resistance to neomycin created problems for selection of the *lacZ-G418*^{r} reporters in a co-transformation event of *HugusA-Hyg*^{r} and *lacZ-G418*^{r}. Faint staining was observed in the cells carrying the *SAgusA* genes driven by both *Gα4* and *Gα5* promoter, but the staining was not obvious enough to be able to use it in co-expression assays. The faint staining was possibly due to the colored end-product of Gus enzyme activity being nuclear localized.

Discussion

The phylogenetic tree of the seven *Dictyostelium* Gα subunits suggests that the*Gα4* and *Gα5* genes may have evolved from each other more recently than other pairs of Gα subunit genes and this relationship is further supported by similarities in intron positioning and expression (58, 62). However, the contrasting phenotypes between the null mutants or the overexpression mutants in aggregate size and tip morphogenesis suggest that the developmental roles of these genes are quite distinct and in some ways antagonistic. The inhibition of folic acid responsiveness by the overexpression of the *Gα5* gene in early development suggests that the Gα5 subunit can interfere with the Gα4-mediated signal transduction pathway. This inhibition might possibly occur at the level of Gα4 function

since it affects cGMP accumulation, one of the earliest steps in the pathway. The inhibition of folic acid responsiveness is not due to repression of *Gα4* expression, but the expression of other components in this pathway could possibly be affected by Gα5 function. The inhibition of tip morphogenesis by folic acid does not seem to be altered by *Gα5* expression, even though *Gα5* gene expression increases upon aggregate formation. This diminishing effect of *Gα5* expression on folic acid responses during the progression of development might be due to a separation of *Gα5* and *Gα4* expression into different subsets of cells.

The localization of Gα mutants in chimeric organisms and the tip morphogenesis of clonal Gα mutants suggest that an antagonistic relationship exists between Gα4 and Gα5 function in regards to cell fate. The localization of *gα5* null and $G\alpha4^{HC}$ mutants to the central prespore region in chimeras might represent a cell type preference that would be consistent with the impaired tip morphogenesis exhibited by clonal aggregates of these mutants. Conversely, the localization of *gα4* null and $G\alpha5^{HC}$ cells to the posterior region of chimeric organisms might indicate a cell type preference that is conducive to prestalk cell development and tip morphogenesis. The cell-autonomous localization of these mutants suggests that the *Gα4* and *Gα5* subunits promote different cell fates. This idea is further supported by the morphological phenotypes associated with chimeric organisms composed of different Gα mutants. The development of *gα4 /gα5* or $G\alpha4^{HC}/G\alpha5^{HC}$ chimeric organisms results in relatively normal-sized aggregates that are not delayed in tip morphogenesis, suggesting some complementation exists with respect to deficiencies in different cell types. In contrast, chimeric organisms consisting of *gα5* null and $G\alpha4^{HC}$ cells or *gα4* null and $G\alpha5^{HC}$ cells exhibit greater deficiences in tip morphogenesis or other development processes.

The display of both *gα4* null specific and *gα5* null specific phenotypes in strains lacking both *Gα* genes suggests that these phenotypes are not merely due to an imbalance of Gα4 and Gα5 function during development. Therefore, Gα4 and Gα5 subunits are not

likely to exert their function by competing for common signaling components in the same cell. Even in the double *Gα4/Gα5* overexpression mutant, many of the aberrant phenotypes are still present which further supports that normal development is not just a balance of Gα4 and Gα5 function. However, the blending of morphogenetic and gene expression phenotypes in the double Gα subunit overexpression mutant suggests that excessive subunit expression might interfere with both signal transduction pathways. Therefore, the phenotypes produced by the overexpression of one Gα subunit might be in part due to interference with the signal transduction pathway of the other Gα subunit.

The different functions of the Gα4 and Gα5 subunits during *Dictyostelium* development appear to result from both structural and expression differences between these subunits. The inability of the Gα4 or Gα5 subunit to compensate for the loss of the other when expressed from heterologous promoters implies that structural distinctions prevent these subunits from efficiently substituting for each other in their respective pathways. However, structural distinctions that altered even a single protein-protein interaction with another signaling component (e.g., a receptor, effector, etc.) might be sufficient to prevent proper signaling. Functional differences also appear to result from distinctions in the expression of these subunits since the *Gα4* gene is unable to produce *Gα4*^{HC} associated phenotypes when expressed from the *Gα5* promoter. The expression of the *Gα4* gene from the *Gα5* promoter does not appear to be as well tolerated as the expression of the *Gα4* gene expressed from its own promoter since the *pGα5 ::Gα4orf* gene fusions were never found to be abundantly expressed in any transformant. The high frequency of *gα4* null cells becoming aggregation deficient when carrying the *pGα5 ::Gα4orf* gene fusions also suggests that this form of *Gα4* expression might be stressful to cells. Lack of sequence similarity in the promoters of these two genes also suggest the possibility of differential expression (data not shown). However, the ability of the *pGα5 ::Gα4orf* gene fusions to complement the *gα4* null phenotype suggests that at least some overlap in expression can occur. Some of this overlap might occur during vegetative growth when both genes are

expressed at low levels. Attempts to compare the spatial expression patterns of the *Gα4* and *Gα5* genes by co-expressing reporter genes from their promoters have not been successful due to our inability to obtain transformants with both reporter genes at sufficiently high copy number.

The phenotypic distinctions of *Gα4* and *Gα5* mutants suggests that the Gα4 and Gα5 subunits play functionally different roles during development and this difference appears to be in part due to structural and expression differences. While the sequence similarity between these subunits is greater than for other *Dictyostelium* Gα subunit pairs, the percentage of identity is still much lower than many pairs of Gα subunits found in mammals (69). The distinctions in Gα4 and Gα5 function suggest that less related *Dictyostelium* subunits might also have distinct functions. Therefore, the absence of developmental phenotypes in Gα1, Gα7, or Gα8 null mutants is not likely due to functional redundancy but perhaps because these subunits are not essential to the growth or development of *Dictyostelium* cells under laboratory conditions.

CHAPTER 6

CONCLUSIONS

The aim of the experiments outlined in this study was to characterize the Gα5 signal transduction pathway and study its specificity in the development of *Dictyostelium discoideum.* Many of the components that are involved in the Gα5 signal transduction pathway are unknown. However, previous experiments done in this laboratory had provided an insight into the probable function of the Gα5 protein (62). The Gα5 G protein plays a role in the temporal regulation of tip morphogenesis, but how it regulates this developmental process it still unclear.

One of the means by which events downstream of the Gα5 G protein can be studied is to identify and utilize the external biomolecule that is capable of activating Gα5. The first study described in chapter 3 was aimed at trying to identify an external signal that triggers the Gα5 pathway. Chemotaxis assays and radioimmunoassays were used to study the response of the Gα5 G protein to different biomolecules. cAMP was thought to be the most likely biomolecule that would trigger the Gα5 signal transduction pathway. Assays done to evaluate the response of Gα5 mutants and wild-type cells to cAMP gave inconclusive results as both the *gα5* null cells and the $G\alpha5^{HC}$ strain had lower responses to cAMP than the wild-type cells. Since cAMP is an activator of other signaling pathways, such as the Gα2 mediated pathway, Gα2 independent responses of Gα5 to cAMP were tested in *gα2* null *gα2/gα5* double null and $g\alpha2/G\alpha5^{HC}$ mutant cells. cAMP responses in these *gα2* null background cells were at very low levels and hence no conclusive results were obtained.

Preliminary data was obtained with responses of the Gα5 mutants and wild-type cells to other known activators of signal transduction pathways, such as lysophosphatidic acid and platelet activating factor, in *Dictyostelium*. The results suggested that lysophophatidic acid or related biomolecules may elicit responses in cells via the Gα5 signal transduction pathway at a particular stage in development, maybe at the time of tip formation. Also, the Gα5 signal transduction pathway may affect the signal transduction pathway activated by platelet activating factor. An overexpression of *Gα5* seemed to repress cellular responses to folic acid, indicating that the Gα5 protein may have a negative effect on the signal transduction pathways regulated by folic acid. However, folic acid did not seem to affect the rate of tip morphogenesis, a developmental phenotype regulated by the Gα5 G protein, indicating that folic acid was not a negative regulator of the Gα5 signal transduction pathway. Although a signal that could positively affect cellular responses by activating the Gα5 signal transduction was not identified, potential activating biomolecules were tested. Also, it seems likely that the folic acid activated Gα4 signal transduction pathway may be negatively regulated by the Gα5 G signal transduction pathway at a level downstream of Gα4 function.

There may be multiple signals activating the Gα5 signal transduction pathway at different stages in the development of *Dictyostelium*. Thus it may be necessary to look for responses of the cells to different signals at different stages of development. Another possibility is that the responses of cells to the external signal may not involve chemotaxis or accumulation of secondary messengers. Other assays such as light scattering assays and GTPase assays could be used as a more sensitive means of looking for responses to external signals.

The second part of the research project was aimed at trying to characterize the Gα5 subunit. Earlier experiments done in the lab had suggested that *Gα5* expressing cells play a dominant role in regulating the rate of tip morphogenesis when they are developed in chimeras with cells that do not have the*Gα5* gene. The role of the Gα5 subunit in possible

intercellular signaling, and its effect on developmental gene expression and regulation of rate of tip morphogenesis, was studied by analyzing chimeric organisms. This was done by mixing wild-type cells and Gα5 mutant cells independently with *gα5* null cells carrying cell-type specific reporter constructs. The results indicated that the Gα5 function may be important for the production of an intercellular signal which regulates prestalk specific gene expression. One of the questions which remains to be answered is whether this induction of prestalk gene expression by the *Gα5* expressing cells is due to a direct increase in cell differentiation, or whether it is an effect of an increase in cell sorting/migration, which in turn helps in cell differentiation. Insight into the effect of the intercellular signal produced by the *Gα5* expressing cells on other cells may help elucidate some of the players and downstream effectors of this pathway.

Two Gα5 site specific mutants were also characterized in an effort to understand the role of Gα5 in the development of *Dictyostelium*. Both the mutations were in the conserved G-3 region of the Gα subunits and have been well studied in many other systems. The first of these was the $G\alpha5^{Q199L}$ mutation, associated with a constitutively active Gα5 subunit. The second was the $G\alpha5^{G197T}$ mutation. The latter mutation in the mammalian $G\alpha_s$ subunit has been associated with a decrease in adenylyl cyclase activity, termed as a dominant negative phenotype. Analysis of both the mutations in the wild-type cells indicated that at a low copy, they did not have any effect on the cells. At a higher copy, they were lethal to the cells, indicating these *Gα5* mutations were probably stimulating some inappropriate processes that were incompatible with vegetative growth. The results obtained with the use of a repressible promoter to control the expression of the $G\alpha5^{Q199L}$ allele were inconclusive due to high basal levels of expression even when the promoter was repressed. Although no dramatic phenotype is observed in the cells carrying a low copy number of the mutant alleles, an analysis of cellular responses and subtle changes (if any) in morphology associated with other signal transduction pathways in these cells, might provide some information about the intracellular effect of the mutations. These

studies might also indicate whether the *Gα5* mutations are interfering with other signal transduction pathways in the cells.

Studies done with cells carrying a high copy number of analogous mutant alleles in the Gα4 subunit, $G\alpha4^{Q200L}$, had indicated that these alleles too were also lethal to the cells. Recent studies in the lab indicate that at a certain threshold level, wild-type cells carrying this mutant allele show an aggregation deficient phenotype, similar to a *gα2* null mutant phenotype. A similar mutation in the Gα2 subunit also shows the same aggregation deficient phenotype, indicating that both these mutant alleles may be affecting some other signal transduction pathway important for aggregation, such as the Gα2 signal transduction pathway. Hence it is possible that the $G\alpha5^{Q199L}$ allele may also produce a similar phenotype when it is not present at a very high copy number in the cells. This phenotype might have been missed due to selection for a high copy number of the mutant allele in the cells. Further studies done on cells carrying the $G\alpha5^{Q199L}$ allele will help in the analysis of the effect of this mutation on the cells.

Finally, a study was done to determine the specificity of Gα subunit interactions and G protein mediated signaling pathways in *Dictyostelium*. The two most closely related Gα subunits in *Dictyostelium*, Gα4 and Gα5 were analyzed and compared to examine their functional specificity. This was done using mutational analysis of the two subunits with respect to their roles in developmental morphogenesis, gene expression, chemotactic movement, second messenger production and intercellular signaling. These studies suggested that these related Gα subunits function in parallel pathways that antagonistically promote cell localization, morphogenesis and cellular differentiation. If these two subunits interact with common signaling components, it seems unlikely that they do so in the same set of cells. Considering the fact that Gα4 and Gα5 are the most closely related subunits, and that they do not perform completely redundant functions, it seems unlikely that there are other pairs of Gα subunits that are redundant in function to each other. Hence *Dictyostelium* Gα subunits might be important in determining pathway specificity in

response to the external signal, while other components such as the Gβ subunit and downstream effectors such as the adenylyl and guanylyl cyclases and MAP kinases appear to act in many signal transduction pathways without pathway specificity.

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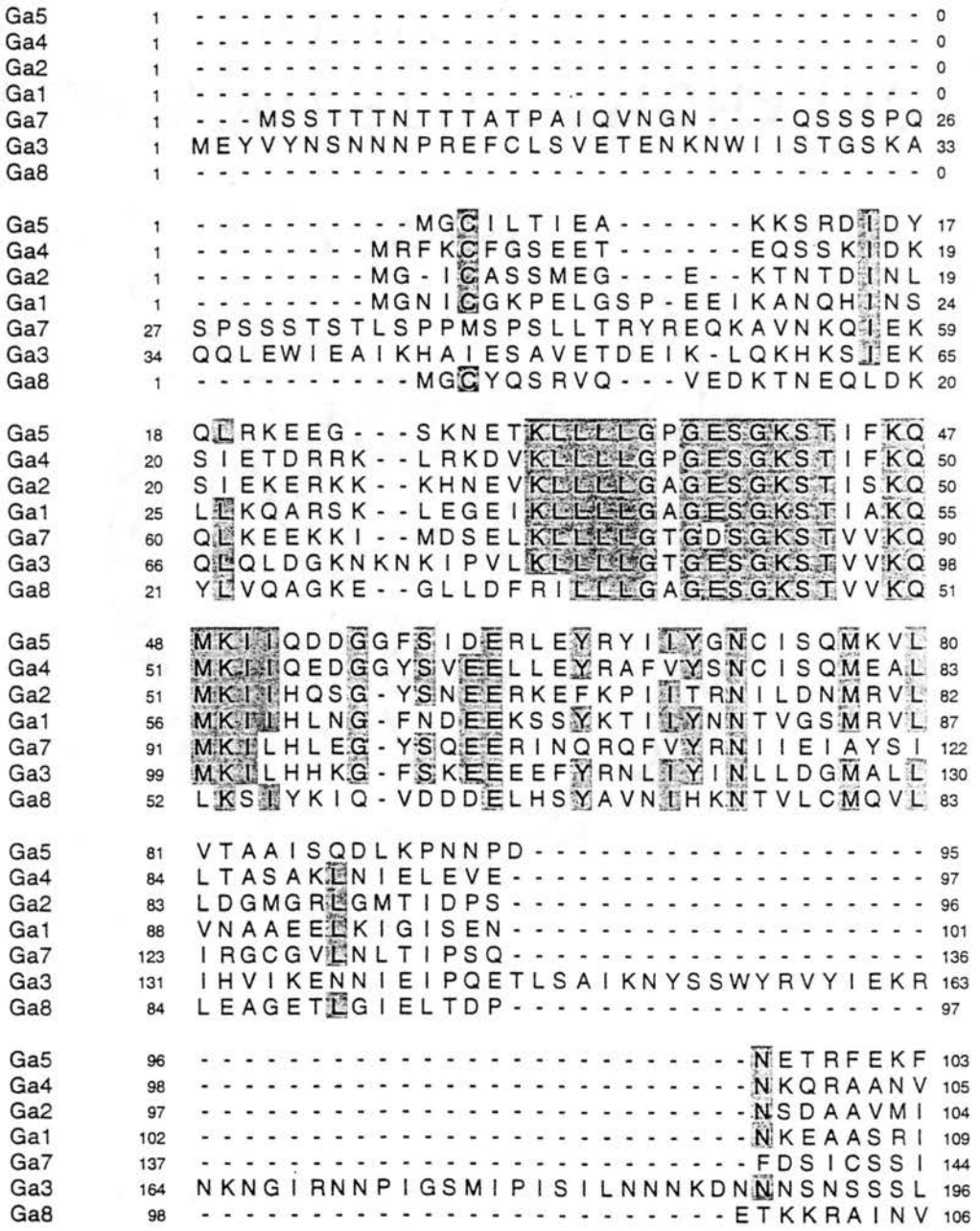
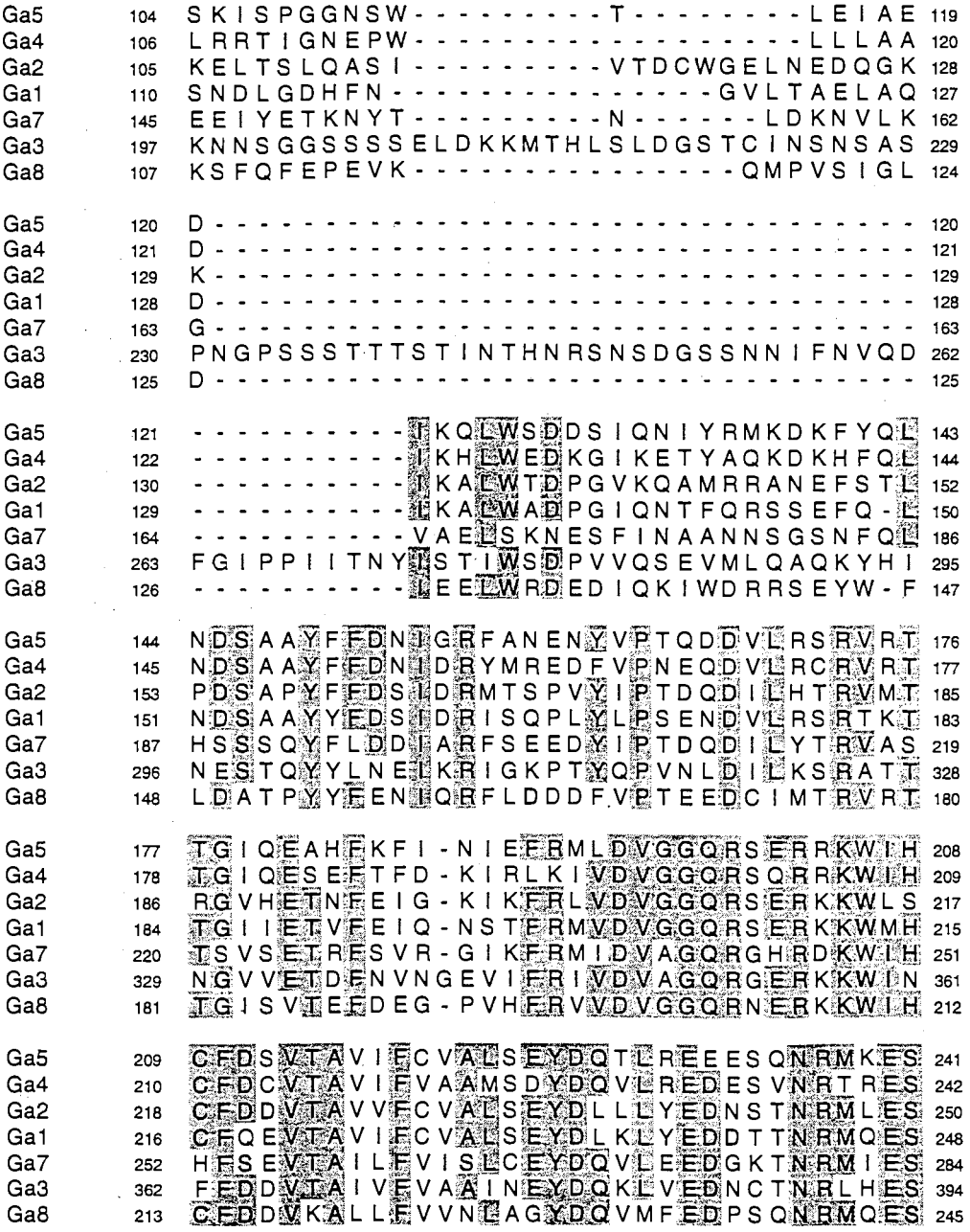
APPENDIX

Amino Acid Sequence Alignment of Known Gα Subunits of *Dictyostelium discoideum*

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Ga7 145 EEIYETKNYT---------N------LDKNVLK 162
Ga3 197 KNNSGGSSSSSELDKKMTHLSLDGSTCINSNSAS 229
Ga8 107 KSFQFEPEVK---------------QMPVSIGL 124
Ga5 120 D---------- 120
Ga4 121 D---------- 121
Ga2 129 K---------- 129
Ga1 128 D---------- 128
Ga7 163 G---------- 163
Ga3 230 PNGPSSSTTTSTINTHNRSNSDGSSNNIFNVQD 262
Ga8 125 D---------- 125
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Ga1 129 ----------IKALWADPGIQNTFQRSSEFQ-L 150
Ga7 164 ----------VAELSKNESFINAANNSGSNFQL 186
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Ga8 126 ----------LEELWRDEDIQKIWDRRSEYW-F 147
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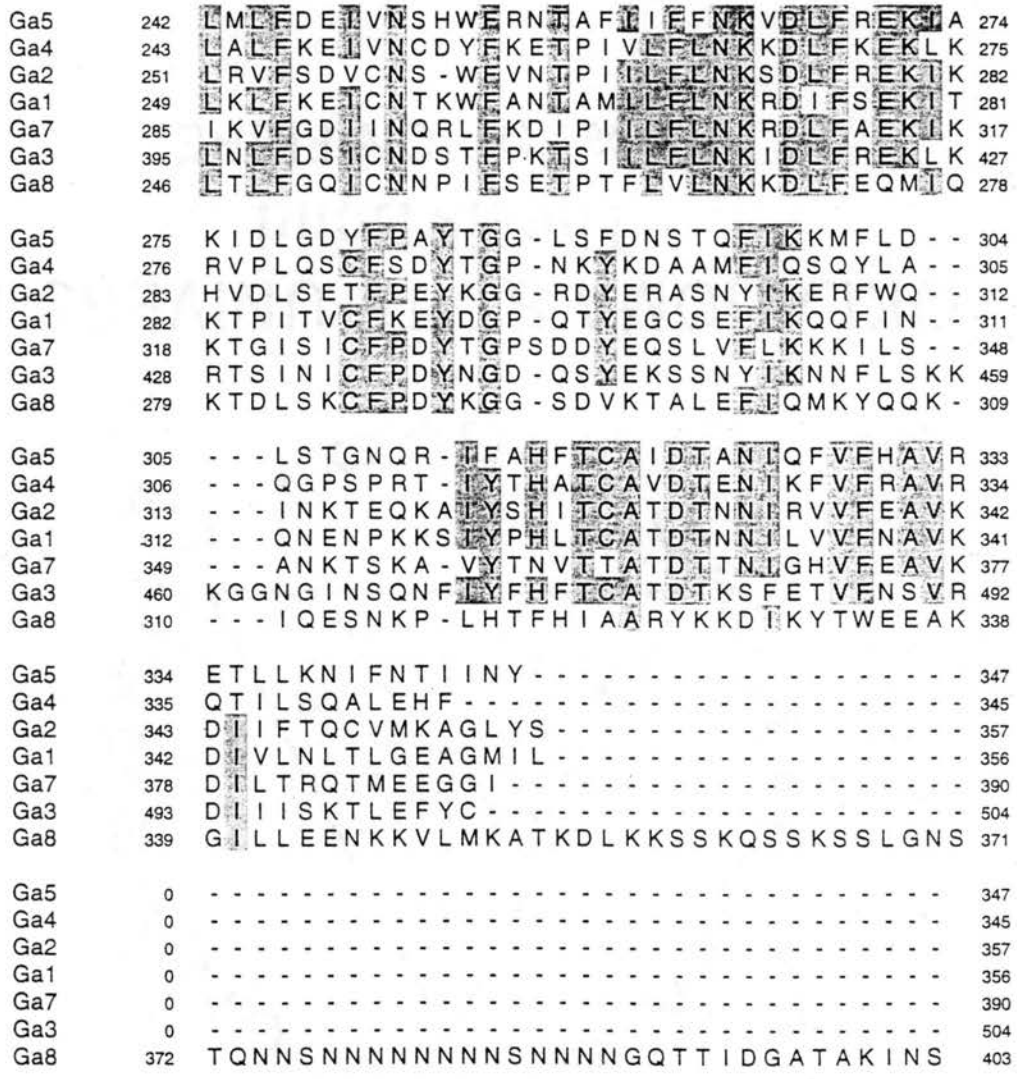
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Ga4 243 LALFKEIVNCDYFKETPIVLFLNKKDLFKEKLK 275
Ga2 251 LRVFSDVCNS-WEVNTPIILFLNKSDLFREKIK 282
Ga1 249 LKLFKETCNTKWFANTAMLLFLNKRDIFSEKIT 281
Ga7 285 IKVFGDIINQRLFKDIPIILFLNKRDLFAEKIK 317
Ga3 395 LNLFDSICNDSTFPKTSILLFLNKIDLFREKLK 427
Ga8 246 LTLFGQICNNPIFSETPTFLVLNKKDLFEQMIQ 278
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Ga4 276 RVPLQSCFSDYTGP-NKYKDAAMFIQSQYLA-- 305
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Ga7 318 KTGISICFPDYTGPSDDYEQSLVFLKKKILS-- 348
Ga3 428 RTSINICFPDYNGD-QSYEKSSNYIKNNFLSKK 459
Ga8 279 KTDLSKCFPDYKGG-SDVKTALEFIQMKYQQK- 309
Ga5 305 ---LSTGNQR-IFAHFTCAIDTANIQFVFHAVR 333
Ga4 306 ---QGPSPRT-IYTHATCAVDTENIKFVFRAVR 334
Ga2 313 ---INKTEQKAIYSHITCATDTNNIRVVFEAVK 342
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Ga7 349 ---ANKTSKA-VYTNVTTATDTTNIGHVFEAVK 377
Ga3 460 KGGNGINSQNFIYFHFTCATDTKSFETVFNSVR 492
Ga8 310 ---IQESNKP-LHTFHIAARYKKDIKYTWEEAK 338
Ga5 334 ETLLKNIFNTIINY-------------------- 347
Ga4 335 QTILSQALEHF---------------------- 345
Ga2 343 DIIFTQCVMKAGLYS------------------- 357
Ga1 342 DIVLNLTLGEAGMIL------------------- 356
Ga7 378 DILTRQTMEEGGI--------------------- 390
Ga3 493 DIIISKTLEFYC----------------------- 504
Ga8 339 GILLEENKKVLMKATKDLKKSSKQSSKSSLGNS 371
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Ga4 0 ---------- 345
Ga2 0 ---------- 357
Ga1 0 ---------- 356
Ga7 0 ---------- 390
Ga3 0 ---------- 504
Ga8 372 TQNNSNNNNNNNNSNNNNGQTTIDGATAKINS 403

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