THE ANALYSIS OF A PUTATIVE MAP KINASE DOCKING MOTIF IN *DICTYOSTELIUM DISCOIDEUM* Gα2 PROTEIN

By

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THE ANALYSIS OF A PUTATIVE MAP KINASE

DOCKING MOTIF IN *DICTYOSTELIUM DISCOIDEUM*

Gα2 PROTEIN

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Abstract: Some G protein alpha subunits contain a mitogen-activated protein kinase (MAPK) docking site (D-motif) near the amino terminus that can impact cellular responses to external signals. The *Dictyostelium* Gα2 subunit is required for chemotaxis to cAMP during the onset of multicellular development and the subunit contains a putative D-motif in a region analogous to that in other Gα subunits. The Gα2 subunit D-motif was altered ($G\alpha2^{D-}$) to examine its potential role in chemotaxis and multicellular development. In $g\alpha2^{-}$ cells the expression of the $G\alpha2^{D-}$ or wild-type Gα2 subunit from high copy number vectors rescued cell aggregation but blocked the transition of mounds into slugs. This phenotype was also observed in parental strains with a wild-type Gα2 locus indicating that the heterologous Gα2 subunit expression interferes with multicellular developmental progress. Expression of the $G\alpha2^{D-}$ subunit from a low copy number vector in $g\alpha2^{-}$ cells did not rescue aggregation whereas the wild-type Gα2 subunit rescued aggregation efficiently and allowed wild-type morphological development. The $G\alpha2^{D-}$ and Gα2 subunit were both capable of restoring comparable levels of cAMP chemotaxis and the ability to co-aggregate with wild-type cells implying that $G\alpha2^{D-}$ expressing cells are defective in intercellular signaling. The ability of cAMP to stimulate the translocation of the GtaC transcription factor was impaired in $G\alpha2^{D-}$ expressing cells compared to Gα2 expressing cells suggesting the putative D-motif is important for developmental gene regulation. These results suggest that the D-motif plays a role in aggregation and some cellular responses to cAMP but not cAMP chemotaxis.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Signal transduction in eukaryotes

Eukaryotes require intricate communication systems to carry out various cellular processes. Therefore, to employ the mechanisms needed to perform different responses, signal transduction pathways and their respective molecules need to have many areas of overlap while keeping extraordinary specificity [1-8]. An initial step in one major class of signal transduction pathways begins when extracellular ligands known as primary messengers, bind and induce conformational changes in GPCRs, or G protein coupled receptors [9]. GPCRs stimulate many downstream responses including changes in metabolism, gene expression, cell differentiation, and cell movement [1-8]. These receptors are made up of seven alpha-helical segments with an amino-terminus extending extracellularly and a carboxyl-terminus residing inside the cell. The loops formed between these helixes denote where serine phosphorylation occurs and where G proteins interact [10,11]. This step allows first messengers to be translated into secondary signals that the cell can utilize, exert and regulate using common signaling proteins like nucleotide cyclases, phosphodiesterases, and transcription factors. Some other vital components that make up several signal transduction pathways are heterotrimeric guanine nucleotide-binding proteins (G proteins) and mitogen-activated protein kinases (MAPK

or MAP Kinase). Both protein families are evolutionarily conserved as G proteins carry GPCR signals inside the cell while MAP Kinases regulate various cellular responses [1,5,12]. It is known that MAP kinases frequently function downstream G proteins, though MAPKs can employ other transducing mechanisms such as the receptor tyrosine kinase in response to certain growth factors or without G proteins entirely. Additionally, iteractions between these signaling proteins can be directly mediated through binding or docking sites or indirectly through scaffolding proteins. The analysis of protein structure and function among homologs has led to the identification of conserved motifs that play important roles in protein-protein interactions. These examples briefly demonstrate the common machinery but complex usage in eukaryotic signal transduction.

1.2 G proteins

G proteins act as molecular on-off switches. They exist as two types, a monomeric molecule, and a heterotrimeric complex consisting of Gα, Gβ, and Gγ subunits. Despite these differences, these enzymes have highly conserved regions and make up the GTPase family [3,9-12]. All GTPases have a guanine nucleotide-binding pocket that binds GTP and enzymatic activity that hydrolyzes it to GDP [12]. GTP-bound enzymes are considered active and GDP-bound enzymes signify inactivity. In heterotrimeric complexes, the Gα subunit is critical for binding G protein complexes to GPCRs, as well as the release and regulation of the Gβγ dimer [13]. Once an extracellular molecule binds to a GPCR it induces the nucleotide exchange in the GPCR-GαGDP-Gβγ network. Following this ligand lock-in, the GαGTP subunit and the Gβγ dimer then disassociate from the receptor and each other and go on to impact downstream effector targets like secondary signal producing enzymes [13]. After a phosphate on GαGTP is cleaved and

used in a subsequent process, Gα is then allowed to return to the transmembrane receptor as a GαGDP-bound making the activation-inactivation cycle complete [14]. As a result of this G protein on-off cycle, many other pathway components go on to carry out their functions. A general model of this process is outlined in Figure 1.

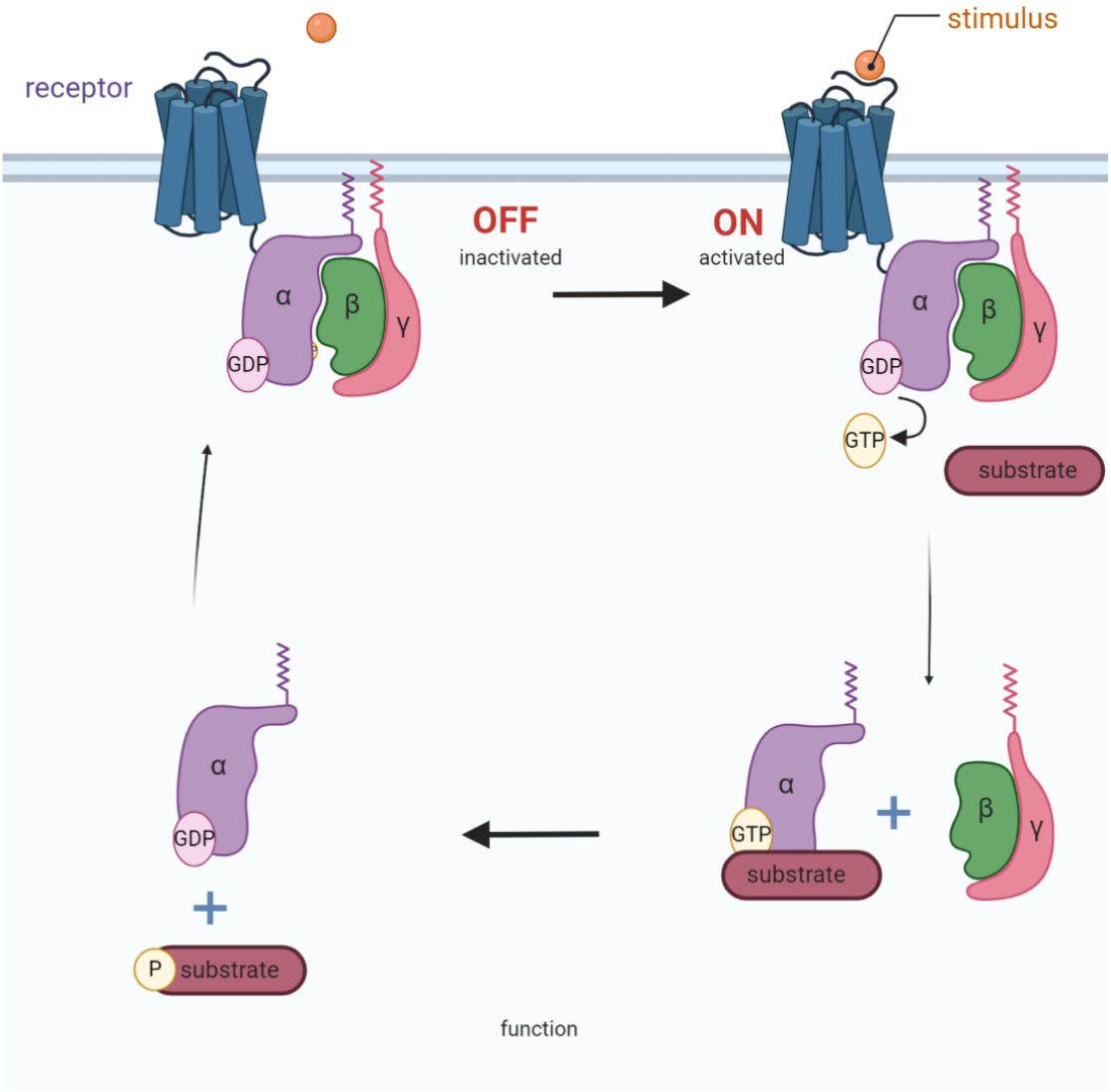


Figure 1: G protein On-Off Cycle. Created with Biorender

1.3 Protein-Protein Interactions, Targets and Intracellular Messengers

There are four common types of heterotrimeric Gα proteins that stimulate immediate targets, $G_{i/o}$, G_q, G_{12}, and G_s [3, 14, 15]. These proteins mediate the production

or release of intracellular messengers such as ions and cyclic nucleotides. For example, $G_q\alpha$ stimulates phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2), to cytosolic inositol triphosphate IP_3 and membrane-bound diacylglycerol (DAG). Once IP_3 is cleaved, it binds and opens the calcium ion channels housed in the endoplasmic reticulum. The increase of cytosolic calcium released by IP_3 is then used by DAG to activate the calcium-dependent kinase, Protein Kinase C [14]. Likewise, $G_s\alpha$ activates adenyl cyclase. With the energy from GTP-bound $G_s\alpha$, adenyl cyclase converts ATP into the secondary messenger cyclic adenosine monophosphate (cAMP). The increase of intracellular cAMP activates the common intermediate, Protein Kinase A which goes on to phosphorylate other proteins with ties to a wide range of cellular processes and cell-type-specific physiological responses [14-16].

1.4 G proteins in mammals and yeast

There are thousands of GPCR genes encoded in mammalian genomes yet, mammalian genes that encode for G proteins only include 16 genes for Gα subunits, 5 for Gβ and 12 for Gγ [3,12-15]. This difference strongly suggests that G proteins can bind to multiple GPCRs and research shows that these proteins have a critical role in converting signals such as neurotransmitters, chemokines, and cellular stressors. Research has also shown that G proteins also mediate some mitogenic processes. For example, $G\alpha_q$/Gα11 Q209L analysis showed that constant low-level expression can lead to transforming mutations in fibroblast cell lines [12]. These transformations have also been studied in their respective GPCRs, and research suggests that the GPCRs are highly tumorigenic as well. This is due to its high transforming nature, especially in response to ligand excess [12]. In another G protein sub-family, uncontrolled Gs-mediated cAMP production can

cause an abundance or inhibition of cell growth in neuronal cells. Similarly, Gα12 oncogenes are tied to increased effector expression causing sarcoma [12].

In response to chemokines, CCR7, CCR8, and other GPCRs on the surface of leukocytes allow for proper immune responses via leukocyte migration and homing. CCR7 recognizes the chemoattractant signal, CCL19, and CCL21 [17-19]. On naïve T-cells, this receptor and its G proteins allow cells to recognize and follow signal gradients in the blood towards secondary lymphoid organs for antigen presentation and subsequent activation. Likewise, neutrophils and other blood-circulating phagocytic cells use G protein signaling to strengthen adherence to endothelial cells during extravasation [19, 20].

In liver stimulatory Gα protein (Gsα) coupled to the β_2-adrenergic receptor elicits a response to epinephrine, a hormone, and neurotransmitter [21]. It does so by cAMP activation of PKA and subsequent activation of glycogen phosphorylase which converts glycogen to glucose for energy. This process is linked to responses involving alertness and skeletal muscle. However, Gsα mutations in this cell-type can lead to several metabolic and endocrine disorders such as polycystic ovarian syndrome, diabetes, and hyperthyroidism [22-24].

Yeast species are other highly studied organisms that utilize G proteins. Interestingly, analyses involving *Saccharomyces cerevisiae* mating response has highlighted a MAPK docking motif on Gpa1, a Gα protein [25-27]. Gpa1 activation initiates Gβγ disassociation for Fus3 activation which causes a response to mating pheromone. However, when Fus3 (MAPK) interacts with activated Gpa1, there is a diminished response for adaptation. Yet, mutations on Gpa1 at the suspected docking site

show budding yeast with a heightened response [26]. In another study, researchers found that active and inactive Fus3 can interact with Gpa1 and that inactive Fus3 can lead to leading to pheromone gradient tracking and morphogenesis control [27]. This interaction suggests the importance of a docking site for MAPK adaptation, regulation surrounding morphogenesis, nucleocytoplasmic localization, and chemoattractant response. This urged researchers to map out and analyze these D-motifs in other organisms.

1.5 MAPKs signaling

Mitogen-activated protein kinases (MAPKs) are a superfamily of enzymes that phosphorylate the hydroxyl group on serine or threonine residues [8]. MAPKs are divided into three main classes: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38s [28-30]. While JNKs and p38 MAPKs are primarily activated by cytokines and stress, ERKs can be activated by mitogenic stimulants, growth factors, and signals that bind G protein-coupled receptors. Conversely, MAPKs are deactivated by mitogen-activated kinase phosphatases (MKPs) [32]. Furthermore, there is the ERK5 subset which is a newly differentiated class that is activated by osmotic and oxidative stress [33,34]. These enzymes are present in most cell types and regulate key roles such as cell differentiation, stress response, and survival.

MAP Kinase pathways typically employ a MAPK cascade consisting of sequential phospho-activation events. As outlined in Figure 1.2, this cascade sometimes involves MAP4K (PAK), but primarily consists of MAP3K (RAF), MAP2K (MEK), and MAPK [7, 35, 36]. As the signal is transduced through this complex cascade, MAPKs operate in both the cytoplasm and the nucleus where they phosphorylate the PXS/TP target site in transcription factors, adaptor proteins, phosphodiesterases, and other

substrates [32, 35]. Due to active site promiscuity, this pathway also depends on docking motifs for enzymes and scaffold proteins to ensure increased specificity and efficiency. To demonstrate, some ERK and p38 MAPK-activated protein kinases have a common docking site ($\Phi_{1\text{-}3}$ X $_{3\text{-}7}\Psi$X Ψ where 'Φ' is a positive residue, Ψ is hydrophobic and 'X' is other), a DEF site (FXF/YP), and an ED site that all contribute to spatial-temporal protein-protein interactions in separate ways [28-36].

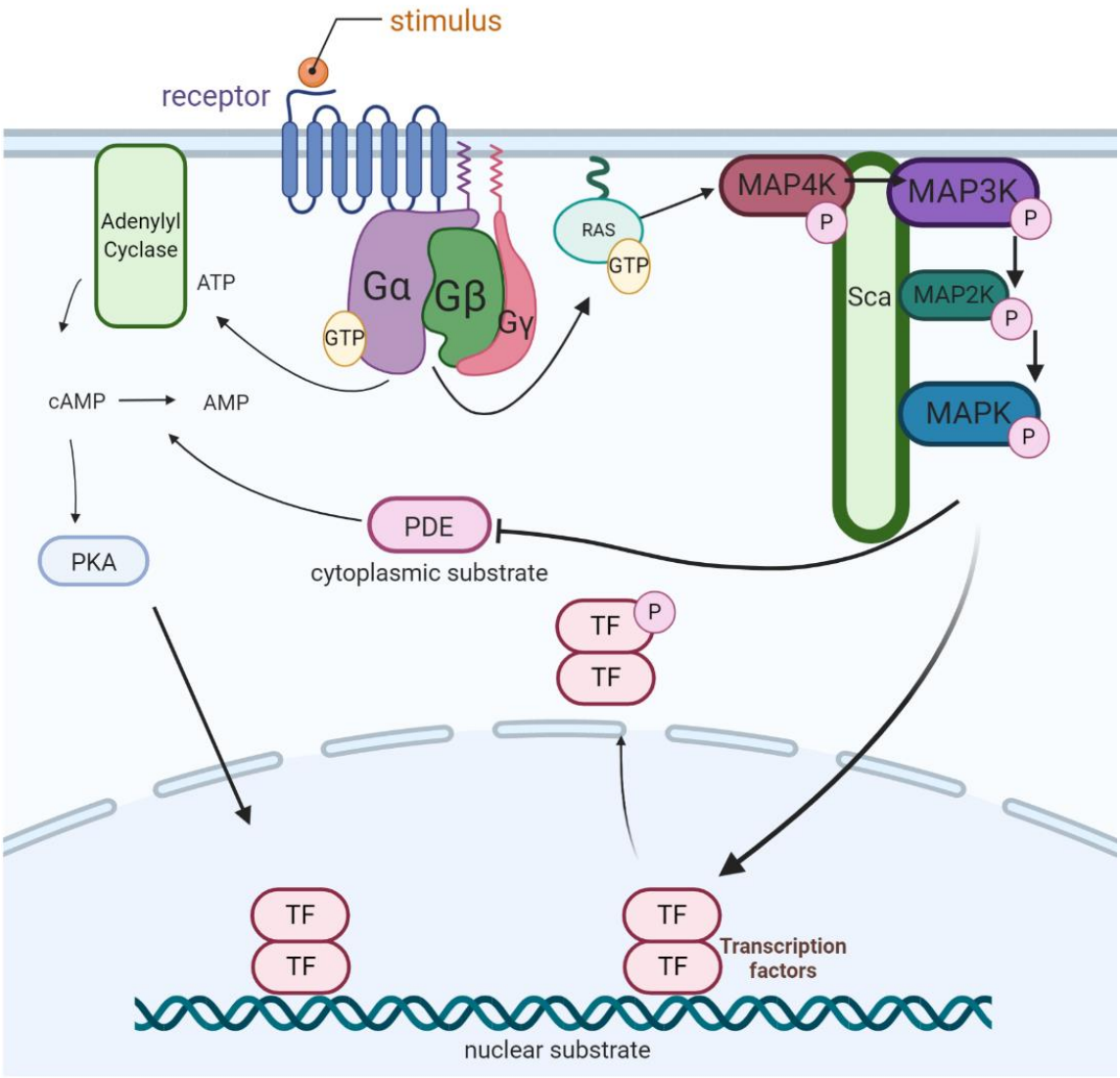


Figure 1.2: General G protein MAPK pathway. Created with Biorender

1.6 *Dictyostelium discoideum* as a model organism

Dictyostelium discoideum is a simple soil amoeba that serves as a model system for eukaryotic biological and biomedical research [37]. *Dictyostelium* studies typically center around fundamental processes like chemotaxis, development, and signal transduction. Single cells are phagocytic in nature and mimic dendritic cells while multicellular aggregates develop into different cell types [38]. Clonal cells are also easily grown in axenic media and on bacterial lawns [39]. In addition, the complete genome of *D. discoideum* is known. This microorganism is simple with respect to other eukaryotes however, it was discovered that there are about 12,500 protein-coding genes compared to 6,000 in yeast [40, 41]. Furthermore, *Dictyostelium* homologs form phylogenetic links connecting both animals and fungi. In the lab amoebae can be transformed with recombinant DNA vectors that can be maintained extrachromosomally or integrate into the genome. This organism has an interesting developmental life cycle that can be analyzed by genetic and biochemical techniques. All these features make *Dictyostelium* research applicable to studying genetic disorders, pathogenesis, and more.

1.7 Life cycle

D. discoideum grows as unicellular solitary amoeba but develops as a multicellular organism. Individual vegetative cells recognize and engulf folic-acid releasing bacteria while nutrient-deprived cells come together to survive harsh environments via an elevated encapsulated spore mass (Fig. 1.3). When starved, stressed cells use cyclic adenosine monophosphate (cAMP) as an intercellular signal to find each other [4, 42-4]. Many other organisms recognize cAMP as a secondary messenger that functions inside the cell rather than an intercellular signaling molecule but *Dictyostelium*

use it both intra- and intercellularly. After several hours, a few cells begin to release cAMP. The cAMP receptors (cARs) on individual cells indirectly activate adenylyl cyclase to make more cAMP as other enzymes relay the signal to other cells [40]. Cells chemotax to cAMP as a mechanism to find neighboring cells but require membrane-associated phosphodiesterases to allow for cAR adaptations [38]. These oscillatory patterns can be seen via streams of cells as they travel up cAMP gradients to assemble into mounds. Once cells form compact aggregates, the multicellular organism rises into a finger-like structure. After standing, they fall into their motile slug stage where they migrate as a unit based on chemotactic, phototactic, and thermotactic capabilities. In their natural habitat, these features would allow them to find the best possible location for spore survival and future germination. Next, the slug comes together in a circular manner once more to form a sombrero or Mexican hat. This stage is similar to the mound stage, but aggregates are more compact and there is a smaller bulb-mound on top of what would become the base of the fruiting body. At this point, cells have solidified their roles as either pre-stalk or pre-spore cells, and thus, the prestalk cells begin to elongate from the center to form a stalk and elevate the spore cells off the substratum. Lastly in the *Dictyostelium* developmental life cycle, there is the fruiting body stage. After ~24 hours, differentiated cells have officially ordered themselves into the base, stalk, and spore mass which form each component of the fruiting body [4, 37, 38, 40].

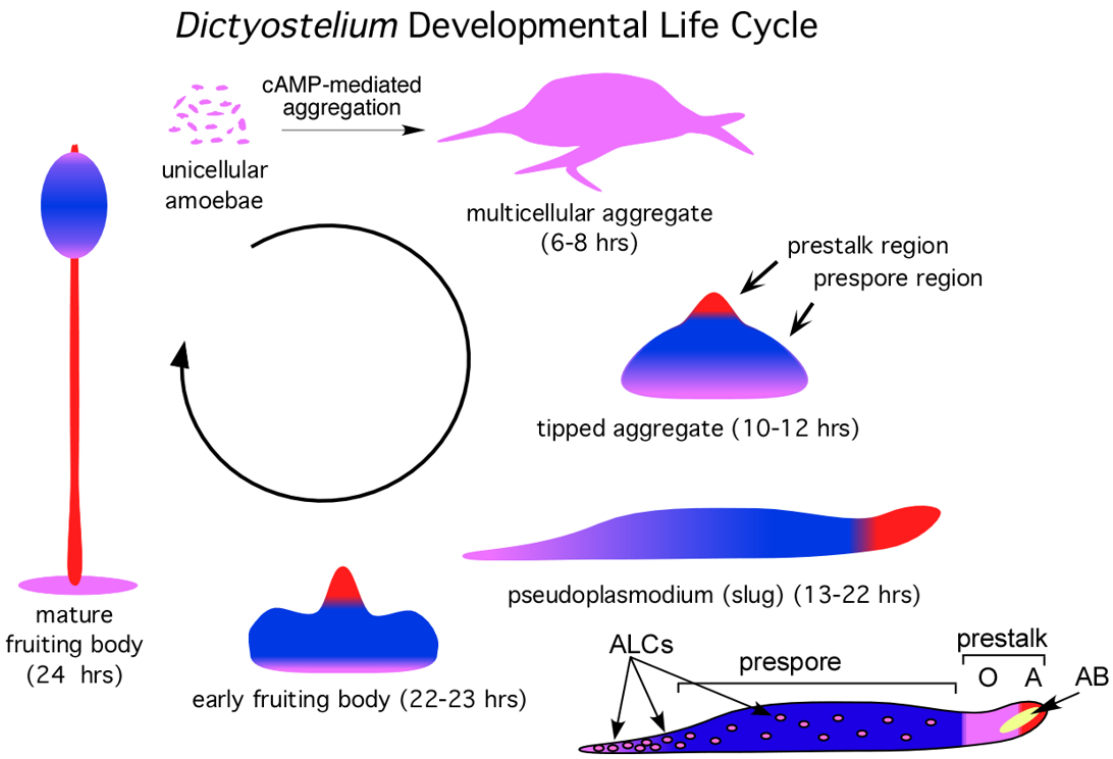


Figure 1.3: Unicellular to multicellular developmental life cycle

1.8 G proteins and ERKs in *Dictyostelium*

D. discoideum has at least 60 putative GPCRs that might regulate cellular responses. Yet, they only have one Gβ and one Gγ subunit that binds to 12 different Gα subunits. In addition to having only one Gβγ dimer, *Dictyostelium* also does not have receptor tyrosine kinases. Regardless, GPCRs and G proteins can mediate activation of Erk1 and Erk2, the only MAPKs in *Dictyostelium* [46,47]. Both ERKs are expressed during growth and development, though erk1$^-$ and erk2$^-$ cells show different developmental phenotypes [4]. Additionally, Erk1 function is less defined, but research suggests that phospho-activation of Erk1 requires the conventional MAP2K, MEK, for dual phosphorylation of the TEY motif while Erk2 does not [48, 49]. Research also shows that folate and cAMP stimulation allow initial Erk2 phosphorylation followed by Erk1, though, Erk2 activators are not well defined. Null Erk1 cells are known to have

altered cAMP signaling and form small aggregates while erk2$^-$ cells do not aggregate at all.

Though the reduced number of MAPKs calls into question specificity and efficiency, docking sites in other proteins such as Gα subunits might allow pathway-specific complexes. *Dictyostelium* Gα3, Gα4, Gα5, and Gα11 all have putative MAPK docking sites, and all but Gα4 have an amino terminus docking motif in a region comparable to that in the yeast Gα, Gpa1 [26, 50-58]. Besides this, Gα modeling predicts that the D-motif is close to the Gβγ dimer and the receptor bind site showing that these proteins might also require activation and disassociation to allow for MAPK docking [50]. Similarly, Gα amino-terminus mutations in *Dictyostelium* have had significant effects on MAPK functionality and phenotype. To clarify, Gα5 inhibits folate chemotaxis, reduces cell size, and accelerates morphogenesis. In an overexpressed Gα5 subunit, docking site alterations can abate precocious gene expression and accelerated tip morphogenesis during development [51]. These same phenotypes are also seen in erk1- cells suggesting that Gα5 D-motif might regulate Erk1 [50]. In addition, Gα4 is required for Erk2 activation and is important for chemotaxis to folate releasing bacterial cells, prespore cell development, and regional segregation, as well as fruiting body morphogenesis [53-57]. However, it is not required in responses to external cAMP stimulation unlike Gα2 [48, 49, 54, 59, 60].

Gα	+xxxxxxHxH

*Sc*Gpa1 ---MGCTVSTQTIGDESDPFLQN**KR**ANDVIEQS**L**Q**L**EKQRDKNEIKLLLLGAGES-

*Dd*Gα2 ---------- MGICASSMEGE**K**TNTDIN**L**S**I**EKERKKKHNEVKLLLLGAGES-

*Dd*Gα5 --------------MGCILTIEA**KK**SRDID**Y**Q**L**RKEEGSKNETKLLLLGPGES-

*Dd*Gα11 --------------MGSQFSVLN**RK**WLIERS**I**M**I**EKRKRRSNKLIKILMMGNENS-

*Dd*Gα4 90---LNIELEVENKQRAANVL**RR**TIGNEPW**L**L**L**AADIKHLWEDKGIKETYAQ-

*Hs*Gα15 MARSLTWRCCPWCLTEDEKAAA**R**VDQEINR**I**L**L**EQKKQDRGELKLLLL

Table 1.1: Alignment of putative MAP kinase docking site. (K/R(1-2)-X(4-8)-I/L-X-I/L) from *Dictyostelium discoideum* (*Dd*), *Saccharomyces cerevisiae* (*Sc*) and humans (*Hs*). Positively charged and hydrophobic residues are indicated in bold. The altered motif in the Gα2 subunit is indicated by the *Dd* Gα2^{D-} sequence.

1.9 *Dictyostelium* Gα2 subunit

Previous research surrounding *Dictyostelium* Gα2 show that the cAMP GPCR, cAR1, is specific to this subunit. [42]. Therefore, Gα2 is required for developmental cAMP signal transduction, chemotaxis and aggregation [59-61]. The Gα2 subunit helps carry out these functions by regulating early developmental genes and by activating adenylyl and guanylyl cyclase [60]. Direct downstream interactions between Gα2 and either MAPK have not been reported. However, research does suggest that though Erk2 is activated by extracellular cAMP for cAR1 and cAR3-associated processes such as chemotaxis, accumulation, and cell differentiation; neither Gα2 nor Gβ are required for Erk2 phospho-activation when stimulated with cAMP (4, 48-50). Another study also suggests that Gα2 has an essential role throughout development with regards to prestalk cell morphogenesis [59]. Like other *D. discoideum* Gα proteins, this subunit has an amino-terminal putative MAPK docking site and is suspected to respond and adapt to cAMP much like yeast

respond and adapt to mating pheromone. These findings have led us to explore the role of this putative D-motif in *Dictyostelium* Gα2 because while several Gα docking sites have previously been analyzed, the protein interaction between Gα proteins and MAP kinases is still not fully understood.

CHAPTER II

METHODOLOGY

2.1 Strains and media

All *Dictyostelium* strains were derived from the axenic laboratory strain, KAx3. This strain also served as the wild-type control. The Gα2 null gene was obtained by inserting *thyA (thy1),* the gene required for thymidine synthesis, into Gα2 cDNA at a *Bcll* site [42]. All constructs were transformed into $g\alpha2^-$ cells using the electroporation technique outlined below. The cells were maintained on either SM+/3 agar covered with *Klebsiella aerogenes* or in axenic HL5 medium (yeast extract and peptone with 50% glucose and an antimycotic) [39]. Probable clones were screened via 1000X G418 (3-8 μg/mL HL5) or 100X blasticidin (3-5 μg/mL HL5) drug selection. Successful clonal strains were transferred from the original electroporation plates to 24 microwell plates and petri-dishes for their duration and subsequent analysis. A co-population of thymidine-deficient JH10 (thyA::PYR5-6) cells and aggregation-deficient mutants were mixed in a 2:1 ratio to collect aggregation deficient spores [51]. Plasmids and strains used are listed in Table 1.1.

2.2 Gα2 mutagenesis, cloning and expression vectors

To make the high-copy complement strain, the wild-type Gα2 open reading frame (ORF) was amplified from a cDNA vector using the oligonucleotides found in Table 2.1. The sequence was then cut at *Xbal-HindIII* and inserted into a pBluescriptSKII- cloning vector and placed into a modified pDXA-GFP (GFP removed) expression vector at a *HindIII-Xbal* site [61]. The complement low copy vector construct was made by excising the Gα2 gene from a *SpeI* site in the high copy vector and inserting it into a pBluescriptSKII vector with the blasticidin resistant gene at *PstI* (pJH1075).

To make a putative D-motif mutation, PCR amplification of Gα2 was done via site directed mutagenesis (Gene Tailor) using the $G\alpha2^{D-}$ oligonucleotides found in Table 2.1. This mutagenized key D-motif residues (KxxxxxLIL) to alanine. A silent mutation was also introduced to form a *BgIll* site to distinguish between the D-motif mutant and wild-type. This was then put into a TOPO cloning vector. The mutations were verified via sequence analysis and the ORF inserted into the modified pDXA-GFP and pJH1075. Both mutant genes are expressed in integrating vectors under a heterologous actin15 promoter. All high copy vectors express a G418 resistant gene and all low copy vectors have blasticidin resistance. The blasticidin resistance vector only needs a single copy for drug resistance while the G418 resistance vector needs multiple copies.

Extrachromosomal expression vectors for Erk1-GFP, $Erk2^{-}$GFP, GFP-GtaC (pHC326) and pTX-GFP2 were previously described (61-63). The GFP-GtaC, Erk1-GFP and $Erk2^{-}$ GFP extrachromosomal expression vectors were used for confocal analysis and pTX-GFP2 was used for chimera assays [47, 62, 63]. All plasmids and strains used are listed in Table 2.2.

Name	Oligonucleotide sequence
Gα2^{D}-sens	CAACCAATACTGATGCTGCAGCATCTATTGAAAAAGAAAG
Gα2^{D}-anti	CAGCATCAGTATTGGTTGCTTCTCCTTCC
Gα2up	GCCGGCAAGCTTAAAAAATGGGTATTTGTGCATCATCAATGGAAGGAG
Gα2down.	CGGCGCTCTAGATTAAGAATATAAACCAGCTTTCATAACACATTG

Table 2.1: Oligonucleotide list

Strain	Genotype
KAx3	Laboratory wild type, background strain, positive control
Gα2^{HC}/ Gα2^{LC}	Gα2 complement G418 resistant (high copy) / Bsr (low copy)
Gα2$^{D\text{-}HC}$ / Gα2$^{D\text{-}LC}$	Gα2 modified D-motif with silent mutation *Bglll* G418r (high copy) / Bsr (low copy)
gα2^{-}	gα2 null. Disrupted with *thyA*, negative control.

Plasmids	Use and Characteristics	
Topo TA	Cloning vector	
pBluescript II SK^{-}	Cloning vector	
pJH1075	Expression vector. Bsr gene (*PstI* frag.) inserted into pBluescript SK^{-} (p1021)	
pDXA-GFP (pJH1057)	Extrachromosomal expression plasmid. pAct15::GFP::G418r	Modified (GFP removed)
pTX-GFP2 (pJH1058)	pAct15::GFP::G418r	
pHC326	Extrachromosomal expression vector. GtaC-GFP with G418r	Chi lab

Table 2.2: Strains and plasmids

* Other researchers constructed gα2^{-} cells, preformed site directed mutagenesis and worked with high copy Gα2 expressing cells. All high copy (HC) experiments were done by NA.

2.3 Electroporation transformation procedure

Desired clones were grown overnight in shaking HL5 medium to mid-log phase (approx. 3×10^6 cells/ml). The cell suspensions were washed in electroporation buffer (12 mM NaH2PO4 adjusted to pH 6.1 with KOH and 50 mM sucrose) and harvested. Cells were resuspended in fresh electroporation buffer and mixed with 25 uL of expression plasmid DNA solution. Cells and plasmid DNA were mixed, electroporated (3.0 uF, 1.3 kV) and transferred to a petri dish with 8 mL HL5. Drug selection of either G418 or blasticidin began 24 hours after electroporation. Visible colonies of drug-resistant transformants were present approximately one week later. These clones were then isolated, screened, and used in analysis. [52, 64].

2.4 Phenotypic screening

Previously selected transformants were grown overnight to 2-5 × 10^7 cells/ml. They were then harvested by dislodging cells from HL5 plates and centrifugation at 1K rpm. The cells were washed twice in phosphate buffer (12 mM NaH2PO4 adjusted to pH 6.1 with KOH), and resuspended in phosphate buffer at 10^7-10^8 cells/ml. After, cell suspension. were plated out in droplets for development on non-nutrient phosphate (1.5% agar) plates or *K. aerogenes* lawns. Cell development was examined via dissecting microscopy [47].

2.5 Chimera assay

Mixed population droplets of either KAx3 and complement or KAx3 and D-motif mutants were plated out for development using the phenotypic screening procedure as described above. Prior to being plated on non-nutrient phosphate plates, mutant cells were mixed in a 1:10 ratio with KAx3. Here each mutant population expressed GFP using pTXGFP2 [63]. Photographs were taken over the course of 24 hours (approx. 9-

and 19-hours post-plating) to track development of these chimeras and the mutant's ability to co-aggregate [47].

2.6 Above-agar chemotaxis assay

Cell droplets were plated out as aforementioned. In addition to this, 1µl of 100 µM cAMP stimulant was spotted 2-3 mm away from the cell droplet. All strains were treated identical and were placed on the same agar plate. Initial images were obtained at the start of the assay and again after 3 hours. Agar indents were made to combine the corresponding images from each time point. Chemotaxis was examined by marking the circumference of individual drops made in the initial time point and using that to measure the leading edge of cells that migrated towards the chemoattractant at the final time point. Each assay analyzed at least five droplets per strain and multiple separate assays were done for each strain. Two-tailed student's t-tests were done to analyze statistical differences $P < 0.05$ was considered to indicate a statistically significant difference [47, 51].

2.7 Confocal fluorescence microscopy

GtaC-GFP expressing cells were grown overnight in fresh medium. Cells were placed on coverslips attached to 60 mm petri dishes containing a 10 mm diameter hole and were allowed to settle for 10 minutes. The initial media and dead cells were removed, and more media was added. After 15 minutes, adhering cells were washed for 30 seconds and covered in developmental buffer (phosphate buffer with 2 mM $MgCl_2$ and 1 mM $CaCl_2$). Settled cells were then stimulated with 100 nM cAMP. The cytoplasmic and nuclear distribution of the GFP-GtaC reporter was recorded using the 60X oil objective over an

8-minute time period with 30 second intervals using spinning disk confocal microscopy. GFP-GtaC videos were analyzed using Fiji (ImageJ. U. S. National Institutes of Health) by calculating the mean intensity of pixels from a selection in the nucleus compared to a selection made in the cytoplasm [62]. GtaC and MAPK-GFP nucleus-cytoplasmic ratio data was normalized by averaging the first and second time points and using that value in proportion to cells at later time points. Outliers were discarded if they fell outside of the interquartile range.

CHAPTER III

RESULTS

3.1 Overexpression of the Gα2 or Gα2^{D-} subunit

To examine the contribution of the putative D-motif in the amino terminal region of the Gα2 subunit, a mutant Gα2 allele in which the signature codons of the motif, encoding the positively charged and large hydrophobic residues, were converted to alanine codons (Table 2.1). Similar alterations have been used to examine putative D-motifs in other Gα subunits [27, 46, 50]. The expression of the wild-type Gα2 or mutant Gα2^{D-} from the relatively constitutive act15 promoter on extrachromosomal vectors conferring G418 drug resistance was initially used to assess whether the Gα2^{D-} subunit conferred the same capability as Gα2 subunit in developmental processes. On non-nutrient agar plates these strains were able to aggregate but most aggregates remained at the mound stage rather than developing further like parental strains containing only an endogenous Gα2 allele (Fig. 3.1). Clonal transformants growing on bacterial lawns created plaques in which cell aggregates were observed for both Gα2 and Gα2^{D-} expressing cells but rather than continuing through the multicellular developmental life cycle the mounds typically disaggregated (data not shown). Only in rare cases for both the Gα2 and Gα2^{D-} strains did the mound continue to develop further into fruiting bodies. To determine if the overexpression of the Gα2 subunits interferes with developmental

progression, the Gα2 and Gα2^{D-} expression vectors were introduced into the parental strain for phenotypic analysis. Clones containing either vector displayed aggregation in response to starvation but the transition between the mound and slug stages of development was delayed by several hours. The developmental phenotype of strains carrying these expression vectors suggests that ectopic expression of the Gα2 or Gα2^{D-} subunit from a high copy number extrachromosomal vector can provide sufficient Gα2 function to complete aggregation but then can delay or block development beyond the mound stage.

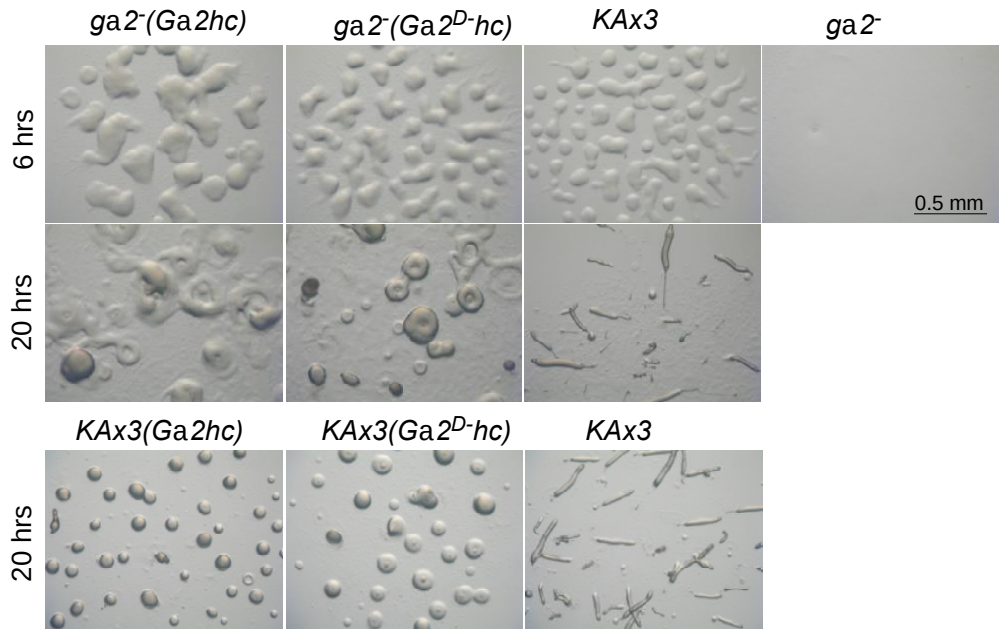


Figure 3.1: Developmental phenotypes in high copy strains. Parental strain (KAx3) and gα2^{-} strains with or without the high copy number Gα2 or Gα2^{D-} vectors were grown in fresh medium for 24 hr. and then washed free of nutrients and plated on non-nutrient plates. Images of developmental morphology were recorded at the times indicated. Only

the 6 hr. image of the gα2^{-} strain is shown because the cells remained aggregation deficient at later times. All images are at the same magnification.

3.2 Gα2^{D-}LC expression does not rescue aggregation efficiently

As an alternative to the high copy number vectors, the Gα2 and Gα2^{D-} subunits were expressed from a previously characterized low copy number integrating vector conferring blasticidin resistance [49]. The expression of Gα2 from this vector in gα2^{-} cells rescued aggregation and subsequent development without a noticeable delay in the transitions from mounds to slugs suggesting the lower copy number of Gα2 vector provides a more physiological relevant level of Gα2 than the high copy number vector (Fig. 3.2). This phenotype was consistently observed for independent clones implying that most random genomic integration sites allow for appropriate gene dosage. In contrast, gα2^{-} cells expressing the Gα2^{D-} subunit from the same vector were defective in aggregation. This aggregation defect was not observed when the Gα2^{D-} vector was expressed in parental cells containing the endogenous Gα2 allele indicating that ectopic expression of the Gα2^{D-} subunit does not cause an aggregation defect. While some small aggregates could be observed during synchronous starvation on non-nutrient plates, most cells did not actively aggregate into mounds suggesting the lower gene copy number does not efficiently complement the Gα2 gene disruption as does the Gα2 subunit. Extended culturing of Gα2^{D-} expressing cells often led to an increase in aggregation capability when plated at higher densities implying that the aggregation defect is an unstable phenotype that can be altered through acquired mutations. However, growth of Gα2^{D-} expressing cells on bacterial lawns remained completely aggregation deficient (Fig. 3.2). Expression of the wild-type Gα2 subunit from the low copy vector was sufficient for

aggregation and development on bacterial lawns. All subsequent analyses of the Gα2 and Gα2^{D-} subunit were conducted in gα2^{-} clones containing the low copy number expression vectors.

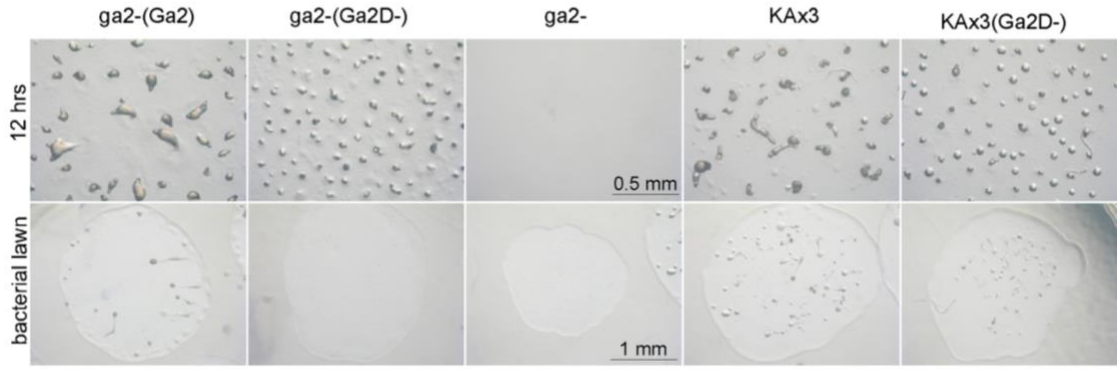
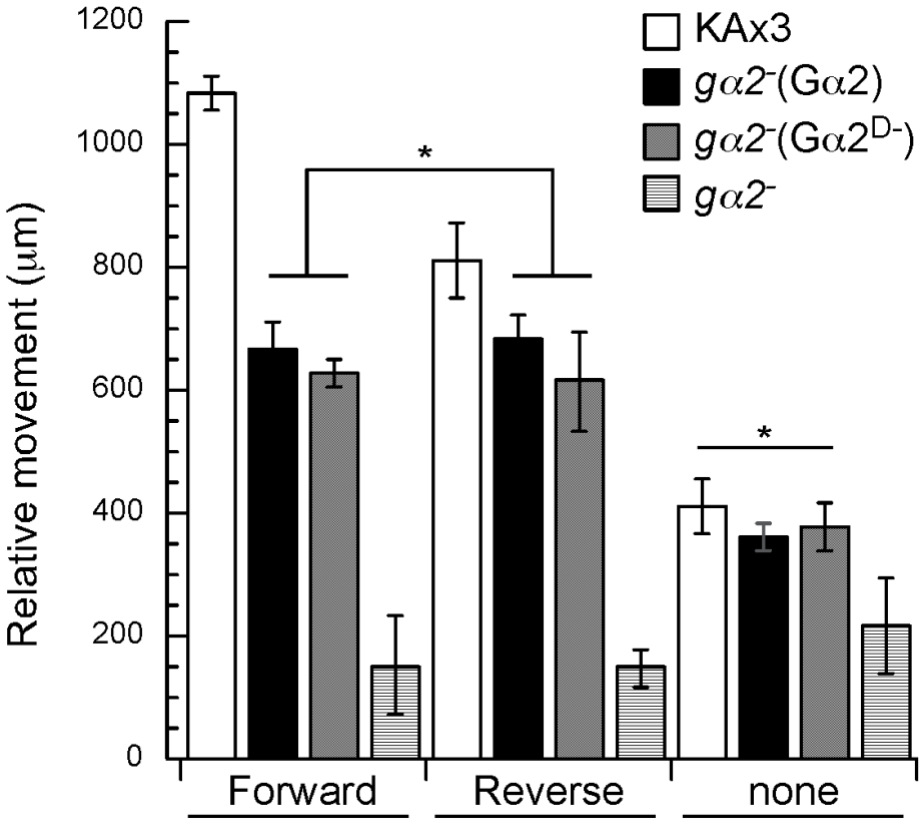


Figure 3.2: Developmental phenotypes in low copy strains. For development on non-nutrient plates strains were grown in fresh medium and treated as described in Figure 3.1. Images were recorded at the time indicated (upper panels – all with same magnification). Strains were also spotted on a lawn of *Klebsiella aerogenes* and images of the plaques and developing aggregates were taken after 4 days (lower panels – all with same magnification). Plaques on the edge of the images are from different strains.

3.3 Alteration of the D-motif results in defective intercellular signaling

The defective aggregation associated with the Gα2^{D-} subunit could result from cell autonomous defective responses to cAMP stimulation or from the inability of the cells to generate intercellular signals such as cAMP. Chemotaxis assays of gα2^{-} cells expressing the Gα2 or Gα2^{D-} genes indicated both subunits allow for increased movement to exogenous cAMP stimulation compared to gα2^{-} cells without either expression vector (Fig. 3.3). In both strains, cells moved greater distances in the presence of cAMP but a

directional bias in this movement to was not evident in either strain compared to that observed for the parental KAx3 strain. The cAMP gradient used in chemotaxis assays is not static because the cAMP diffuses past the cell droplet. This diffusion over time allows for cells to move in all directions from the cell droplet but typically a greater proportion of cells have moved in the direction of the cAMP source. The Gα2 and Gα2^{D-} expressing cells exhibited similar movement in all directions which suggests that chemokinesis rather than chemotaxis is the dominant response.



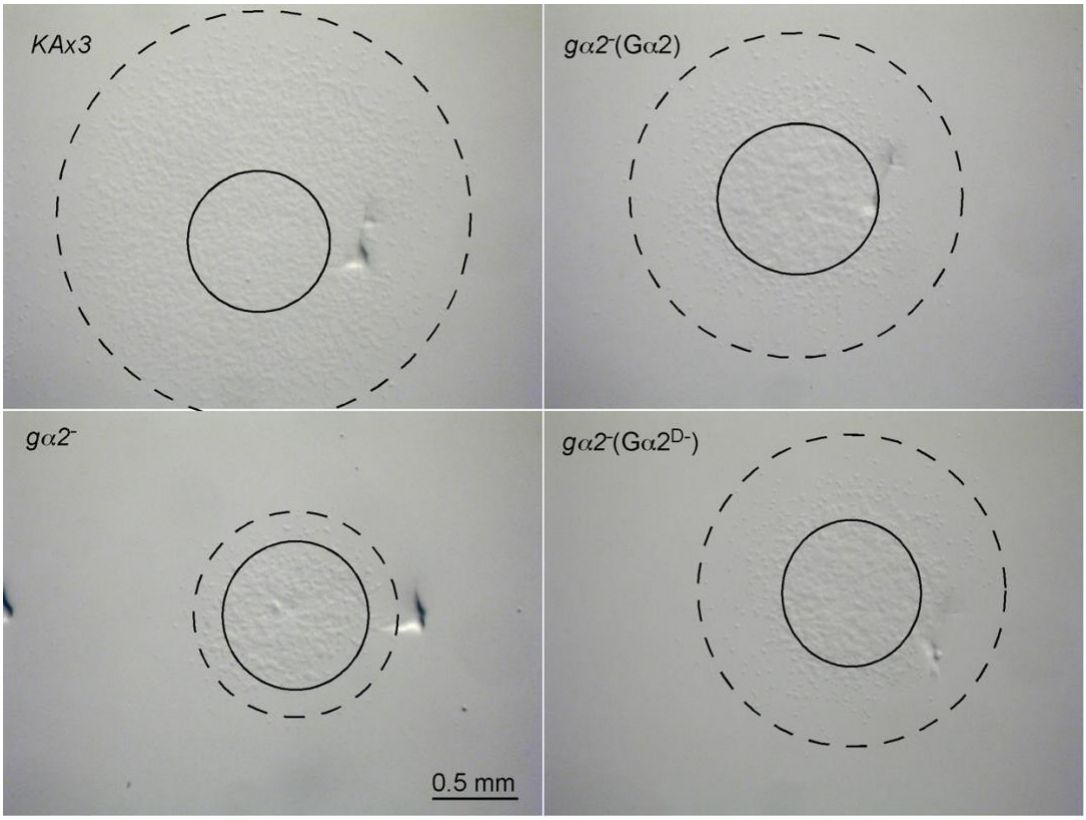


Figure 3.3: Chemotaxis of complement Gα2 and Gα2^{D-} to cAMP. Cells were prepared and assayed for cAMP chemotaxis as described in Chapter II. (A) Distance between the leading edge of cells and the original perimeter of the cell droplet is indicated. Cell movement toward (forward), away from (reverse), or in the absence of cAMP are displayed. Values represent the mean of one chemotaxis assay of six cell droplets for each strain and the error bars represent the standard deviation. Chemotaxis data is representative of at least 3 assays. Differences indicated by an asterisk or distances of all gα2$^-$ strains were assessed by Student's t-test (two tail) and determined to be not significantly different (P>0.05). (B) Images of chemotaxis assays.

Other aggregation defective mutant strains that do not secrete sufficient levels of cAMP have been shown to co-aggregate with wild-type cells in a chimeric population because the wild-type cells can provide sufficient intercellular signaling [66]. Therefore, GFP-labeled Gα2 or Gα2^{D-} subunit expressing cells were mixed with parental KAx3 cells prior to development and the presence of the Gα2 or Gα2^{D-} subunit expressing cells in aggregates was determined using fluorescence microscopy. The Gα2 and Gα2^{D-} subunit expressing cells were capable of co-aggregating with the wild-type cells suggesting wild-type intercellular signaling is sufficient to rescue aggregation of Gα2^{D-} expressing cells (Fig. 3.4). The distribution of the Gα2 and Gα2^{D-} cells in chimeric slugs were similar in that both were underrepresented in the extreme anterior regions but otherwise these cells were found throughout the other regions. Chimeric populations with gα2^{-} cells showed limited co-aggregation with parental KAx3 cells and these are likely cells that have been carried along rather than taking an active role in aggregation.

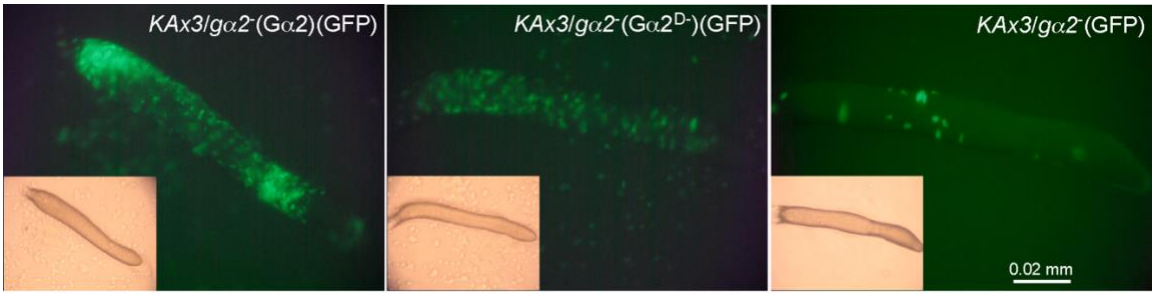
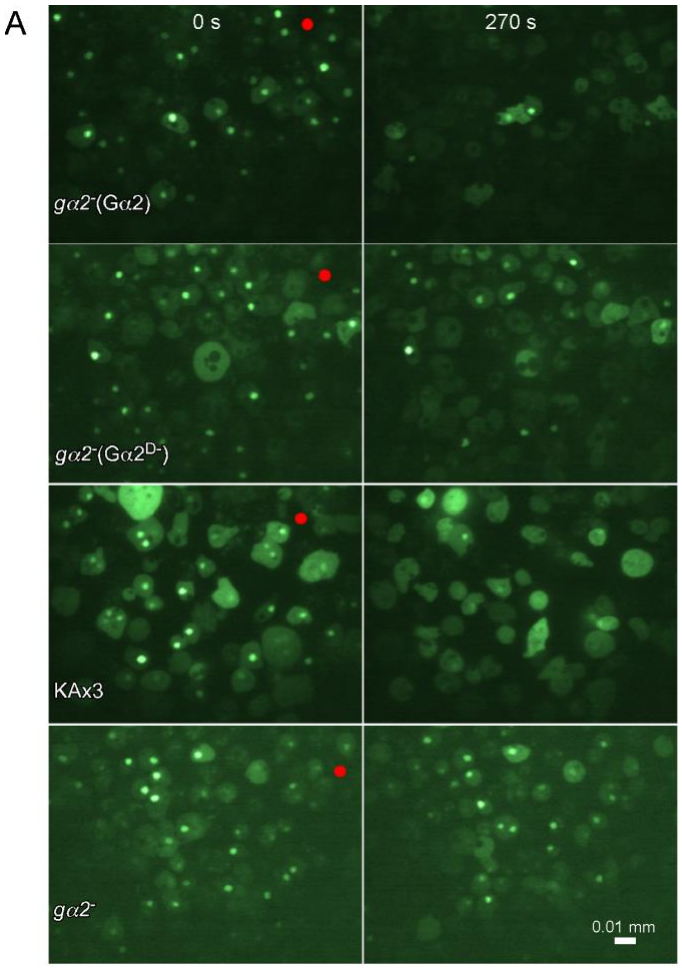


Figure 3.4: Co-aggregation with parental strain. Cells expressing the Gα2 or Gα2^{D-} subunit or no Gα2 subunit were labeled with a GFP expression vector and mixed with KAx3 cells at a ratio of 1:10 before development on non-nutrient plates. Images of developing structures were recorded using fluorescence microscopy. All images are at the same magnification except the brightfield inset images that are at 25% magnification.

3.4 D-motif alteration impacts transcription factor translocation

Many G protein-mediated signaling pathways modulate gene expression through the regulation of transcription factors. A recently characterized response to external cAMP is the transient translocation of the transcription factor GtaC from the nucleus to the cytoplasm. The shuttling of GtaC is thought to be an important process in gene regulation during the aggregation phase of development [62, 66]. The movement of GtaC from the nucleus to the cytoplasm can be monitored using a GFP-GtaC reporter construct [62]. To test whether the putative MAPK docking site on Gα2 impacted the shuttling of the GtaC transcription factor between the nucleus and the cytoplasm, a GFP-GtaC reporter vector was introduced into strains expressing Gα2 or the $G\alpha2^{D-}$ subunit. Both strains exhibited translocation of the reporter from the nucleus to the cytoplasm after cAMP stimulation but the translocation in the $G\alpha2^{D-}$ strain was slower and less extensive than in the Gα2 strain and just slightly greater than in the $g\alpha2^{-}$ strain (Fig. 3.5). This observation suggests that the MAPK docking site on Gα2 contributes to GtaC translocation and therefore is likely to play a role in developmental gene regulation.


A
0 s
270 s
gα2-(Gα2)
gα2-(Gα2D-)
KAx3
gα2-
0.01 mm

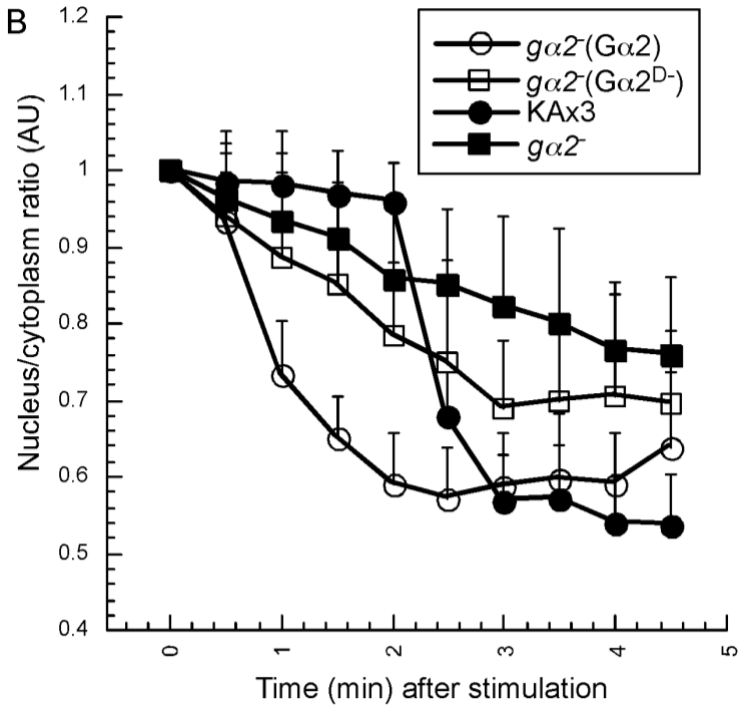
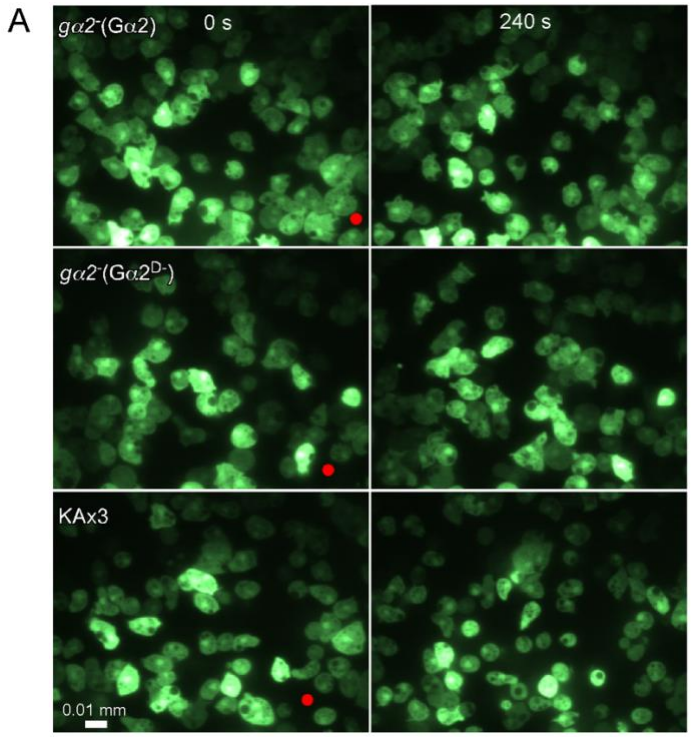

B
gα2-(Gα2)
gα2-(Gα2D-)
KAx3
gα2-
Nucleus/cytoplasm ratio (AU)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0
1
2
3
4
5
Time (min) after stimulation

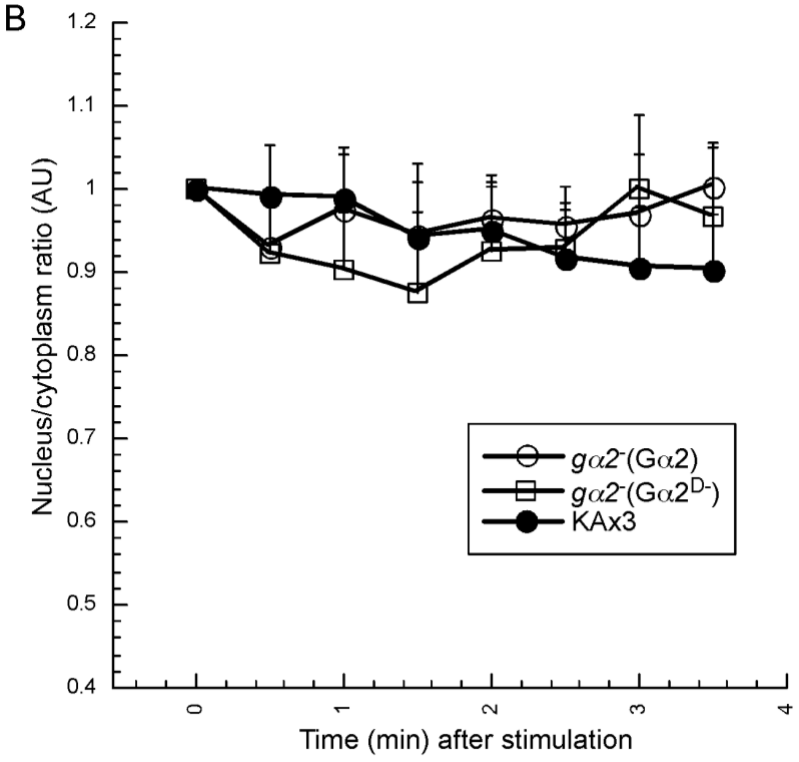
Figure 3.5: Translocation of the GtaC transcription factor. Strains transformed with the GFP-GtaC reporter vector were prepared as described in the Methods section and then stimulated with 100 nM cAMP. Parental KAx3 cells containing the same reporter are shown as a control. (A) Images at the start of cAMP stimulation and 4 min after stimulation are shown using 60x objective oil immersion. (B) Graphical representation of the ratio of nuclear to cytoplasm mean fluorescence in response to cAMP stimulation. Data represent the mean of ratios within middle 50% of values for the strains gα2^{-}(Gα2) (open circles, n=52, 100nM cAMP), gα2^{-} (Gα2^{D-}) (open squares, n=52, 100nM cAMP), and gα2^{-} (closed squares, n=75, 100nM cAMP). Parental strain KAx3 (closed circles, n=25, 100nM cAMP). Error bars represent the standard deviation.

3.5 Alteration of the D-motif does not change MAPK cellular distribution

The nucleocytoplasmic translocation of transcription factors is often dependent on MAPKs or other protein kinases while other studies suggest MAPKs are involved with its regulation (Cai and Hadwiger, unpublished results). In a previous study, we discovered that *Dictyostelium* Erk1 and Erk2 can be found in both the cytoplasmic and nuclear compartments of the amoeba based on the expression and distributions of GFP tagged Erk1 and Erk2 proteins. This study qualitatively indicated that chemoattractant stimulation with either cAMP or folate did not cause major changes in the distribution of the GFP-Erk1 or GFP-Erk2 in cells. We therefore re-examined MAPK distribution quantitatively using confocal fluorescence microscopy. The absence of major changes in the ratio of nuclear/cytoplasmic ratio over the course of cAMP stimulation in the parental KAx3 cells confirmed previous conclusions that the distribution of GFP-Erk2 does not undergo changes in responses to cAMP stimulation (Fig. 3.6). The nuclear/cytoplasmic

ratio of GFP-Erk2 distribution also did not change in Gα2 or Gα2^{D-} expressing cells stimulated with cAMP. The same analysis of GFP-Erk1 was more challenging due to the limited number of cells with a detectable level of fluorescence in all strains. The basis of this limited expression is unknown but could represent toxicity associated with the GFP-Erk1 protein in most cells of the population. Based on the analysis of far fewer cells, the nuclear/cytoplasmic ratio of GFP-Erk1 also did not undergo detectable changes after stimulation with cAMP in parental KAx3 cells (Fig. 3.6). This lack of change was also observed in the very few Gα2 or Gα2^{D-} cells with detectable GFP-Erk1 (Fig. 3.6C). While no major changes in the nuclear/cytoplasmic ratios were observed in response to cAMP stimulation it is possible that a small portion of the MAPKs can translocate between these compartments during the response.


A
gα2⁻(Gα2)
0 s
240 s
gα2⁻(Gα2D⁻)
KAx3
0.01 mm


B
Nucleus/cytoplasm ratio (AU)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0
1
2
3
4
Time (min) after stimulation
gα2⁻(Gα2)
gα2⁻(Gα2D⁻)
KAx3

C

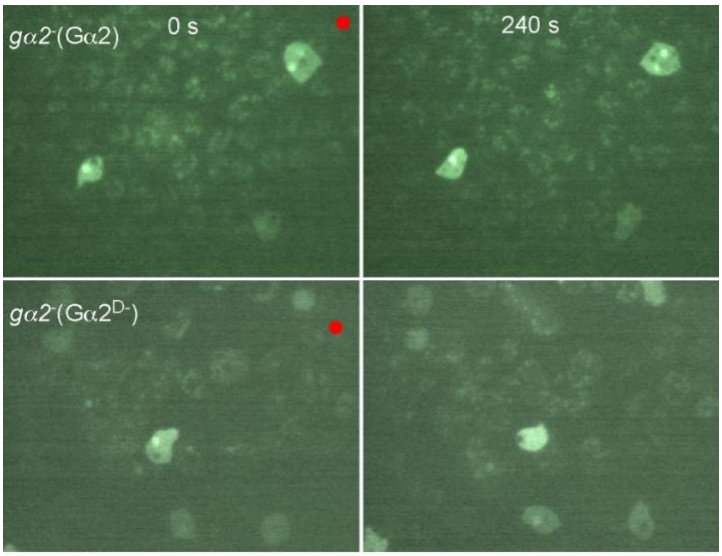


Figure 3.6: Cellular distribution of GFP-Erk2 and GFP-Erk1. Strains transformed with the GFP-Erk2 reporter vector were prepared and analyzed as described in Fig. 3.5. Parental KAx3 cells transformed with the same vector are shown as a control. (A) Representive images at the start of cAMP stimulation and 4 min post stimulation (upper panels) using 60x objective oil immersion. (B) Graphical representation of the ratio of nuclear to cytoplasm mean fluorescence in response to cAMP stimulation. Data represent the mean of ratios within middle 50% of values for the strains gα2^{-} (Gα2) (open circles, n=31), gα2^{-} (Gα2^{D-}) (open square, n=21), KAx3 (closed circle, n=30). (C) Before and after images of GFP-Erk1 using 60X objective oil immersion.

CHAPTER IV

DISCUSSION

The results from this study indicates that the putative MAPK docking motif in the amino terminal region of the *Dictyostelium* Gα2 subunit contributes to the aggregation of cells during the developmental response to starvation. While the requirements for Gα2 subunit function in aggregation and cAMP chemotaxis were established many years ago from the analysis of strains with a disrupted Gα2 locus, the phenotype of cells expressing the $G\alpha2^{D-}$ subunit is distinct in multiple ways. Firstly, aggregation is not completely impaired during synchronous starvation on non-nutrient plates even though aggregation is completely absent in plaques when cells are grown on bacterial lawns. Secondly, the defective aggregation is not directly associated with a loss of chemotaxis to cAMP because the $G\alpha2^{D-}$ expressing cells display movement in response to cAMP in manner similar to that of cells expressing the wild-type allele. This movement in both strains is clearly stimulated by cAMP but the movement does not show the chemotactic directionality observed for cells with the Gα2 subunit expressed from the Gα2 locus. Lastly, cells expressing the $G\alpha2^{D-}$ allele can co-aggregate with wild-type cells suggesting that the altered Gα2 subunit confers aggregation competence when supplied with extracellular signals or interactions. Therefore, the $G\alpha2^{D-}$ subunit is capable of some but not all Gα2 functions and some of these functions are limited to synchronous starvation

B not present during development on bacterial lawns. The impaired translocation of the GFP-GtaC reporter in Gα2^{D-} expressing cells in response to cAMP implies that Gα2 can contribute to this process. An earlier report demonstrated that the translocation of this transcription factor occurs independently of the Gβ subunit that functions to mediate chemotaxis responses to cAMP, suggesting that the signaling pathway that regulates this translocation can occur independently of the Gβ subunit. The delayed translocation of the GFP-GtaC reporter in gα2^{-} cells suggests that the Gα2 subunit is not essential but contributes to the regulation this process. The expression of the Gα2^{D-} subunit impairs this process compared to the wild-type Gα2 control implying the putative MAPK docking site region of Gα2 is important for translocation. GtaC has been previously shown to be phosphorylated in cells responding to cAMP and therefore it is possible that the putative MAPK docking site of the Gα2 subunit impacts the function or distribution of protein kinases that regulate GtaC. The distinction in the pattern of translocation between parental cells and the complemented gα2^{-} cells suggests that heterologous expression of the Gα2 subunit, perhaps increased expression and accelerated the initial translocation. Expression of Gα2 subunit from a multicopy vector could potentially alter the stoichiometry of the subunit with receptors, effectors, or MAPKs and therefore lead to an unusual pattern of GtaC translocation.

The lack of detectable changes in the nuclear/cytoplasmic distribution of GFP-Erk1 or GFP-Erk2 in response to cAMP stimulation suggests that substantial changes in the distribution of MAPKs are not required for the rapid translocation of the GtaC transcription factor. The assessment of MAPK distribution in other organisms have also utilized MAPKs tagged with fluorescent proteins and in some cases, changes in

distribution have been associated with cell stimulation. In mammals the typical MAPKs, Erk1 and Erk2, show increased presence in the nucleus in response to cell stimulation [67]. In the yeast mating response, the Fus3 MAPK becomes enriched in the nucleus and this enrichment is impacted by alterations in the MAPK docking site motif [25, 27]. Interestingly, the Kss1 MAPK can also be activated in response to mating pheromone, but this MAPK increases in the cytoplasm suggesting that MAPKs do not follow a universal mechanism in response to external stimuli [68]. Translocation of a small portion of the MAPKs between these compartments cannot be ruled out and it is possible that the fluorescent protein tagged version of the MAPKs might not be regulated or function as the endogenous MAPKs.

A surprising result from the analysis of high copy number Gα2 expression vectors was the inhibition of developmental progression beyond the mound stage. While not previously reported in the complementation of gα2^{-} mutants, this phenotype is likely the result of higher gene copy number of Gα2 expression vectors because the low copy number vectors with the identical Gα2 genes did not inhibit this developmental transition. The heterologous expression of the Gα2 alleles from the act15 promoter might allow for exceptionally high levels of Gα2 subunit after mound formation and this could lead to disruptions of signaling pathways in developing aggregates. An earlier study has shown that expression of the Gα2 gene from a prespore or prestalk promoter did not alter development [59]. There are approximately 60 G protein coupled receptors in *Dictyostelium* and out of four cAMP receptors, at least two are expressed primarily after aggregation, though research suggests there are more. Excessive Gα2 subunit levels could impair signaling processes at these later stages especially if there are competitions with

other related Gα subunits. High copy number Gα1 subunit expression vectors also result in a delay in the mound to slug transition [69]. The Gα1 subunit is the closest paralog to the Gα2 subunit [55]. Overexpression of the Gα4 subunit, a mediator of folate chemotaxis with the folate receptor, Far1, can delay the aggregation phase and block development after aggregate formation [55, 70]. Chimeric studies indicate that too much Gα4 inhibits the development of prestalk cells that form a tip on the aggregated and lead developmental progression from the mound to a slug [57]. However, overexpression of the Gα5 subunit, most closely related to the Gα4 subunit, has the reverse effect by promoting prestalk cell development and precocious tip formation on mounds [55, 51]. Taking all these phenotypes in account, the high copy expression of the Gα2 subunit must not be a generalized Gα subunit overexpression phenotype but rather a phenotype more specific to an individual subunit.

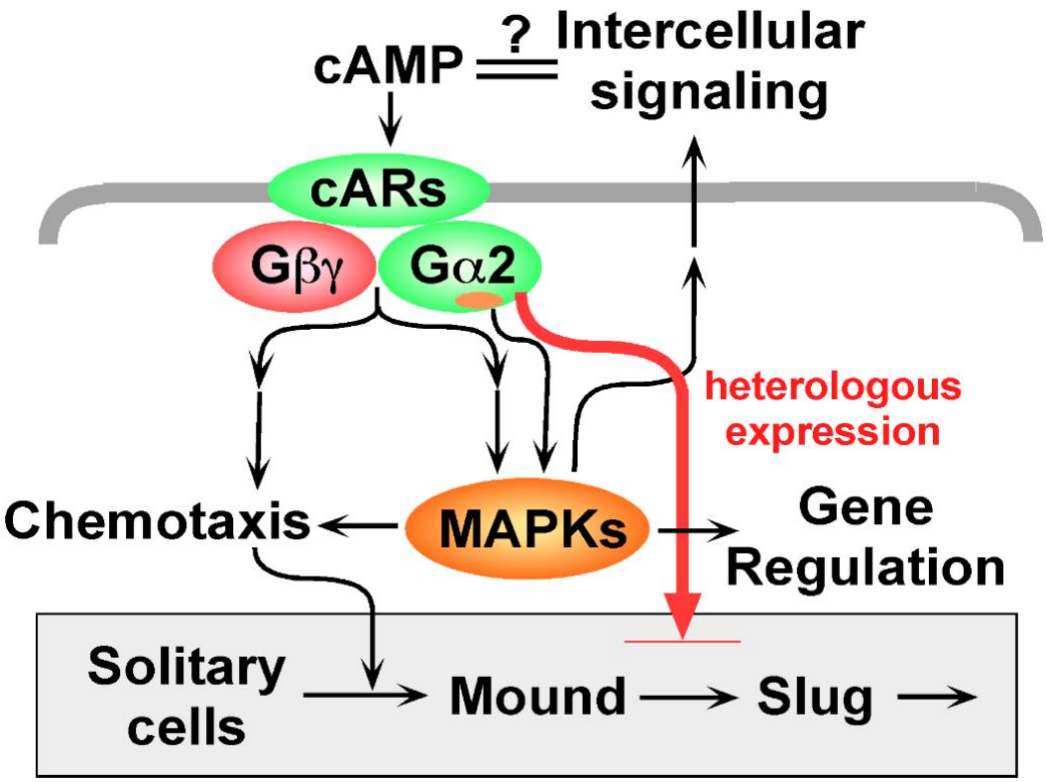


Figure 3.7: Model for the role of the putative Gα2 D-motif in developmental signaling. Stimulation of cAMP receptors (cARs) leads to Gα2 activation and a variety of cellular responses including chemotaxis, gene regulation, and intercellular signaling. Heterologous expression of Gα2 subunits can lead to the inhibition of developmental progression (red arrow). The putative D-motif in Gα2 (orange section) contributes to MAPK regulated processes.

CHAPTER V

CONCLUSION

The aim of this study was to characterize the MAPK docking site in *Dictyostelium* Gα2. Here we analyzed the docking motif role by expressing a $G\alpha2^{D-}$ mutant gene in $g\alpha2^{-}$ cells under low and high copy vectors and examined their ability to cAMP-chemotax, communicate intracellularly, and regulate transcription factor shuttling. The results of this study suggest the region corresponding to the putative D-motif of Gα2 serves important roles in intercellular signaling during aggregation and the translocation of the GtaC transcription factor. The heterologous expression of the Gα2 subunit can also compromise the directed movement of cells to cAMP and inhibit developmental morphogenesis suggesting that G protein subunit stoichiometry is an important factor throughout the developmental life cycle. While not directly contributing to the activation of MAPKs, Gα subunits might contribute to signaling pathways by associating with complexes that contain MAPKs. The presence of D-motif sequences in some of the Gα subunits found in other organisms suggests such Gα subunits possibly play a role in the regulation of cellular responses in organisms other than yeast and Dictyostelid. These results are broadly compatible with previous Gα docking motif studies as prior findings suggest and confirm that Gα subunits have docking sites for MAP kinases. However, based on the analysis conveyed, it can be concluded that the Gα2 D-motif is not

important for all Gα2 mediated processes.

5.1 Limitations

Possible limitations in this study surround chemotaxis assays and translocation analysis methodologies. Over time, above agar chemotaxis assays involve a dispersal of cells as well as diffusion of chemoattractant. This can cause disorientation of cells not related to Gα2 mutant interactions. Similarly, a high cell density could urge leading-edge cells to move away from each other in search of food and could appear to move based on the presence or absence of stimulus. Other studies that use more controlled chemoattractant gradients, such as microfluidics, and distinctive cell tracking could lessen these limitations. In the translocation analysis, there could be limitations surrounding sample selection and size. We attempted to offset outliers by employing a statistical threshold but in doing so forfeited sample cells. For certain strains, variability in fluorescence amongst cells plays a factor while other strains struggled to maintain viable fluorescing cells as outlined in Chapter III. In the future, advanced computational software and mass quantifications could be beneficial. However, the general trends seen in this study suggest that the overall impact of these limitations are low.

5.2 Future research and implications

Dictyostelium, serves as a good model to study the cell biology linked to these pathways. More specifically, use of the GtaC reporter could be used to characterize the remaining Gα-MAPK docking sites. One way our lab tried to use the reporter was by combining chemotaxis and translocation analysis. With the proper equipment, cells could

be exposed to chemoattractant and allowed to chemotax while confocal microscopy would be used to monitor nucleocytoplasmic shuttling.

Future research also could employ studies ranging from *in vitro* co-immunoprecipitation to *in silico* biological network analysis. On a small scale, co-immunoprecipitation is a common technique that could be used to zero in on G protein-MAPK interactions and complexes based on antigen detection for one of the suspected proteins. This assay would allow researchers to then separate and detect proteins in a western blot. Though, this might cause some issues for transient G protein interactions and assays that use less efficient antibodies. To further understand the bigger picture, network analysis could provide a comprehensive view of direct protein-protein interaction for a simple eukaryote like *Dictyostelium*. This is a less common technique that uses nodes to represent proteins while edges signify physical interactions. This methodology shows a detailed view of how information flows in a particular pathway while highlighting the most important protein or gene based on the number of point-contacts. In the past it has been used to discover protein complexes, feedback loops and motifs. This would be for a very expansive yet general interactomic study.

Nevertheless, D-motif characterization offers suggestive evidence for universal protein communication and complexes. Arguably, most mammalian diseases occur when protein-protein interactions have gone awry. Both Gα and MAPKs are ubiquitous and their inability to function properly could lead to neurodegenerative diseases like Parkinson's or Amyotrophic lateral sclerosis to birth defects, cardiovascular disease, and cancer. Therefore, understanding how these proteins interact with one another is imperative for

elucidating signal transduction pathways, their individual protein functions, and could provide insight for the treatment of signal transduction related disorders.

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