THE CHARACTERIZATION OF MAP KINASE REGULATION OF CYCLIC AMP SIGNALING IN *DICTYOSTELIUM*

By

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THE CHARATERIZATION OF MAP KINASE REGULATION OF CYCLIC AMP SIGNALING IN *DICTYOSTELIUM*

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Abstract: cAMP signaling plays a critical role in cell development and chemotaxis of *Dictyostelium discoideum*. Regulation of the MAP kinase protein Erk2 and phosphodiesterase RegA activity eventually controls the cAMP-mediated cell activities in *Dictyostelium*. Previous studies suggest that Erk2 might be down regulating its substrates, including RegA, to obtain control of cAMP levels and cAMP-associated functions. Conventional MAPKs interact with its substrate via consensus docking motif found in its substrates. Mutational studies show that the putative MAPK docking motif in RegA is not essential for downregulation of RegA function by Erk2. Alteration of docking motif enhanced the Erk2-RegA interactions. *Dictyostelium* Erk2 is an atypical MAP kinase; therefore, it might regulate its substrates by a different mechanism. Genetic epistasis tests suggest that the MAP kinase Erk1 might positively regulate RegA function. Erk1 activation occurs as a secondary response to Erk2 activation suggesting that Erk1 might function in an adaptation response by promoting RegA function and decreasing cAMP levels. The *Dictyostelium* Gα2 subunit is highly expressed during the aggregation stage of development and the loss of Gα2 function results in the inability of cells to chemotax to cAMP and to aggregate. A putative MAPK docking motif in the amino terminus of the *Dictyostelium* Gα2 subunit suggests this subunit might interact with MAPKs in a manner similar to that observed for a Gα subunit in yeast during mating responses. Alteration of the MAPK docking motif in Gα2 impairs aggregation but enhances cell movement. These phenotypes suggest that the docking site might facilitate the interaction of Gα2 with MAPKs in the adaptation response to cAMP.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION TO cAMP SIGNALING, PHOSPHODIESTERASES, MAP KINASES AND G-PROTEINS....1
1.1. Cyclic adenosine monophosphate (cAMP) is an important regulator of cellular activities....1
1.2. RegA phosphodiesterase regulates intracellular cAMP....2
1.3. Structure of RegA phosphodiesterase....4
1.4. MAP kinases regulate many proteins including phosphodiesterase RegA in *Dictyostelium*....5
1.5. MAP kinase docking motifs are found in RegA and Gα proteins....7
1.6. MAP kinase proteins interact with Gα proteins....7
1.7. G protein function and Gα subunits....8
1.8. *Dictyostelium discoideum* development, differentiation, and its importance in biological research....10
1.9. Implication of our study in other systems....14
1.10. Outline of the dissertation....15
1.11. References for chapter I....17

II. *ACANTHAMOEBA* AND *DICTYOSTELIUM* USE DIFFERENT FORAGING STRATEGIES....28
2.1. Abstract....28
2.2. Introduction....29
2.3. Results....32
2.3.1. Comparison of amoeboid chemotaxis to folate....32
2.3.2. Comparison of amoeboid chemotaxis to bacteria....37
2.3.3. *Acanthamoeba* dispersal....41
2.3.4. Comparison of G protein Gα subunits....43
2.3.5. Comparison of other G protein subunits....46
2.4. Discussion....49
2.5. Conclusion ….....52
2.6. Methods....53
2.6.1. Strains, growth conditions....53
2.6.2. Chemotaxis assays....53
2.6.3. G protein ortholog analysis....55
2.7. Acknowledgements....55
2.8. Supplemental data....56

Chapter Page

2.9. References for chapter II 56

III. *DICTYOSTELIUM* ERK2 IS AN ATYPICAL MAPK REQUIRED FOR CHEMOTAXIS 66
3.1. Abstract 66
3.2. Introduction 67
3.3. Materials and methods 70
3.3.1. Strains and development 70
3.3.2. Recombinant DNA constructs and amplifications 71
3.3.3. Chemotaxis assays 72
3.3.4. Analysis of bacterial cell engulfment 72
3.3.5. Reporter protein translocation 73
3.3.6. Immunoblot analysis of MAPKs 73
3.3.7. MAPK ortholog analysis 75
3.4. Results 75
3.4.1. Disruption of the *erk2* gene 75
3.4.2. Erk2$^-$ cells have growth defects on bacterial lawns 77
3.4.3. Loss of Erk2 impairs bacterial engulfment 80
3.4.4. Erk2 is required for folate chemotaxis 81
3.4.5. Loss of Erk2 does not affect folate detection & early signaling events 84
3.4.6. Erk2 is required for development and cAMP chemotaxis 86
3.4.7. Loss of Erk2 impairs Erk1 activation in folate chemotactic response ..88
3.4.8. Erk2 sequence is related to atypical MAPKs 89
3.5. Discussion 91
3.6. Supplemental data 96
3.7. References for chapter III 101

IV. MAPK REGULATION OF THE REGA PHOSPHODIESTERASE IN *DICTYOSTELIUM* 111
4.1. Introduction 111
4.2. Methods 114
4.2.1. Strains and mutants 114
4.2.2. Growth and culture conditions 115
4.2.3. Cloning and mutagenesis 115
4.2.4. Transformation and selection of clones 115
4.2.5. Phenotype study and chimera study 116
4.2.6. Immunoprecipitation and western blot 116
4.3. Results 117
4.3.1. Putative MAPK docking sites in *Dictyostelium* RegA. 117
4.3.2. Over-expression of *regA* with alter D-motif delays development 118
4.3.3. Expression of *regA*$^{D-}$ from the endogenous *regA* promoter did not rescue the phenotypic defect of *regA*$^-$ cells.....120
4.3.4. *regA*$^{D-}$ cells display a bias in their distribution in chimeric aggregates122

Chapter Page
4.3.5. Putative D-motif is not essential for ERK2 and RegA interactions.....125
4.3.6. Erk1 genetic epistasis test shows RegA and Erk1 are likely to function in same pathway 127
4.4. Discussion 129
4.5. Supplementary data 133
4.6. References for chapter IV 134

V. STUDY OF PUTATIVE MAPK D-MOTIF IN *DICTYOSTELIUM* Gα2 PROTEIN 138
5.1. Introduction 138
5.2. Methodology 140
5.2.1. Strains and mutants 140
5.2.2. Phenotype study 141
5.2.3. Cloning and mutagenesis 141
5.2.4. Transformation of *Dictyostelium* cells 141
5.2.5. cAMP chemotaxis 142
5.2.6. Western blot for Gα2/ $G\alpha2^{D-}$ subunit levels 142
5.3. Results 142
5.3.1. *Dictyostelium* Gα2 protein has a putative MAP kinase D-motif 142
5.3.2. Expression of *gα2* or $g\alpha2^{D-}$ genes from high copy number vectors impacts the progression of development 143
5.3.3. Expression of $G\alpha2^{D-}$ protein from a low copy number vector does not rescue aggregation 146
5.3.4. Presence of wild-type cell signaling does not rescue aggregation of cells expressing the $G\alpha2^{D-}$ subunit from a low copy number vector 147
5.3.5. Low-copy $g\alpha2^{D-}$ cells exhibit increased cell movement 148
5.4. Discussion 150
5.5. References for chapter V 153

VI. CONCLUSIONS AND FUTURE DIRECTIONS 156
6.1. References for chapter VI 163

REFERENCES 167

LIST OF TABLES

Table Page

2.1. Percent identity of amoeboid Gα subunits to Dd Gα4 subunit 45

LIST OF FIGURES

Figure Page

1.1. *Dictyostelium discoideum* life cycle 10
2.1. Chemotaxis of *Dictyostelium* and *Acanthamoeba* to folate 34
2.2. Migration maps of *Dictyostelium* and *Acanthamoeba* in folate chemotaxis assays 36
2.3. Chemotaxis of *Dictyostelium* and *Acanthamoeba* to bacteria 39
2.4. Migration maps of *Dictyostelium* and *Acanthamoeba* in chemotaxis to *Klebsiella aerogenes* 40
2.5. *Acanthamoeba* movement at different cell densities in the presence of bacteria 42
2.6. Phylogenetic tree of *Dictyostelium* and *Acanthamoeba* G protein Gα subunits .. 45
2.7. Phylogenetic trees of G protein Gβ subunits/Racks and Gγ subunits of some amoebozoan and other selected eukaryotes 48
2.8. Model of *Acanthamoeba* movement 49
S2.1. Movie of *Dictyostelium* movement in the presence of folate 56
S2.2. Movie of *Acanthamoeba* movement in the presence of folate 56
S2.3. Movie of *Dictyostelium* movement in the presence of *K. aerogenes* 56
S2.4. Movie of *Acanthamoeba* movement in the presence of *K. aerogenes* 56
S2.5. Movie of *Acanthamoeba* movement near a droplet of *K. aerogenes* 56
3.1. Disruption and knock-in complementation of the *erk2* locus 76
3.2. *Dictyostelium* growth 79
3.3. Engulfment of bacteria 81
3.4. Chemotaxis of MAPK mutants to folate 83
3.5. Early chemotactic signaling in response to folate 85
3.6. Development and cAMP chemotaxis 87
3.7. Phosphorylation of MAPKs 89
3.8. Phylogenetic analysis of MAPKs 90
3.9. Model of Erk2 mediated signaling pathways 92
S3.1. Verification of genomic insertions 96
S3.2. Nuclei staining of axenic shaking cultures 97
S3.3. Chemotaxis of MAPK mutants to folate 98
S3.4. Movie of wild-type cell movement in the presence of folate 99
S3.5. Movie of *erk2*$^-$ cell movement in the presence of folate 99
S3.6. Movie of *erk2*$^-$ mutant complemented with Erk2 expression vector cell movement in the presence of folate 99

Figure Page

S3.7. Movie of *erk1*$^{-}$*erk2*$^{-}$ cell movement in the presence of folate 99
S3.8. Movie of *erk1*$^{-}$*erk2*$^{-}$ mutant complemented with Erk2 expression vector cell movement in the presence of folate 99
S3.9. Wild-type (WT) and *erk1*$^{-}$ cell chemotaxis to folate 100
S3.10. Images of typical *erk1*$^{-}$*erk2*$^{-}$ colonies 100
4.1. Insertion of complementary *regA/regA*$^{D-}$ gene under endogenous *regA* promoter 115
4.2. Sequence alignment of catalytic domain of *Dictyostelium* phosphodiesterase RegA with mammalian phosphodiesterases 118
4.3. Comparison of RegA sequences between different species of Dictyostelids.....118
4.4. Developmental phenotype of high copy *regA/regA*$^{D-}$ cells 119
4.5. Fruiting body structure of high copy *regA/regA*$^{D-}$ cells 119
4.6. Late stage development phenotype of cells expressing *regA/regA*$^{D-}$ under endogenous *regA* promoter 121
4.7. Fruiting body of *regA/regA*$^{D-}$ cells 122
4.8. Chimera study of GFP tagged *regA/regA*$^{D-}$ cells 124
4.9. Co-immunoprecipitation of Erk2 with RegA/RegA^{D-} protein 126
4.10. Genetic epistasis test to show relation between *erk1* and *regA* 128
4.11. Genetic epistasis test to show relation between erk1 and regA 129
4.12. Proposed model for regulation of RegA by Erk1 and Erk2 respectively 132
S4.1. Alignment of catalytic domain RegA phosphodiesterase with different mammalian phosphodiesterases 133
S4.2. PCR verification of *regA/regA*$^{D-}$gene integration into *regA* locus 133
S4.3. Model of catalytic domain of RegA phosphodiesterase 134
5.1. Protein sequence alignment of different *Dictyostelium* Gα subunits and *Saccharomyces* Gpa1 143
5.2. Early stage developmental phenotypes of cells expressing the Gα2 and Gα2^{D-} subunits from high copy number vectors.....144
5.3. Late stage developmental phenotypes of cells expressing the Gα2 and Gα2^{D-} subunits from high copy number vectors 144
5.4. Detection of Gα2 and Gα2^{D-} subunits expressed from high copy number expression vectors 145
5.5. Detection of Gα2 subunit at a different times of starvation 145
5.6. Developmental phenotypes of parental KAx3 cells expressing the Gα2 and Gα2^{D-} subunits from high copy number vectors 146
5.7. Developmental phenotypes of cells expressing the Gα2 and Gα2^{D-} subunits from low copy number vectors 147
5.8. Development of chimeric populations and cell fate 148
5.9. Above agar cAMP chemotaxis assays 149
5.10. A model representing possible role of *Dictyostelium* Gα2 protein in chemotaxis 152

CHAPTER I

INTRODUCTION TO cAMP SIGNALING, PHOSPHODIESTERASES, MAP KINASES AND G PROTEINS

1.1. Cyclic adenosine monophosphate (cAMP) is an important regulator of cellular activities

cAMP is an essential intracellular cell signaling molecule and it is required in many cellular responses, such as cell differentiation, development, proliferation, and chemotaxis [1]. cAMP production in the cell is stimulated when the extracellular signals activate a G protein-coupled receptor (GPCR). The G protein associated adenylyl cyclase, downstream of GPCRs, produces cAMP when activated. The produced cAMP diffuses through the cytoplasm and binds to effector proteins. Protein kinase A (PKA), a heterotetramer protein, is a widely known effector protein activated by cAMP. The activated protein kinase then phosphorylates other proteins essential for gene expression and other cellular responses to extracellular signals. PKA is activated when cAMP binds to its regulatory subunits releasing the catalytic subunits that phosphorylate target proteins [2, 3]. cAMP-PKA activity is made more specific by subcellular localization of the PKA by A-kinase anchoring proteins, AKAPs [4]. cAMP is an important intracellular and extracellular signaling molecule for *Dictyostelium discoideum*. In *Dictyostelium*

extracellular cAMP interacts with cAMP receptors, cARs, and these receptors are expressed during different stages of development.

Starved *Dictyostelium* cells secrete the cAMP with oscillating pulses. This secreted extracellular cAMP acts as a chemoattractant when it binds to cARs in surrounding *Dictyostelium* cells. This step causes *Dictyostelium* cells to aggregate together and initiate the development process. Intracellular cAMP is also essential for aggregation and cell differentiation at later stages. These cAMP-dependent processes are mediated through PKA activity [5, 6]. Phenotypes of PKA mutants show that cAMP is required for proper regulation of *Dictyostelium* morphogenesis. The level of cAMP can be regulated in *Dictyostelium* in various ways. The extracellular cAMP can be turned over by membrane-associated phosphodiesterases, PdsA, and intracellular cAMP is broken down mostly by the phosphodiesterase RegA. The degradation of extracellular cAMP by PdsA is vital to allow cells to adapt and return to the excitable state for cAMP sensing. This regulation of cAMP is essential for the early stage of development of *Dictyostelium*. Similarly, cAMP production is also regulated by PKA via feedback inhibition of adenylyl cyclase[6–9]. PKA is essential for activating several proteins, some of which regulate development genes [10].

1.2. RegA phosphodiesterase regulates intracellular cAMP

cAMP and cGMP are two important cyclic nucleotides regulating several signal transduction processes in *Dictyostelium*. Intracellular and extracellular cyclic nucleotides are controlled by a pool of seven different phosphodiesterases in *Dictyostelium.* Three of these enzymes belong to class I phosphodiesterases and the four others belong to class II phosphodiesterases. Some phosphodiesterases are either specific to cAMP or cGMP and

others have dual specificity to both types of cyclic nucleotides. Phosphodiesterases with the same specificity can be spatially segregated to regulate different pools of cyclic nucleotides.

The cAMP-specific RegA phosphodiesterase is the primary regulator of intracellular cAMP level in *Dictyostelium* [11–13]. The breakdown of the bound cAMP causes PKA regulatory subunit to bind the PKA catalytic subunit resulting in the loss of catalytic activity [14]. The cAMP-specific action of RegA is supported by the fact that when cells are treated with phosphodiesterase inhibitors this leads to cAMP accumulation [15]. Phosphodiesterases hydrolyze the phosphodiester bond in cAMP and cGMP. The mechanism of hydrolysis is deduced from the catalytic mechanism of mammalian phosphodiesterase isoforms. The catalytic domain has two metal ions as well as several participating amino acids that are critical for the hydrolysis [16]. RegA phosphodiesterase receives upstream signals for regulation of cAMP level and PKA activity. RegA activity is also regulated by several mechanisms, which includes phosphorylation modification by an upstream histidine kinase, feedback phosphorylation regulation by PKA and phosphorylation modification by upstream MAP kinase Erk2 [9, 17]. These different phosphorylation modifications either down regulate or up regulate the catalytic activity of RegA[18, 19]. RegA is essential for normal functioning of several processes in *Dictyostelium*. Disruption of the *regA* gene causes accelerated spore formation, small aggregates during development and suppression of lateral pseudopods [20, 21].

The loss of RegA function is found to rescue the spore formation in some G protein mutants as well as in leaky *erk2* mutants [9, 22]. Similarly, a mutation at a putative MAP kinase phosphorylation site in RegA prevented a down regulation of phosphodiesterase [9]. These findings indicate that upstream G proteins and Erk2 regulate RegA, directly or indirectly.

The information obtained from the RegA protein sequence and structure, in our study, supports the presumed regulation of RegA by Erk2.

1.3. Structure of RegA phosphodiesterase

Insights of RegA phosphodiesterase structure can be drawn from mammalian cAMP-specific phosphodiesterase structure. RegA phosphodiesterase consists of two domains: a regulator domain and a catalytic domain [18]. A phosphate group transfer to the regulatory domain is found to increase the phosphodiesterase activity by 20 fold [23]. Mammalian phosphodiesterases (PDEs) have variable regulatory domains. These regulatory domains interact with different proteins that determine their spatial and functional specificity. The regulatory domains may be variable in length giving rise to different isoforms. The variability in length of the regulatory domain can orchestrate completely different outcomes of phosphodiesterase activity. Unlike, the regulatory domain, the catalytic domain is highly conserved.

Phosphodiesterase catalytic domains are typically located in the C-terminal. Some phosphodiesterases also have regulatory elements in the catalytic domain [24, 25]. cAMP-specific human PDE4 catalytic region interacts with a MAP kinase for the modulation of phosphodiesterase activity. The PDE4 catalytic region has two different docking sites (D-motifs), that flank a specific phosphorylation site. The phosphorylation of this site down regulates the catalytic activity of PDE4 [19, 26, 27]. Our preliminary studies reveal that RegA catalytic domain has a putative MAP kinase-docking site but without the FQF motif. RegA also has a specific threonine residue (T676) that is possibly a MAP kinase phosphorylation site. The change of this particular threonine to alanine (T676A) caused a significant change in the level of cAMP in *Dictyostelium* cells [9].

Crystal structure of mammalian PDEs catalytic domain reveals that the catalytic region is composed of several α helices that form a deep hydrophobic pocket. Two different bivalent metal ion centers are found in this hydrophobic pocket. One of the metal ions is zinc, while the identity of the next metal ion is unknown. Each metal ion forms bonds with different amino acid residues, AMP and water molecules in a distorted octahedral configuration. Binuclear catalysis is the mechanism proposed for the breaking down of cAMP to AMP. Water or hydroxide molecules bound to metal ions make the nucleophilic attack on the target phosphorous atom of cAMP. Specificity of the enzyme depends on the orientation of the specific amino acid, especially the glutamine residue, in the hydrophobic pocket [28– 30].

1.4. MAP kinases regulate many proteins including phosphodiesterase RegA in *Dictyostelium*

MAP kinase pathways are three-tiered phosphorylation cascades of protein kinases, which transmit signals from Ras-GTP to effector proteins. This system, sometimes called Ras-Raf-MEK-Erk signal transduction, regulates a variety of cell processes including cell migration, cell cycle, immunity, differentiation, growth, metabolism, and chemotaxis. MAP kinases are also important contributors in several pathological processes including cancer, bacterial pathogenesis, autoimmunity, and inflammation [31, 32]. The MAP kinase lies at the lower end of this three-tiered system and is activated by a dual phosphorylation of a specific tyrosine and serine or threonine residue by an upstream MAP kinase MEK (MAP2K). MEK is phosphorylated and activated by upstream MEK kinase (MAP3K). MAP kinases are serine/threonine kinases that phosphorylate specific serine/threonine residues followed by proline residue in its substrates [32, 33]. Besides the typical MAP kinases system, the

mammalian system also has an atypical MAP kinase. Atypical MAP kinases do not have a known upstream activating kinase protein.

MAP kinases are present in lower eukaryotes like fungi and dictyostelids where they control diverse functions [35, 36]. Social amoeba *Dictyostelium discoideum* has two different MAP kinases; Erk1 and Erk2, which share 37% of sequence identity. Both MAP kinases are expressed during vegetative and developmental stages [37]. However, only one MAP kinase kinase (MEK1) protein is known to exist in *Dictyostelium*. Previous studies have suggested that Erk1 but not Erk2 activity is dependent on MEK1. There is a lack of information regarding upstream kinases that regulate MEK1 [38, 39]. *Dictyostelium* MAP kinases are essential for different functions. MAP kinase Erk2 is necessary for chemotaxis, adenylyl cyclase activation during aggregation, morphogenesis, and cell type-specific gene expression during later stages of development [40]. The role of *Dictyostelium* Erk1 is partially known. Disruption studies have shown that *erk1*$^{-}$ cells form small aggregates during development and exhibit defective cAMP chemotaxis. Besides, Erk1 activation occurs as a secondary response to Erk2 activation [41, 42]. Upstream proteins in response to many substances, including cAMP and folate, activate both MAP kinases [36]. In mammalian system, MAP kinases regulate cAMP level in cells indirectly by down regulating phosphodiesterases [19, 27]. In *Dictyostelium discoideum*, leaky *erk2*$^{-}$ cells, show a reduction in the concentration of cAMP. Disruption of *regA* gene in the *erk2*$^{-}$ cells results in an increase in cAMP levels [9]. Our preliminary phenotypic study of these mutants also supported the genetic relationship between *regA* and e*rk2*. The aggregation defect of leaky *erk2*$^{-}$ cells was rescued by disruption of *regA*, suggesting that Erk2 possibly down regulates RegA.

1.5. MAP kinase docking motifs are found in RegA and Gα proteins

The substrates of MAP kinases are identified by the presence of a serine/threonine residue followed by a proline residue. The targeted serine/threonine residue is also flanked by another proline, a few residues upstream. The MAP kinase substrates also have docking sites for interactions with MAP kinases [43]. A MAP kinase consensus D-motif (docking motif) has one or two positively charged residues followed by a spacer region and then a hydrophobic region. An FQF motif can also be found in several MAP kinase substrates [26, 44, 45]. The *Dictyostelium discoideum* RegA phosphodiesterase sequence has a putative D-motif in its catalytic domain towards the C-terminal region, extending between the residues 588-596 (RRSVVQLIL). Unlike the mammalian phosphodiesterases, a FQF motif is not present in RegA. We also found that this D-motif region is highly conserved across other dictyostelids further supporting a possible role in MAP kinase-RegA interactions.

MAP kinase D-motifs are found in other proteins including several Gα subunits in *Dictyostelium discoideum*. *Dictyostelium* Gα5 and Gα4 subunits have a MAP kinase D-motif. Changes in the Gα4 D-motif alters Erk2 and Gα4 interactions and affects morphogenesis during culmination stages of development. The Gα5 D-motif has a role in aggregation size, developmental gene expression, and cell size [41, 46]. The same study showed that another Gα subunit, Gα2, also has a putative MAP kinase D-motif at its N-terminal end.

1.6. MAP kinase proteins interact with Gα proteins

Several studies have shown that in yeast, *Saccharomyces cerevisiae*, the pheromone responsive Gα subunit, Gpa1, interacts with MAP kinase Fus3 resulting in the regulation of MAPK function. The effect of this interaction includes MAPK Fus3 sub-cellular localization, pheromone-induced morphogenesis and a gradient tracking process dependent on Fus3. The

interaction between Fus3 and Gpa1 is essential for adaptation response to mating pheromone. Interestingly, both active and inactive Fus3 can interact with Gpa1. This interaction controls chemotrophic fates in pheromone responding cells [47, 48]. In *Dictyostelium discoideum*, *in vivo* interactions between MAP kinase Erk2 and Gα4 subunit were demonstrated by pull-down assays. The interaction is necessary for late-stage morphogenesis. The Gα4 subunit contains a MAP kinase-docking site at its N-terminal region, the mutation of which caused a reduction in Erk2-Gα4 interaction[46]. Another Gα subunit, Gα2, also has a putative MAP kinase D-motif at its N-terminal region, indicating a possible interaction between MAP kinases and the Gα2 subunit.

1.7. G protein function and Gα subunits

G proteins (guanine nucleotide-binding proteins) are a family of signaling proteins that transmits the signal from membrane-associated G protein-coupled receptors (GPCRs) into the cell. The role of the GPCR domain outside the cell receives the environmental clues in the form of different signaling molecules. The G proteins have three different subunits; α, β, γ. During the inactivated state (GDP bound) the Gα subunit and the Gβγ dimer are linked together to form a heterotrimeric complex. An activated GPCR can promote the exchange of GDP with GTP in the Gα subunit. This binding of GTP causes the Gα subunit and Gβγ dimer to separate. Each part can now interact with effector proteins and transmit the signal downstream. Dynamics of G proteins was first observed in *Dictyostelium* using fluorescence resonance energy transfer (FRET) and total internal reflection fluorescence microscopy (TRIFM). The results showed that the *Dictyostelium* Gα2 subunit and Gβγ dimer dissociate in response to cAMP stimulation. The receptor and its associated G protein did not form a stable complex if the stimulating signals are absent [49, 50]. Intrinsic GTPase activity converts

the GTP to GDP in the Gα subunit resulting in the reformation of heterotrimeric Gα-Gβγ complex and the termination of the signal [51–53]. The G protein subunits are coded by many genes resulting in the diversity of these subunits and specificity in their function. In humans, 16 different genes codes for 21 Gα subunits, 5 different genes code for 6 Gβ subunits and 12 genes encode Gγ subunits. However, the diversity of GPCRs far exceeds the diversity of G proteins, suggesting that a G protein might interact with several different GPCRs [52, 54, 55]. *Dictyostelium discoideum* has 12 different genes encoding Gα subunits and a single gene for a Gβ and a Gγ subunit. In *Dictyostelium*, activated heterotrimeric G proteins are involved in generating second messengers (cAMP, cGMP, etc.) and regulating many cellular activities. The Gβγ subunit is thought to be important for adenylyl cyclase activation [56–58]. The G protein response to extracellular cAMP is one of the most studied responses in *Dictyostelium*. The extracellular cAMP stimulates the GPCR, cAR1, leading to the Gα2βγ complex dissociation. Other downstream signaling includes Ras activation, phosphatidylinositol-3 phosphate kinase (PI3K) activation, the accumulation of PIP3, and the activation of TORC [59].

Among the several *Dictyostelium* Gα subunits, Gα2 is critical for the developmental life cycle. Disruption of the *gα2* gene blocks aggregation process, while expression of some mutant G*α2* alleles cause abnormal stalk differentiation during culmination stage. The serine 113 residue of Gα2 is phosphorylated in response to cAMP stimulation and mutation of this site lead to an aggregation defective phenotype [60–63]. The Gα2 protein also has putative MAP kinase docking site at its N-terminal end. None of the previous mutational studies investigated this docking site. Therefore, in this study, we tried to understand the role of this MAP kinase docking motif site in development and differentiation of *Dictyostelium*.

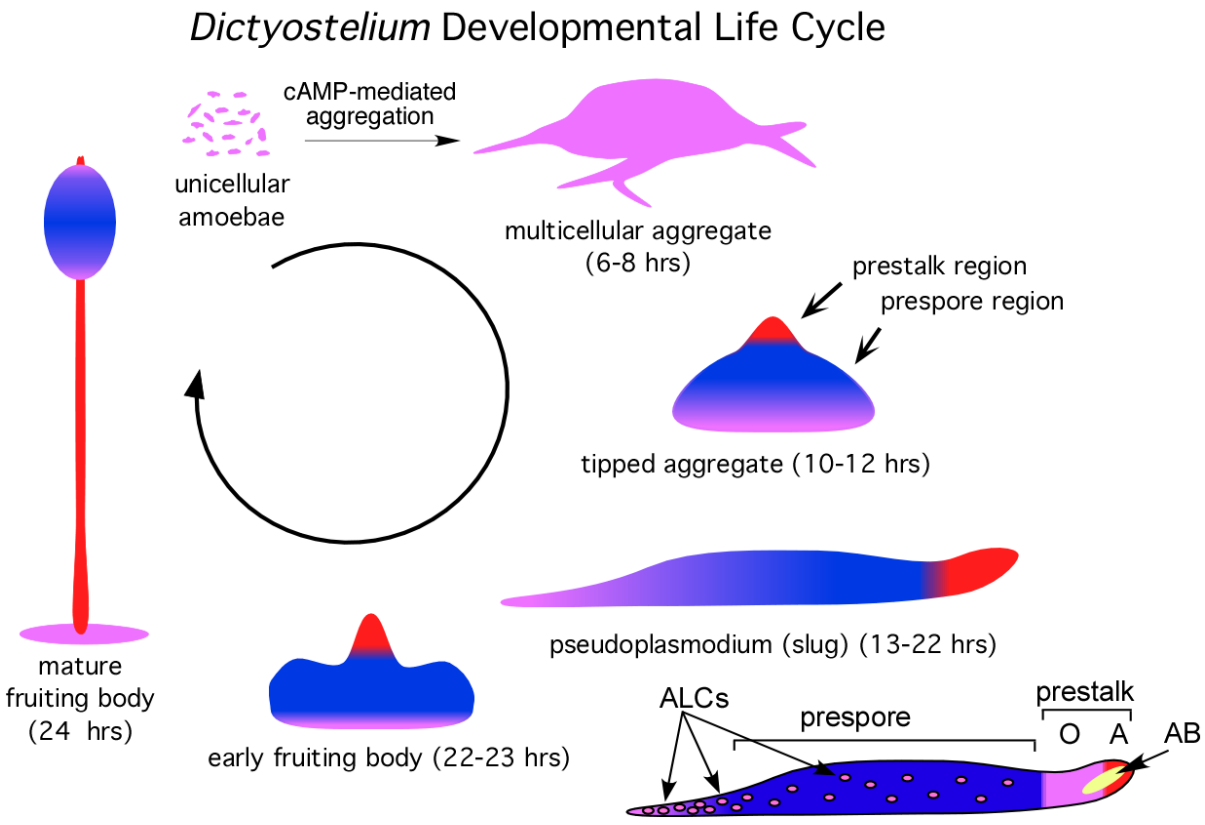

Fig. 1.1. *Dictyostelium discoideum* life cycle. *Dictyostelium* grows as solitary amoebae when conditions are favorable. Nutritional stress causes them to form multicellular aggregates through cAMP chemotaxis. Cells differentiate into prespore and prestalk cells in the slugs. The slugs further develop to form mature fruiting bodies.

1.8. *Dictyostelium discoideum* development, differentiation, and its importance in biological research

Dictyostelium discoideum is a free-living social amoeba found in soil. It grows as a single-celled organism and feeds on bacteria. These amoebae divide by binary fission, and upon starvation, *Dictyostelium* enters into a phase of multicellular development and differentiation. The development cycle is completed after the formation of fruiting bodies within 24 hours. End of exponential growth phase is sensed by quorum sensing factors that detect cell density and prepares them for the starvation phase. Pre-starvation factor (PSF) and condition medium factors (CMF) accumulate in the immediate environment and induce the

synthesis of cAMP receptors (cAR1s) and adenylyl cyclase (ACA). Starvation leads to the migration of cells towards each other, forming loose aggregate mounds [64, 65]. The extracellular pulses of cAMP coordinates the migration and aggregation. cAMP promotes the polymerization of globular actin (g-actin) to filamentous actin (f-actin) [66]. Each cAMP-stimulated *Dictyostelium* secretes additional cAMP and this relayed signal is responsible for the directional flow of neighboring cells towards a center. The cAMP secretion occurs in a pulsatile pattern because of the breakdown of cAMP by a membrane-associated phosphodiesterase. The breakdown is necessary for the adaptation of cAR1 and resetting the cAMP sensing mechanism to allow continued responses. cAMP waves are produced synchronously every 6-8 minutes. The streaming cells finally form a loose aggregate of cells. Extracellular cAMP produced by ACA binds the cAR1 receptor and further activates G proteins downstream. Protein kinase A (PKA) is activated via Erk2. Internal RegA phosphodiesterase is inhibited by Erk2, leading to the activation of PKA. ACA and Erk2 are down regulated by PKA feedback inhibition, enabling RegA to lower cAMP levels and reset cAMP signaling [67–69].

Mound and slug formation processes follow the aggregation stage. During aggregation, cell markers of prespore and prestalk were found in cells indicating that differential adhesion and sorting is probably responsible for differentiation at later stages. One such example is the variable chemotaxis rate of prespore and prestalk cells towards cAMP [70–72]. The tip of the mound/slug produces cAMP, which guides the migration and orientation of the slug and also directs cell sorting. Cells express different types of cAMP receptors during the post aggregation stages. cAMP receptor cAR2 is expressed by prestalk cells and cAR3 is expressed by cells at the posterior part of a slug, prespore cells. Deletion of

cAR2 arrests cells at the mound stage showing the importance of cAMP signaling at post aggregation stages of development [73, 74]. The G-box binding factor (GBF) is an essential transcriptional activator of several post aggregation genes. Some of these genes are expressed in specific cell types [75, 76]. The cells also express tiger genes (*tgrC1* and *tgrB1*). These proteins act like human major histocompatibility complex (MHC) and ensure maximum homogeneity in prespore cells[77]. Differentiation-inducing factor 1 (DIF1) which is produced from the posterior side of the slug promotes stalk cell differentiation and migration. Two genes, *ecmA* and *ecmB,* are induced by DIF1. These two genes are expressed in different regions of the slug. The *ecmA* gene is expressed in prestalk, pstAO region. The *ecmB* gene is expressed in the central part of the prestalk, PstAB, region and the anterior basal region of the slug. Additionally, both genes are expressed in many cells that are among the prespore cells. The *ecmA* and *ecmB* expressing cells later form a stalk, basal disc, upper and lower cup of spore sac in the fruiting body. The stalk cell differentiation is found to be regulated by cyclic di-GMP [78, 79].

A polyketide molecule, 4-methyl-5-pentylbenzene-1, 3 diol (MPBD), initiates the culmination stage. MPBD induces terminal differentiation in prestalk cells and causes the production of spore differentiation factor 1 (SDF-1) for terminal differentiation of sporogenous cells [80, 81]. Several other factors also regulate the terminal differentiation process including glutamate, SDF-2, cytokines, and cyclic diGMP. After culmination is triggered, the slug forms a structure with the prestalk AO region at the top and the prestalk region B at the bottom. The prestalk AB region forms a tube-like structure and cells become vacuolated. More prestalk cells enter into the tube and this leads to an increase in the length of the stalk. During this process, the prespore cells get lifted off the substratum. The prestalk cells

eventually get vacuolated and die. SDF-1 and SDF-2 continue the maturation of prespore into spores [65, 82].

Dictyostelium is used as a model organism for several processes including cell motility, phagocytosis, chemotaxis, some aspects of developmental biology, and cell adhesions. It is also used to study several human diseases and to study host-pathogen interactions [83]. *Dictyostelium* has an interesting lifestyle. The multicellular development stages provide opportunities for studying cell-signaling processes during development. The unicellular cell stage serves as a good model for studying chemotaxis, phagocytosis, and pinocytosis [84]. The genome of *Dictyostelium* is amenable for genetic studies. An estimated 13500 genes are distributed on six haploid chromosomes in *Dictyostelium*. The number of genes found is closer to that in *Drosophila melanogaster* and much higher than the 6000 genes found in *Saccharomyces cerevisiae*. The haploid genome provides easy access to gene manipulation. The success rate for gene disruption using homologous recombination is high. *Dictyostelium* aggregates shows the expression of different marker genes in different cell subtypes. These subtypes show a difference in cell movement pattern and respond to different signaling molecules. This process of differential motility and response to messenger molecules is analogous to development in higher animals [85].

Dictyostelium is a lower eukaryotic organism and is less complex than higher eukaryotes. Therefore, it offers a less complicated system for intracellular cell signaling studies. Concerning our study, *Dictyostelium* provides a less complicated model to study MAP kinase and phosphodiesterase interactions. Two different MAP kinase proteins exist in *Dictyostelium*, Erk1, and Erk2, offering a simpler system to study MAP kinases. In the mammalian system, complexities arise due to many MAP kinases with functional

redundancy and cross talking between the pathways [86]. *Dictyostelium* has several phosphodiesterases. RegA is the major intracellular cAMP-specific phosphodiesterase. The phosphodiesterase RegA has no isoforms, unlike the mammalian species of phosphodiesterases, making the system easy to study. This simplicity makes, *Dictyostelium* a suitable model organism for studying MAP kinase-phosphodiesterase interaction.

1.9. Implication of our study in other systems

Phosphodiesterases are important regulators of many cellular processes. Anti-phosphodiesterase drugs are administered in several diseases conditions to reduce the severity associated with diseases. Phosphodiesterases can be regulated at different times and locations by a variety of proteins. Therefore, understanding of the specific regulation of phosphodiesterase in a given set of conditions might lead to new treatment strategies for some diseases. Study of Erk2-RegA phosphodiesterase interactions in a simple *Dictyostelium* model removes the complexities that would have resulted from studying such interactions in higher eukaryotes. *Dictyostelium* Erk2 is an atypical MAP kinase protein [39]. Further studies are needed to understand the role and mechanism of human atypical MAP kinases in cell signaling. *Dictyostelium* Erk2 provides might provide important insights into the role of atypical MAP kinases as well as their interactions with other proteins. *Dictyostelium* is also a model for understanding development. *Dictyostelium* researchers found that *Dictyostelium* development provides insights into some aspects of development in higher animals and insects[78]. Erk2-RegA interactions occur in response to extracellular cAMP during development. This interaction shows a significant impact on development processes.

1.10. Outline of the dissertation

Our research objective is to investigate the role of a few signaling proteins during early and late stages of *Dictyostelium discoideum* development. *Dictyostelium* is an amoeba and has many conserved signaling pathways that are present in higher eukaryotes and other eukaryotic pathogens. We hope that the insights from our research work can be applied to cell signaling in humans and the pathogenic amoebae *Acanthamoeba castellanii*.

In the second chapter, we are focused on foraging strategy differences between *D. discoideum* and *A. castellanii*. We investigated the chemotaxis of these two amoebae towards folate and bacteria. *Acanthamoeba* did not show chemotaxis towards the chemoattractants, and the motility was found to be random. *Dictyostelium* shows strong chemotaxis response to the chemoattractants. We reason that such behavior of *Acanthamoeba* can be explained from an evolutionary perspective and the absence of a G protein subunit gene in its genome. These findings are published in *Protist* [87]. The principal investigator performed chemotaxis experiments and gene comparisons between amoebas. The other authors performed chemotaxis data analysis.

The third chapter focuses on the role of MAP kinase Erk2 in chemotaxis. We made a complete disruption of *erk2* gene. Previous phenotypic studies of e*rk2* mutants were based on leaky *erk2* mutants. These leaky mutants showed a substantial reduction but not a loss of Erk2 activity. The principle investigator did the *erk2*, *erk1* and *mek1* gene disruptions. Research collaborators and other authors performed the chemotactic response and the biochemical analyses. As an author, my contribution confirmed the *erk2* gene disruption by immunoblot experiments. We concluded that Erk2 is essential for chemotaxis, cell

movement, and growth. The study also suggests that *Dictyostelium* Erk2 belongs to atypical MAP kinase group of kinases.

Chapter 4 focuses on the interaction between the RegA phosphodiesterase and MAP kinases during developmental stages of *Dictyostelium discoideum*. This study is based on genetic evidence, which indicated that Erk2 down regulates RegA activity. Furthermore, analysis of RegA protein sequence shows the presence of a putative MAP kinase-docking motif in its catalytic domain. This led to the hypothesis that Erk2 interacts with RegA via this D-motif. The principle investigator performed the preliminary study involving *regA*, *erk2*, and *erk1* gene disruptions and the genetic epistasis studies. The author did the site-directed mutagenesis, cloning, transformation, and phenotype study. The author also did the western blot and immunoprecipitation studies. The results show that alteration of the D-motif modulates the regulation of RegA activity by Erk2. Expression of the D-motif altered *regA* at physiological levels cannot rescue the defective phenotypes of $regA^-$ cells. High-level expression of D-motif altered *regA*, however, did rescue the defective phenotypes. The co-immunoprecipitation study showed that the MAP kinase D-motif in RegA is not essential for physical interaction between RegA and Erk2. In contrast, mutation enhances the interaction between $RegA^{D-}$ and Erk2.

In Chapter 5, we explored the role of putative MAP kinase D-motif found in the *Dictyostelium* Gα2 protein. This study is based on findings in the yeast *Saccharomyces cerevisiae*, where a Gα protein, Gpa1, interacts with MAP kinase protein Fus3 to allow an adaptation response to pheromone [47]. The *Dictyostelium* Gα2 protein is required for cAMP-mediated aggregation and the loss of this protein leads to an aggregation defect during development. The D-motif altered Gα2, $G\alpha2^{D-}$, when expressed in $g\alpha2^-$ cells at a low level,

did not rescue aggregation. The *gα2*$^{D-}$ gene when expressed at high level in the *gα2*$^{-}$ cells, rescued the aggregation phenotype. The wild-type *gα2* gene recovered aggregation at both high and low levels of expression. The results show that the loss of function caused by the alteration of MAP kinase D-motif in Gα2 can be rescued when the mutated gene is overexpressed. The principle investigator constructed the *gα2*$^{-}$ cells, designed a few PCR primers and did the background study. The author did the site-directed mutagenesis, cloning, transformations, phenotypic analyses, chemotaxis assays, western blots and other studies.

We studied the importance of MAP kinase D-motifs in two different proteins in *Dictyostelium discoideum*. We found that the putative D-motifs in RegA and Gα2 proteins are essential for their function. However, the function can be rescued when the mutated protein is overexpressed.

1.11. References for chapter I

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CHAPTER II

ACANTHAMOEBA AND *DICTYOSTELIUM* USE DIFFERENT FORAGING STRATEGIES

This chapter presented here is slightly modified from the following published article: Kuburich, N. A., Adhikari N., & Hadwiger, J.A. (2016). *Acanthamoeba* and *Dictyostelium* Use Different Foraging Strategies. *Protist*, 167 (6) 511-525, Reprinted with permission.

2.1 Abstract

Amoeba often use cell movement as a mechanism to find food, such as bacteria, in their environment. The chemotactic movement of the soil amoeba *Dictyostelium* to folate or other pterin compounds released by bacteria is a well-documented foraging mechanism. *Acanthamoeba* can also feed on bacteria but relatively little is known about the mechanism(s) by which this amoeba locates bacteria. *Acanthamoeba* movement in the presence of folate or bacteria was analyzed in above agar assays and compared to that observed for *Dictyostelium*. The overall mobility of *Acanthamoeba* was robust like that of *Dictyostelium* but *Acanthamoeba* did not display a chemotactic response to folate. In the presence of bacteria, *Acanthamoeba* only showed a marginal bias in directed movement whereas *Dictyostelium* displayed a strong chemotactic response. A comparison of genomes revealed that *Acanthamoeba* and *Dictyostelium* share some similarities in G protein signaling components but that specific G proteins used in

Dictyostelium chemotactic responses were not present in current *Acanthamoeba* genome sequence data. The results of this study suggest that *Acanthamoeba* does not use chemotaxis as the primary mechanism to find bacterial food sources and that the chemotactic responses of *Dictyostelium* to bacteria may have co-evolved with chemotactic responses that facilitate multicellular development.

2.2. Introduction

Amoeboid organisms exist in various environments where they interact with many other microbial organisms. *Dictyostelium* and *Acanthamoeba* are two amoebae of general interest because *Dictyostelium* has been used as a model for eukaryotic cell movement and differentiation and *Acanthamoeba* has been documented as a human pathogen [1-8]. Both amoebae inhabit soil environments and feed on bacteria and other microbes [9-11]. Cell movement is a critical feature to allow these cells to find locations where bacteria exist. Mechanisms of cell movement have been extensively studied in *Dictyostelium discoideum* but relatively little is known about the movement of *Acanthamoeba castellanii* [12,13]. Since both organisms can inhabit similar environments it is possible that they share similar foraging mechanisms [14]. However, under extreme starvation conditions where food cannot be found the fates of these amoebae are very different. *Dictyostelium* is a social organism that can form multicellular aggregates when starved and these aggregates undergo an elaborate developmental program to produce a fruiting body with spores that can remain dormant in the absence of nutrients [15]. In contrast, starved *Acanthamoeba* form solitary cysts that allow survival in nutrient poor conditions [9,16]. The formation of dormant spores and cysts are developmental fates for these amoebae if the foraging efforts fail and therefore foraging and dormancy are likely

to be tightly regulated with respect to each other. *Dictyostelium* chemotactic responses have been regarded as an important model for understanding directed cell movement and the underlying signal transduction pathways [4]. Many *Dictyostelium* studies have focused on the chemotactic responses to cAMP that occur during the aggregation phase of the developmental life cycle. After several hours of starvation, *Dictyostelium* increase the expression of cAMP surface receptors and become competent for cAMP chemotaxis [17-20]. The stimulation of cAMP receptors triggers a G protein-mediated signaling pathway that results in chemotactic movement [21-23]. The response also includes a release of extracellular cAMP so that cells can find each other during the aggregation process. Signaling through these cAMP receptors continues during the multicellular phases of development and contributes to cell sorting within the aggregate and the differentiation of cells into the stalk or spores of the fruiting body [24-26]. In contrast, chemotactic movement associated with foraging is present during vegetative growth and enhanced during the first few hours of starvation [27-30]. This chemotactic response requires receptors for pterin-like compounds such as folate. Foraging cells exhibit substantial meandering during chemotaxis and do not display the elongated morphology typical of aggregating cells. However, both cAMP and folate chemotaxis responses require G proteins that couple to cell surface receptors and many of the downstream cellular responses are quite similar including the transient accumulations of cAMP and cGMP, cytoplasmic influx of calcium, and the activation of regulatory proteins such as mitogen activated protein kinases (MAPKs) (ERK1 and ERK2) [21,28,31,32].

The *Dictyostelium discoideum* genome encodes more than 60 G protein coupled receptors but relatively few of them have been genetically characterized [33]. Four cAMP

receptors have been identified and two of these play a role in the cAMP chemotaxis involved with aggregated formation [17]. Several other receptors have been genetically analyzed, including some close paralogs of the cAMP receptors, and recently a receptor responsible for folate chemotaxis has been identified [34-37]. In regards to G proteins, folate responses require the Gα4 G protein subunit and cAMP responses require the Gα2 subunit [28,38,39]. Interestingly, the Gα4 subunit is also required for cellular localization and morphogenesis during multicellular development [40]. Both folate and cAMP chemotaxis responses require the single Gβ subunit encoded by the genome [41]. A single Gγ subunit that contributes to the heterotrimeric structure has also been identified [42]. These G protein mediated signal transduction pathways for *Dictyostelium* chemotaxis share many similarities to signaling pathways in chemotactic mammalian cells (e.g., neutrophils) suggesting that many chemotactic signaling components have been evolutionarily conserved in eukaryotes [4].

Compared to *Dictyostelium*, very few studies have been conducted on *Acanthamoeba* cell movement. These studies have assayed *Acanthamoeba* movement toward a variety of different compounds and to bacteria [12,13]. The results of these studies suggest that *Acanthamoeba* have variable responses to bacteria and compounds such as cAMP and formylated peptides. These studies did not compare *Acanthamoeba* cell movement to known chemotactic cells such as *Dictyostelium* or mammalian neutrophils. These studies were also conducted before the sequencing of any *Acanthamoeba* genomes and so comparisons of signaling proteins were not considered. The recent sequencing of the *Acanthamoeba castellanii* genome now provides an opportunity to compare genes that are potentially involved with chemotactic signaling in *Acanthamoeba* with those

genes that have been characterized in chemotactic organisms [43]. A recent study has reported similarities in the cAMP-specific phosphodiesterase, RegA, found in both *Acanthamoeba* and *Dictyostelium*. RegA regulates the development of spore formation in *Dictyostelium* and the development of cysts in *Acanthamoeba* [44]. Like *Dictyostelium*, the *Acanthamoeba* genome also encodes many putative G protein coupled receptors and G protein subunits genes that could be potential contributors to their ability to find food sources and undergo cell differentiation [43,45].

In this study we compared the ability of *Acanthamoeba* and *Dictyostelium* to forage for nutrient sources. Chemotactic assays to folate and bacteria were used to determine if these organisms possess similar mechanisms to find nutrients in similar environments. Analyses of these two organisms under identical conditions suggest that these organisms have evolved different mechanisms to find bacteria. The genomes of these organisms were also compared for G proteins that potentially contribute to these responses. The difference in foraging strategies used by these amoebae is supported by the difference in G protein subunits encoded in the *Acanthamoeba* and *Dictyostelium* genomes.

2.3. Results

2.3.1. Comparison of amoeboid chemotaxis to folate

Acanthamoeba and *Dictyostelium* both feed on bacteria as a food source suggesting that *Acanthamoeba* might have chemotactic responses to folate as previously described for *Dictyostelium* [46]. To determine if *Dictyostelium discoideum* and *Acanthamoeba castellanii* have similar chemotactic responses to folate, both species were examined in an above agar chemotaxis assay. Both amoebae displayed robust cell

movement as indicated by the maximum migration distance in the presence of folate (Fig. 2.1A, B). The migration distance for *Dictyostelium* was greatly reduced in the absence of folate but *Acanthamoeba* displayed a similar migration distance in the presence or absence of folate suggesting *Acanthamoeba* cell movement was not dependent on the folate. This idea is further supported by the *Acanthamoeba* chemotaxis index of 1.1 compared to *Dictyostelium* chemotaxis index of 1.6 in the presence of 10 μM folate (Fig. 2.1C). A chemotaxis index of one is expected for nonchemotactic (i.e., random migration) cells because the number of cells moving toward and away from the chemoattractant source is approximately equal. The greater than one chemotaxis index for *Dictyostelium* is consistent with previous studies that demonstrate this amoeba is chemotactic to folate [28,29,47]. *Dictyostelium* can inactivate folate as a chemoattractant through a deamination reaction and so as folate diffuses beyond the cell droplet a folate gradient can develop on all sides of the cell droplet [48]. This effect can explain the increased *Dictyostelium* movement in all directions as the assay proceeds. In comparison, *Acanthamoeba* moved robustly in all directions regardless of the presence of folate. No significant differences in migration were displayed by *Acanthamoeba* when exposed to different concentrations of folate, even up to 10 mM (data not shown), but *Dictyostelium* movement increased in distance and cell density at higher folate concentrations. *Acanthamoeba* also began moving beyond the original cell droplet perimeter much earlier than *Dictyostelium* consistent with *Acanthamoeba* movement not being dependent of the formation of a folate gradient.

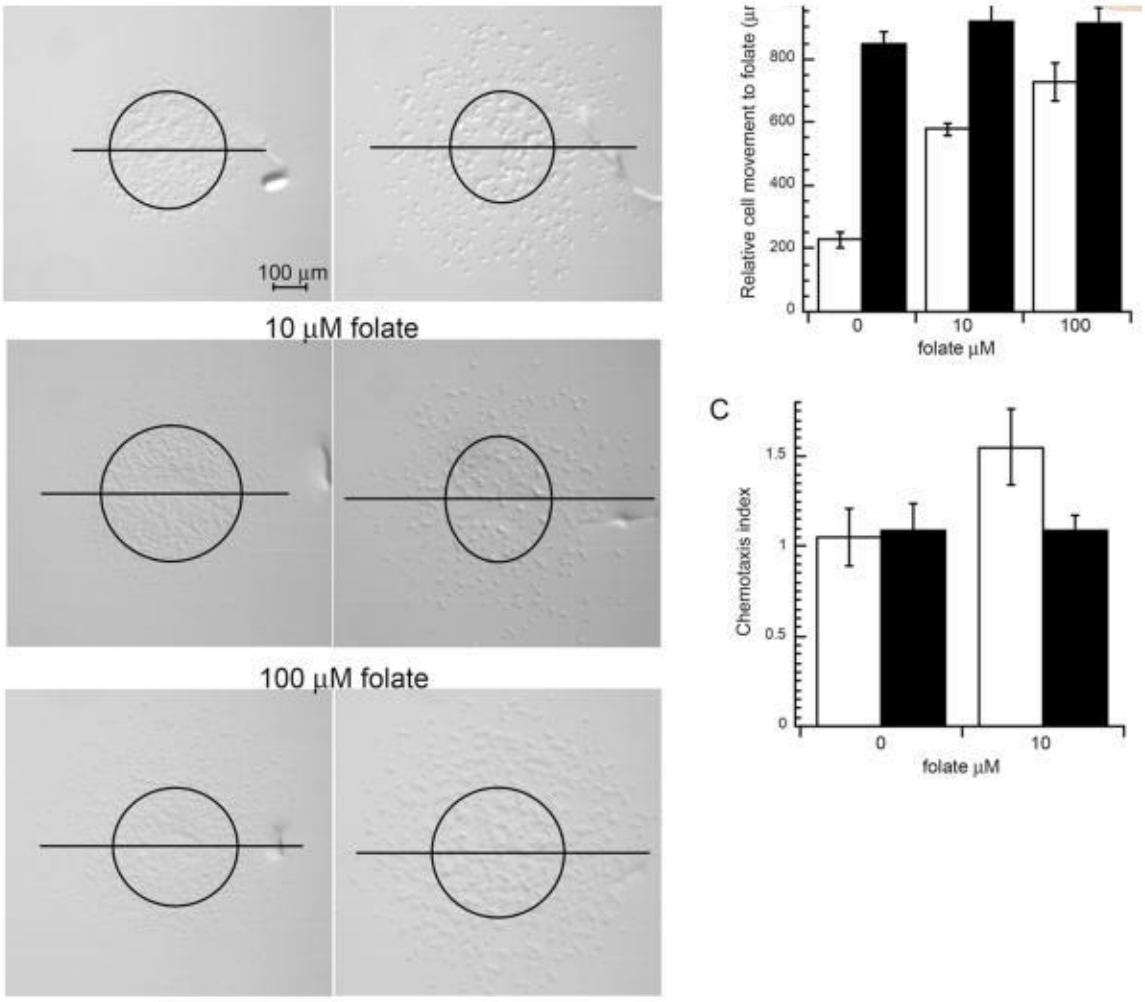

Fig. 2.1. Chemotaxis of *Dictyostelium* and *Acanthamoeba* to folate. Chemotaxis assays were set up as described in the methods section. (A) Images of *Dictyostelium* (*Dd*) and *Acanthamoeba* (*Ac*) 2.5 hour after plating with no folate, 10uM folate, or 100 uM folate. Images are orientated with the folate chemoattractant diffusing from the upper side of the image. Representative images are shown. Each chemotaxis assay typically included the analysis of 6 droplets (minimum of 4) and each assay was repeated at least 2 times. Circles on the image represent the approximate cell droplet perimeter at the time of plating and the horizontal lines bisect the upper and lower halves of the circle. In cases where migrating cells moved beyond a single field of view multiple images were collected to account for all migrating cells. (B) Distance traveled of the leading edge of migrating *Dictyostelium* (open bars) and *Acanthamoeba* (black bars) toward the source of folate. Data are the mean distance measured for 6 droplets of cells. For each concentration of folate this chemotaxis assay was repeated 6 times and the data from one representative assay is shown.

Unpaired Student's *t*-test *p* values for assays with or without folate were determined (*Dictyostelium* assays $p << 0.001$ and *Acanthamoeba* assays $p > 0.03$). (C) Chemotaxis index of *Dictyostelium* (open bars) and *Acanthamoeba* (black bars) assayed with folate (10 µM) exposure or without (0 µM). The chemotaxis index was determined by the number of cells outside the original droplet perimeter on the side facing the source of folate divided by the number of cells outside the perimeter facing away from the source of folate. Data is the mean chemotaxis index from 6 droplets of cells. This assay was repeated at least 3 times and data shown represents a typical assay. Error bars represent the standard deviation of the mean. Unpaired Student's *t*-test *p* values for chemotactic index assays with or without folate were determined (*Dictyostelium* $p = 0.0006$ and *Acanthamoeba* $p = 1.0$).

Time-lapse photography of *Acanthamoeba* and *Dictyostelium* was also used to monitor cell movement in the presence of folate. Cell tracking software was used to map the movement of individual cells over time (Fig. 2.2, S1, S2). Cells located on the edge facing the folate source were chosen because they had little or no contact with other cells. The average distance traveled by *Dictyostelium* and *Acanthamoeba* during the 33 min period was comparable (*Dd* 35.6 $\pm$ 5.9 and *Ac* 29.3 $\pm$ 8.9). Many of the *Dictyostelium* and *Acanthamoeba* cells displayed movement with noticeable meandering and both populations contained some cells that did not move. This heterogeneity in cell movement is typical of *Dictyostelium* populations in response to folate [47]. Cell tracking was also used to assess directionality for *Dictyostelium* and *Acanthamoeba*. Migration directionality can be defined as the displacement (direct distance from start point to end point) divided by the total path length of the cell and so cells traveling in a relatively straight line (high persistence) have directionality values near one and cells that meander more have lower values [49]. The average directionality values for *Acanthamoeba* (0.84 $\pm$ 0.12) and *Dictyostelium* (0.83 $\pm$ 0.14) were very similar suggesting that both amoebae had similar levels of meandering. However, most *Dictyostelium* displayed a bias in cell movement toward the source of folate. In contrast, *Acanthamoeba* movement continued

in all directions without being biased by the folate gradient. The *Acanthamoeba* movement was similar to that previously described for *Dictyostelium* in the absence of a chemoattractant and this movement has been defined as "random motion" because cells exhibit a stochastic combination of persistence and meandering [50].

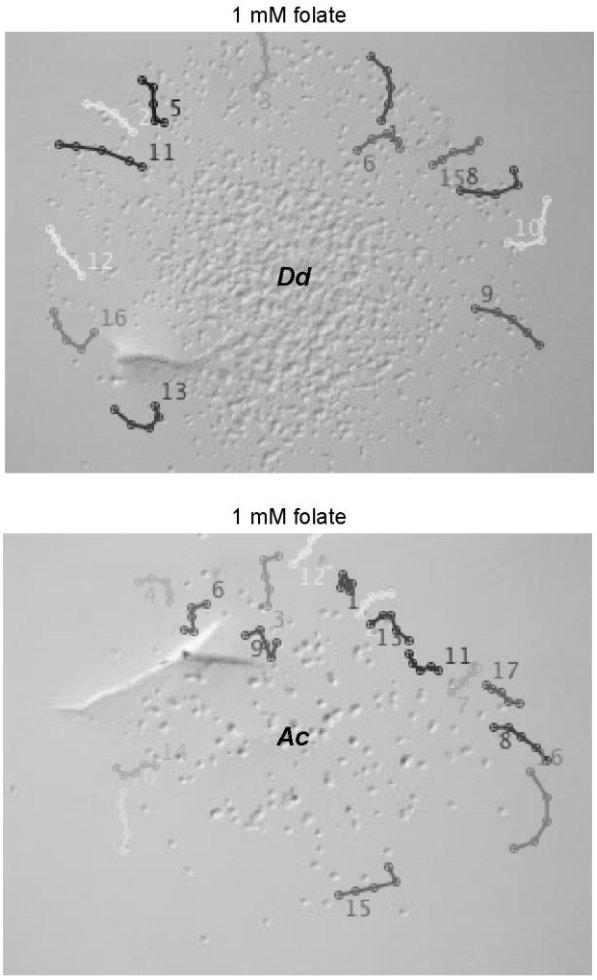

Fig. 2.2. Migration maps of *Dictyostelium* and *Acanthamoeba* in folate chemotaxis assays. Time-lapse photography of *Dictyostelium* (*Dd* upper image) and *Acanthamoeba* (*Ac* lower image) in response to 1 mM folate (source diffusing from the upper side of each image) after approximately 2 hours after plating. Only the final image is shown but images were collected every 20 s for 33 min as described in the methods section. Tracks of individual cells were traced using 5 time points (approximately every 6 min) using MTrackplugin in ImageJ and overlaid on the final image. The number for each track is located near the tail of each track. Cells near the

leading edge and with relatively few cell-cell interactions were chosen for track analysis. Movies containing all time-lapse images are included in the Supplementary Figures S1, S2.

2.3.2. Comparison of amoeboid chemotaxis to bacteria

Although *Acanthamoeba* did not display a chemotactic response to folate it is possible that other molecules released from bacteria might serve as chemoattractants. Therefore, chemotaxis assays were conducted with a variety of bacterial species. Chemotaxis to *Klebsiella aerogenes* and *Escherichia coli* was analyzed because these species are often used in the laboratory setting as a food source for *Dictyostelium*. *Acanthamoeba* was also found to grow efficiently on lawns of these bacteria. Bacteria were harvested and washed with phosphate buffer to remove factors associated with the growth medium. As with the folate chemotaxis assays, *Dictyostelium* displayed a significant chemotaxis index but *Acanthamoeba* did not (Fig. 2.3). However, *Acanthamoeba* exhibited robust cell movement in all directions allowing some of the cells to reach the bacterial droplet. Both *Dictyostelium* and *Acanthamoeba* that reached bacterial droplets were capable of consuming the bacteria (data not shown).

A previous report has suggested that *Acanthamoeba* can chemotaxis to *Pseudomonas aeruginosa* with a chemotactic index of 1.6 in an under agar chemotaxis assay with substantially different parameters than our above agar assay [13]. Our above agar assay with *Acanthamoeba* in the presence of *P. aeruginosa* produced a comparable chemotaxis index of 1.5 (Fig. 2.3). This response was significantly less than the chemotactic response of *Dictyostelium* under the same conditions. Although the chemotaxis response of *Acanthamoeba* to *P. aeruginosa* was marginally statistically significant the robust movement of *Acanthamoeba* in all directions suggests that any chemotactic contributions to cell movement were secondary to the mechanism underlying

the process of cell dispersal. *Dictyostelium* displayed a chemotactic response to *P. aeruginosa* that was similar to its response to other bacteria suggesting that *Dictyostelium* might use a similar mechanism to detect all bacteria. Time-lapse recordings of amoebae migration to bacteria (*K. aerogenes*) were very comparable to those observed for the amoeboid movement to folate (Fig. 2.4, S3, S4). Both amoebae displayed meandering movement and the average migration distances during the 33 min period were similar (*Dd* 31.1 + 7.1 and *Ac* 28.8 + 4.2, relative pixel units). The average directionality values for *Dictyostelium* (0.90 $\pm$ 0.10) and *Acanthamoeba* (0.92 $\pm$ 0.12) were also comparable to each other and similar to those observed in response to folate. In many cases, the *Dictyostelium* and *Acanthamoeba* movement significantly slowed when the cells reached the bacterial mound but some *Acanthamoeba* were capable of tunneling further into the bacterial mound (Fig. S4). This tunneling behavior suggests that some cells might not immediately switch from foraging to feeding when making contact with bacteria. However, *Acanthamoeba* were never observed leaving the bacterial mound suggesting the foraging movement did not continue indefinitely in while in contact with the bacteria. The larger cell size of *Acanthamoeba* compared to *Dictyostelium* might possibly contribute to the ability of individual cells to physically penetrate further into the bacterial mound.

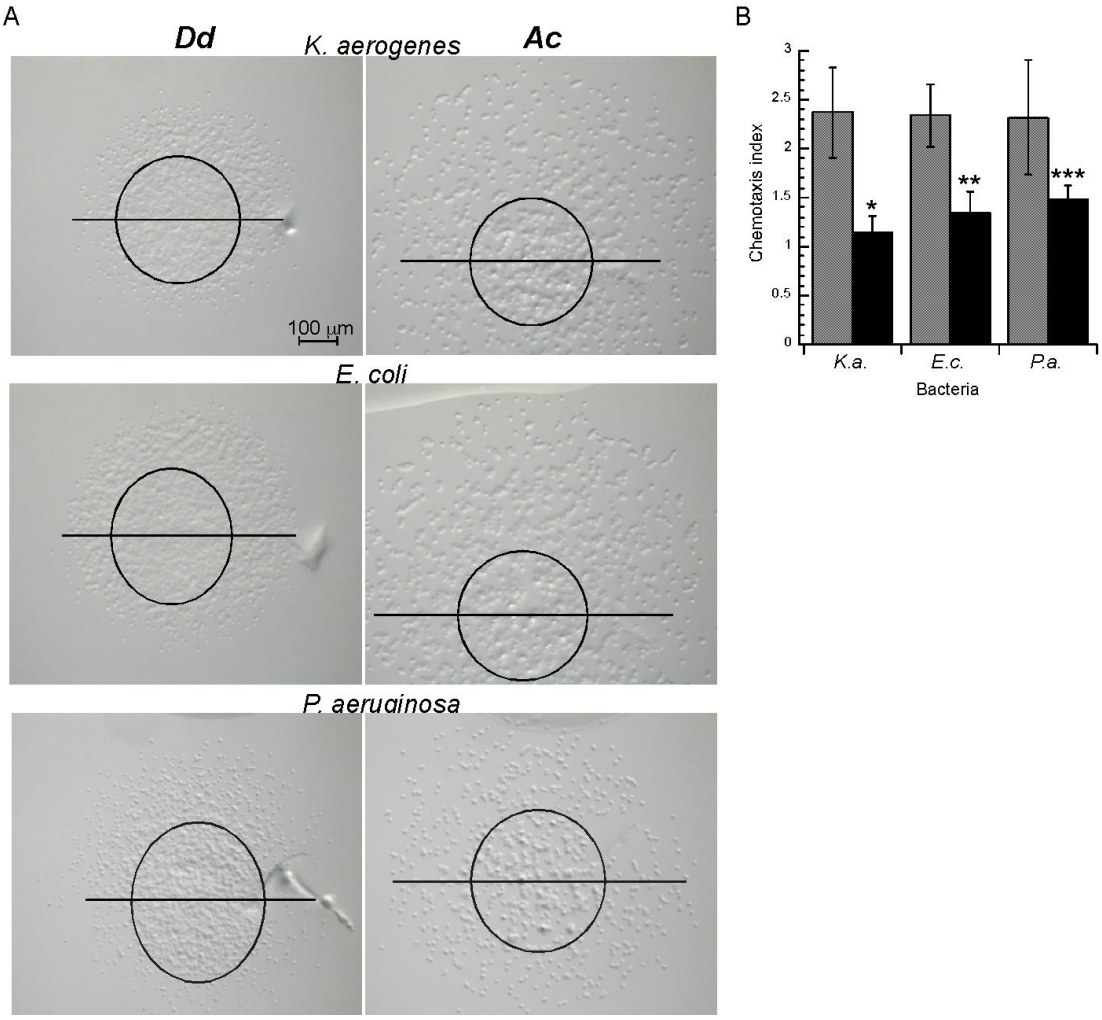

Fig. 2.3. Chemotaxis of *Dictyostelium* and *Acanthamoeba* to bacteria. Chemotaxis assays were set up as described in the methods section. (A) Images of *Dictyostelium* (*Dd*) and *Acanthamoeba* (*Ac*) 2.5 hour after plating with *Klebsiella aerogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Images are orientated with the bacterial source orientated on the upper side of the image. Representative images are shown from an assay that included 6 droplet of either *Dictyostelium* (*Dd*) or *Acanthamoeba* (*Ac*). Each chemotaxis assay that included a minimum of 4 cell droplets was repeated at least 2 times. Original droplet perimeters and bisector lines were determined as described in Fig. 2.1. In cases where migrating cells moved beyond a single field of view multiple images were collected to account for all migrating cells. (B) Chemotactic index of *Dictyostelium* (open bars) and *Acanthamoeba* (black bars) to bacterial droplets. Data is the mean chemotaxis index from 6 droplets of cells. This assay was repeated at least 3 times and data shown represents a typical assay. Error bars represent the standard deviation of the mean. Unpaired Student's *t*-test *p* values for chemotactic index assays with or without bacteria was

determined (all *Dictyostelium* assays $p < 0.001$ and *Acanthamoeba* assays $*p = 0.6$, $**p = 0.05$, $***p = 0.04$).

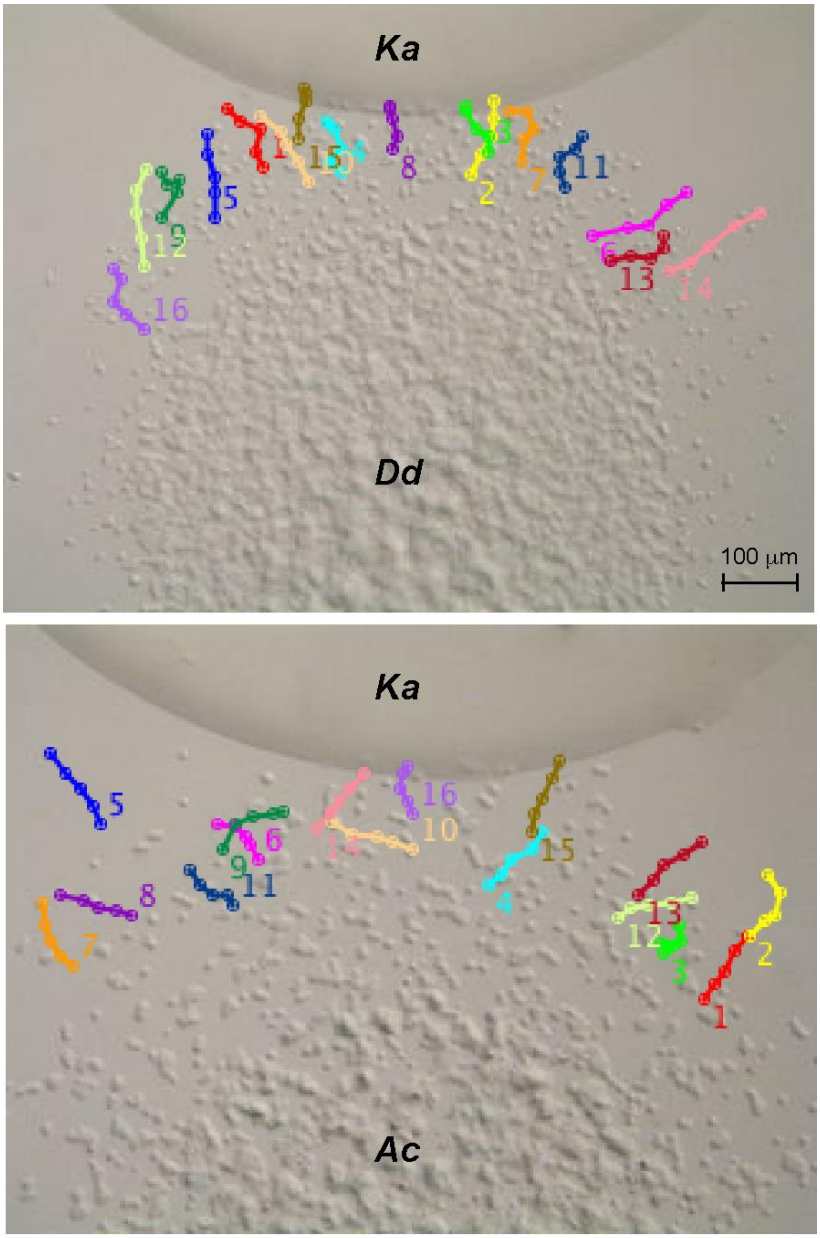

Fig. 2.4. Migration maps of *Dictyostelium* and *Acanthamoeba* in chemotaxis to *Klebsiella aerogenes*. Time-lapse photography of *Dictyostelium* (*Dd* upper image) and *Acanthamoeba* (*Ac* lower image) in response to *K. aerogenes* droplets (positioned on the upper side of each image) after approximately 2 hours after plating. Only the final image is shown but images were collected and tracks were traced as described Fig. 2.2. The number for each track is located near the tail of each track. Cells near the leading edge and with relatively few cell-cell interactions

were chosen for track analysis. Movies containing all time-lapse images are included in the Supplementary Figures S3, S4.

2.3.3. *Acanthamoeba* dispersal

The dispersal of *Acanthamoeba* might possibly be driven through a chemorepulsion mechanism that could potentially mask a response to a chemoattractant at high cell densities. Chemorepulsion has been observed for other amoebae including *Dictyostelium* and *Entamoeba* in response to molecules produced during vegetative growth. Although growth medium was washed away prior to the chemotaxis assays, the amoeba might still be capable of releasing chemorepellent molecules. To examine if chemorepulsion contributes to the movement of *Acanthamoeba*, chemotaxis assays were repeated using different densities of *Acanthamoeba* because chemorepulsion is expected to correlate with increased amoeba density. High (10^8 cells/ml) and low (5×10^6 cells/ml) density *Acanthamoeba* suspensions were used in chemotaxis assays to *K. aerogenes* (Fig. 2.5A). Chemotaxis indices were not determined for *Acanthamoeba* at the high cell density because of the difficulty of counting individual cells but at the lower cell density *Acanthamoeba* had a chemotaxis index of 1.2 ± 0.4, comparable to the higher cell density assays described in Figure 2.3. Chemorepulsion, particularly at high cell densities, might be expected to disperse cells in a uniform radial pattern due the decreasing level of autocrine factors in all directions away from the cell droplet and also the potential repulsion between individual cells. However, *Acanthamoeba* displayed a disorderly pattern of dispersal (i.e., uneven distribution of migrating cells) at both high and low densities suggesting that dispersal is the result of random motion rather than chemorepulsion. Furthermore, *Acanthamoeba* at relatively low cell densities near droplets of bacteria were also capable of moving toward or away from the bacteria (Fig. 2.5B, 5S). This observation suggests that random motion can occur in close proximity of bacterial cells where potential chemoattractant and chemorepellent concentrations are expected to be relatively high and low, respectively. These results suggest that *Acanthamoeba* movement is not primarily determined by chemotaxis or chemorepulsion.

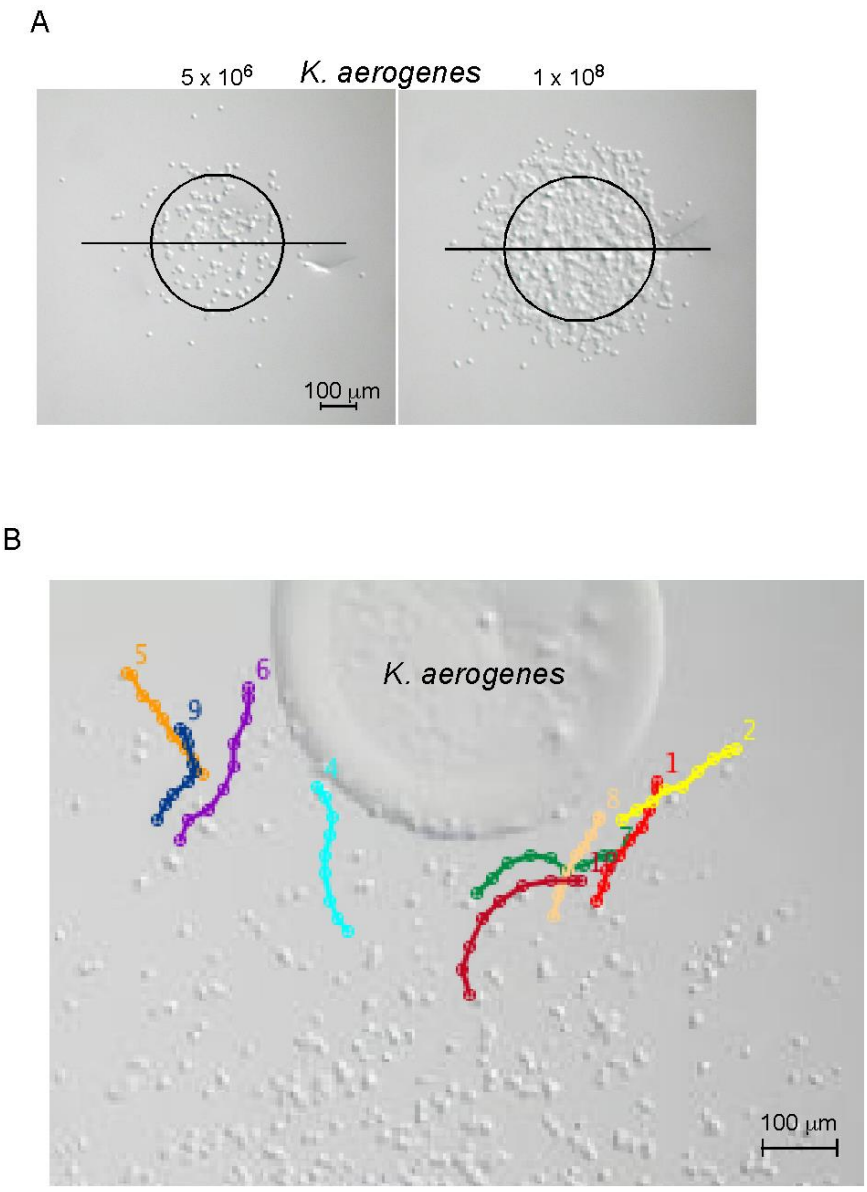

Fig. 2.5. ***Acanthamoeba*** **movement at different cell densities in the presence of bacteria.** Chemotaxis assays were set up as described in the methods section. (A) Images of *Acanthamoeba* (*Ac*) movement 2 hours after plating at different densities (5 x 10^6 or 1 x 10^8 cells/ml suspensions) on agar plates near droplets of *Klebsiella aerogenes* (positioned on the upper side of each image). Representative images are shown from an assay that included 5 droplets of *Acanthamoeba* (*Ac*) for each cell density and each assay was repeated 3 times. Original droplet perimeters and bisector lines were determined as described in Fig. 2.1. (B) Time-lapse photography of *Acanthamoeba* in response to *K. aerogenes* droplet (positioned on the upper side of each image) after approximately 4.5 hour after plating. Only the final image is shown but images were collected and tracks were traced as described Fig. 2.2. The number for each track is located near

the tail of each track. Cells moving near but not directly toward the bacterial droplet were chosen for track analysis. Movies containing all time-lapse images are included in the Supplementary Fig. S5.

2.3.4. Comparison of G protein Gα subunits

The chemotaxis assays to folate and bacterial sources suggest that the foraging strategy of *Acanthamoeba* is different than that of *Dictyostelium*. The basis of this difference could possibly be differences in the signaling components encoded by each organism's genome. Chemotaxis and other chemoresponsive processes (e.g., chemotrophic growth) in eukaryotes are typically associated with G protein-mediated signaling pathways [4]. Given that the *Dictyostelium discoideum* Gα4 subunit is required for chemotactic responses to folate and bacteria, the sequence of this subunit was used to search for homologous proteins in *Acanthamoeba* and other amoebae. *Dictyostelium* discoideum and related Dictyostelid species (*Dictyostelium purpureum*, *Polysphondylium pallidum*, *Dictyostelium fasciculatum*, and *Actyostelium subglobosum*) all possessed a single closely related Gα4 ortholog with sequence identity greater than 90% (Table 2.1). A previous report has indicated that *Dictyostelium purpureum* and *Polysphondylium pallidum* have chemotactic responses to folate and while the other species have not been tested it is likely they also respond to folate given the conservation of the Gα4 subunit [29]. The most closely related *Acanthamoeba* Gα subunit to the *Dictyostelium* Gα4 subunit was a Gα5 ortholog but this subunit has a much lower sequence identity (54%) than the Gα4 orthologs in other amoebae. The *Dictyostelium discoideum* Gα5 and Gα2 subunits also have a much lower sequence identity (52% and 44%, respectively) with respect to the Gα4 subunit and this is indicative of their functional differences. The Gα2 subunit, like the Gα4 subunit, mediates a chemotactic response but this response is to cAMP. A

phylogenetic analysis of the 12 *Dictyostelium* and 5 *Acanthamoeba* Gα subunits indicated that other than a similarity between Gα5 orthologs (60% identity), these amoebae do not share closely related Gα subunits (Fig. 2.6). Out of the other 4 *Acanthamoeba* Gα subunits, 3 of them shared greatest sequence similarity with each other rather than *Dictyostelium* Gα subunits. The remaining *Acanthamoeba* Gα subunit, Gα3, had sequence similarity with the *Dictyostelium* Gα7 subunit. As previously reported, most *Dictyostelium* G subunits, except for Gα1-Gα2 pair and the Gα4-Gα5 pair share little sequence similarity with each other outside of the highly conserved sequences associated with guanine nucleotide binding. Even the most closely related pairs of *Dictyostelium* Gα subunits, Gα4-Gα5 and Gα1-Gα2, do not show functional redundancy in chemotactic or developmental phenotypes [21,51].

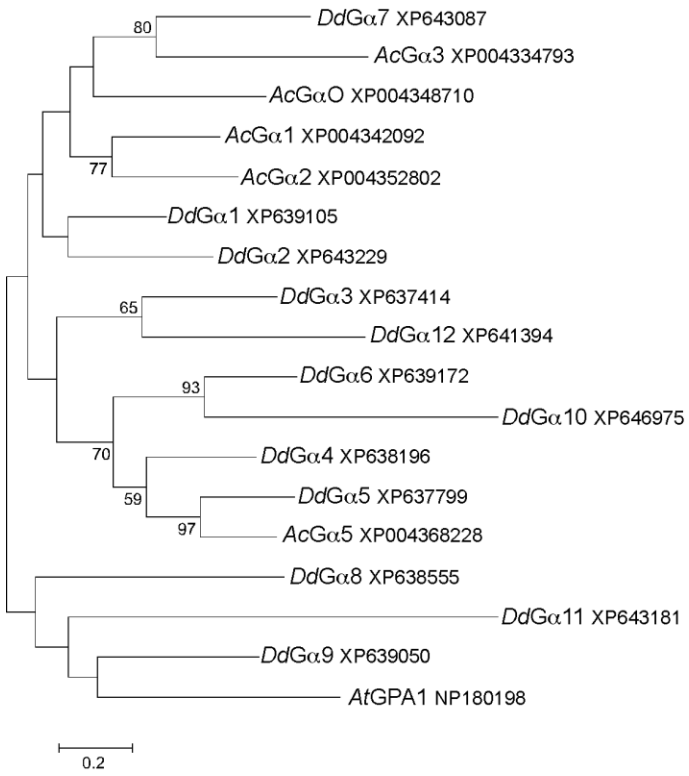

Fig. 2.6. Phylogenetic tree of *Dictyostelium* and *Acanthamoeba* G protein Gα subunits. BLAST searches were used to identify Gα subunit homologs in *Dictyostelium discoideum* (*Dd*) and *Acanthamoeba castenalli* (*Ac*) genomes. A phylogenetic tree was generated as described in the methods section. The *Arabidopsis thaliana* G subunit (*At*GPA1) was used as an out-group. Each sequence has an accession reference number.

Organism	Gα subunit	% identity
Dictyostelium purpureum	Gα4	99
Polysphondylium pallidum	Gα4	94
Dictyostelium fasciculatum	Gα4	91
Acytostelium subglobosum	Gα4	93
Acanthamoeba castellanii	Gα5	54
Polysphondylium pallidum	Gα5	53
Dictyostelium discoideum	Gα5	52
Dictyostelium discoideum	Gα2	44

Table 2.1. Percent identity of amoeboid Gα subunits to the *Dd* Gα4 subunit.

2.3.5. Comparison of other G protein subunits

In *Dictyostelium*, the sole Gβ subunit is required for chemotaxis to both cAMP and folate indicating that both Gα2 and Gα4 complex with the Gβ in these chemotactic responses. The *Dictyostelium* Gβ subunit sequence was used to search for orthologs in *Acanthamoeba* but the search did not reveal any closely related proteins as suggested by the low range of sequence identities (20-29%). This contrasts *Dictyostelium* Gβ searches in other eukaryotes such as humans where Gβ orthologs were identified with much higher sequence identities (60-70%). Several of the *Acanthamoeba* proteins detected in the sequence similarity searches were identified as Gβ-like proteins because they contain WD-repeats like those found in Gβ subunits. One of these *Acanthamoeba* WD-repeat proteins has significant similarity to proteins commonly known as Rack homologs (Receptor associated with protein C kinase) that exist in a diverse range of eukaryotes. The *Acanthamoeba* and *Dictyostelium* Rack proteins share 59% identity but none of the other *Acanthamoeba* WD-repeat proteins shared more than 34% sequence identity with the *Dictyostelium* RACK protein. Phylogenetic analysis of Gβ related proteins from *Dictyostelium* and *Acanthamoeba* and some other eukaryotes indicated that Gβ subunits and Rack proteins form distinct clades suggesting these WD repeat proteins represent two different classes of proteins (Fig. 2.7A). The *Acanthamoeba* Rack protein shares comparable relatedness with higher eukaryotes as it does with other amoeboid species. G proteins which couple to cell surface receptors are typically heterotrimeric proteins that contain a Gγ subunit. The Gγ subunit binds tightly to Gβ subunit and remains tightly associated with this subunit upon activation of the Gα subunit. A *Dictyostelium* Gγ subunit has been identified and shown to couple with the Gβ subunit but its requirement

in chemotactic movement has not been established [42]. The *Dictyostelium discoideum* Gγ subunit was used to search for related proteins in *Acanthamoeba* but the search did not identify any closely related proteins. Attempts with human or yeast Gγ to find related proteins in *Acanthamoeba* were also unsuccessful but the low sequence conservation and the small protein size make the searches for Gγ subunit homologs more challenging than homologs to other G protein subunits in distantly related organisms. However, Gγ subunits from other dictyostelids were detected. A phylogenetic analysis of Gγ sequences indicates that the dictyostelids species have closely related Gγ subunit compared to those found in other unrelated organisms (Fig. 2.7B). The phylogenetic tree suggests higher sequence similarities between Gγ subunits within a given phylum.

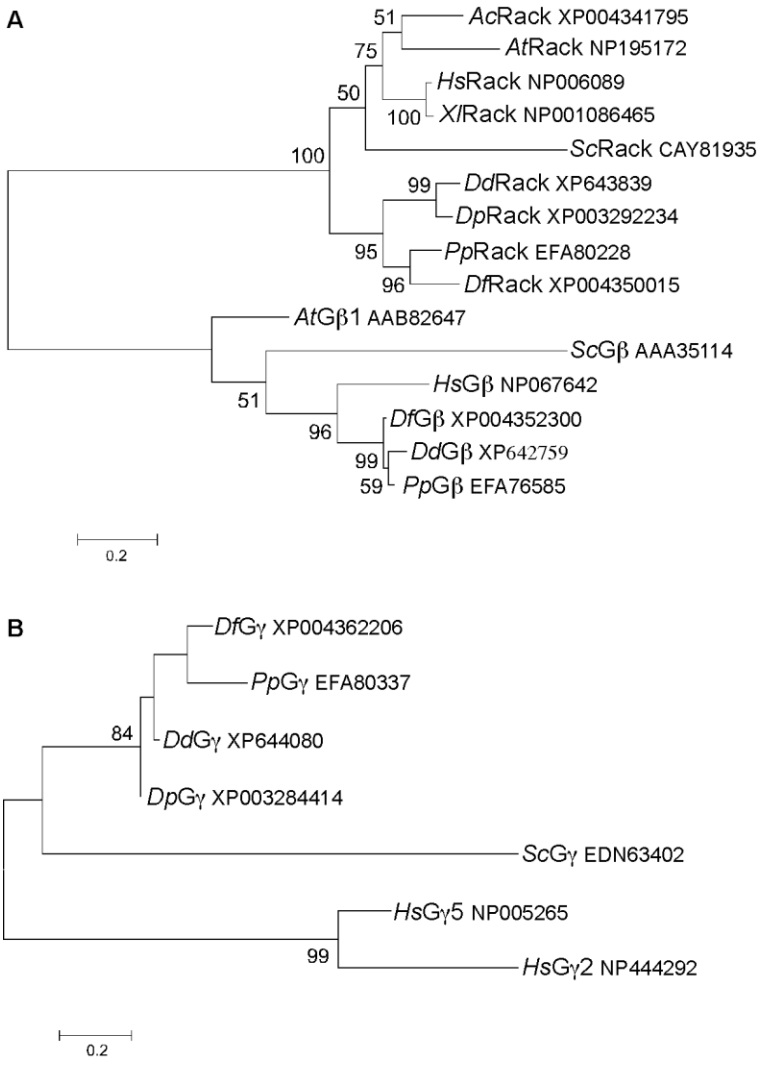

Fig. 2.7. Phylogenetic trees of G protein Gβ subunits/Racks and Gγ subunits of some amoebozoan and other selected eukaryotes. (A) Phylogenetic tree of Gβ and Rack subunit homologs identified through BLAST searches. Only one Gβ subunit of the multiple Gβ subunits from the human (*Hs*) genome was selected for comparison purposes. (B) Phylogenetic tree of Gγ subunit homologs identified through BLAST searches. Phylogenetic trees were generated as described in the methods section. Species represented include *Dictyostelium* discoideum (*Dd*), *Acanthamoeba castenalli* (*Ac*), *Homo sapien* (*Hs*), *Xenopus laevis* (*Xl*), *Arabidopsis thaliana* (*At*), *Dictyostelium purpureum* (*Dp*), *Polysphondylium pallidum* (*Pp*), *Dictyostelium fasciculatum* (*Df*), and *Saccharomyces cerevisiae* (*Sc*) genomes. Each sequence has an accession reference number.

2.4. Discussion

The results of this study suggest that *Acanthamoeba* forage for bacterial food sources using a mechanism distinct from *Dictyostelium*. *Dictyostelium* displayed directed movement to both folate and bacterial sources even though the migration paths included substantial meandering. *Acanthamoeba* displayed a slight bias in the movement toward some bacterial sources but the robust movement in all directions overshadowed any directed movement to the bacterial source suggesting that random motion rather than chemotaxis represents the primary foraging mechanism of *Acanthamoeba* (Fig. 2.8). Cell dispersal through random motion is likely to increase the chance that some members of an *Acanthamoeba* population find new food sources because the population can cover a larger area compared to a population directed by chemotaxis. Cell dispersal through random motion has also been described for *Dictyostelium* in the absence of a chemotactic signal [52].

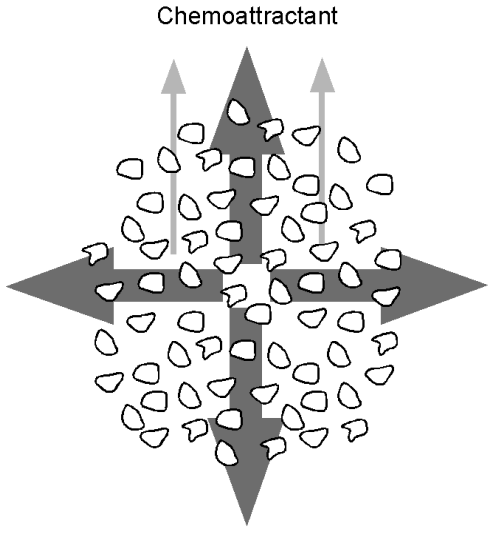

Fig. 2.8. Model of *Acanthamoeba* movement. Nutrient deprivation primarily results in random motion allowing cells to disperse in all directions (thick dark gray arrows). Movement toward a

chemoattractant (thin light gray arrows) is very weak compared to other amoebae such as *Dictyostelium.*

The efficient dispersal of *Acanthamoeba* at even low cell densities suggests that chemorepulsion is not an important contributor for *Acanthamoeba* dispersal in the conditions tested in this study but such a mechanism could possibly operate in populations of vegetatively growing cells. Under growing conditions *Dictyostelium* use an autocrine signal, AprA, as a chemorepellent and other amoebae, such as *Entamoeba*, use ethanol production as a chemorepellent [53,54]. While the dispersal of cells reduces the competition for food in a localized area, it might also have important consequences with respect to cell fate. *Acanthamoeba* that are unsuccessful in foraging enter into a dormant state (encystation) as solitary cells, allowing survival until local food conditions change. In contrast, *Dictyostelium* enter into a multicellular state to form dormant cells (spores) if foraging fails and so mechanisms that promote cell dispersal would likely work in opposition to the cell aggregation process. Therefore, social amoebae such as *Dictyostelium* might limit cell dispersal mechanisms to allow for more efficient cell aggregation.

The lack of *Acanthamoeba* chemotaxis to folate is consistent with the absence of a Gα4 subunit ortholog in the genome given that this subunit is highly conserved among species that can chemotax to folate. While most of the reported folate-responsive amoebae belong to the Dictyostelid classification, a recent study indicates that *Vahlkampfia*, classified in a different subphylum, can also chemotax to folate [14,55]. It remains to be determined if the *Vahlkampfia* genome contains a Gα4 subunit homolog. Many amoebae, including *Acanthamoeba*, possess a Gα5 subunit homolog that is closely related to Gα4 subunits but phenotypic characterization of the *Dictyostelium gα5*$^{-}$ mutants

indicates that this subunit is not required for folate chemotaxis [51]. Rather, the Gα5 subunit appears to act in opposition to the Gα4 subunit as suggested by the increased folate responsiveness of cells that lack the Gα5 subunit and the decreased folate responsiveness of cells that overexpress the Gα5 subunit [56]. The functional relationship of the Gα4 and Gα5 subunits is not fully understood but Gα subunit chimeric studies suggest that the functional differences are not solely due to receptor coupling but rather downstream signaling [57].

The absence of a Gβ subunit gene in the current *Acanthamoeba castellanii* genomic data is very surprising given that several putative Gα subunits are present. Biochemical and genetic characterization of Gβ subunits in other organisms suggest the Gβ subunits can have a variety of interactions and roles in downstream signaling and in some cases they provide the primary role in signaling to downstream responses [58,59]. Not detecting a Gβ subunit gene could possibly result from incomplete coverage of the genome by the sequencing analysis. The *Acanthamoeba castellanii* genome project did not report the depth of sequencing coverage but did indicate 94% coverage of the transcriptome [43]. Searches for Gβ subunits were also conducted using recently deposited *Acanthamoeba* sequences in the AmoebaDB database (AmoebaDB and MicrosporidiaDB:functional genomic resources for Amoebozoa and Microsporidia species). In several *Acanthamoeba* species, including *Acanthamoeba castellanii*, these searches revealed partial gene sequences with 50-60% identity to known Gβ subunits suggesting that Gβ homologs are present in the *Acanthamoeba* genus. It is also possible that other WD-repeat proteins might provide Gβ subunit function. The presence of the Gβ-like Rack protein in *Acanthamoeba* offers the possibility that this protein could

function in G protein signaling pathways. Studies in the yeast *S. cerevisiae* indicate that the Gpa2 Gα subunit does not couple with the single Gβ or Gγ subunit in this organism [60]. Instead the yeast Rack homolog, Asc1, has been reported to serve in place of a Gβ subunit for the Gpa2 signaling pathway [61]. The Gpa2/Asc1 G protein pathway mediates the sensing of glucose to control cell growth and division in yeast but thus far this pathway has not been implicated in cell polarity or chemotrophic growth, unlike the Gpa1/Gβ pathway [62]. While Rack proteins may mediate G protein signaling it is possible that these proteins might not be capable of contributing to chemotactic or chemotrophic responses like Gβ subunits. The Rack ortholog in *Dictyostelium* cannot compensate for the chemotactic deficiencies that occur in the absence of the Gβ subunit but that does not exclude the possibility that the Rack protein might function in signaling pathways that do not require Gβ proteins [63].

2.5. Conclusion

While *Acanthamoeba* and *Dictyostelium* might exist is similar environments and consume common bacteria, these amoebae use very different approaches to finding their food sources based on their cell movement. It is interesting that both amoebae share many families of proteins that participate in G protein-mediated signal transductions but yet very little overlap exists with specific signaling proteins such as specific Gα subunits. Perhaps some of the differences in signaling proteins are the result of social or solitary strategies for surviving starvation. Chemotactic responses of *Dictyostelium* to folate and bacteria might have co-evolved with chemotactic responses to cAMP because many similarities exist between these responses. These similarities include the activation of MAPKs and other kinases and transient changes in the level of cyclic nucleotides that are

important for regulating cell morphology and gene expression [21,31,64]. The use of similar signaling proteins downstream of G protein function for both foraging and cell aggregation could save time and critical energy reserves in the switch between foraging and the cell aggregation process. In addition, the relatively close proximity of cells with each other during chemotactic foraging, a process somewhat analogous to herding, can also help expedite the aggregation process if needed. A potential drawback to "herding" is that it increases the competition between cells for food sources that might be found. However, if food is not found then the close proximity of cells is beneficial because it reduces the distance cells need to migrate to form a multicellular aggregate. In contrast to social amoeba, *Acanthamoeba* does not require cell aggregation to form cysts and so an investment in chemotactic signaling mechanisms might not be warranted. These differences in developmental fates among social and non-social amoebae might provide some of the basis for the different foraging strategies.

2.6. Methods

2.6.1. Strains, growth conditions

The axenic *D. discoideum* strain KAx3 and the *A. castellanii* strain ATCC 30010 were used in this study. Both amoebas were grown in HL5 medium [65]. *K.aerogenes* was grown on SM+/3 medium and *Escherichia coli* and *Pseudomonas aeruginosa* were grown on L broth [66]. Folate solutions were adjusted to pH 7 using 100 mM $NaHCO_3$.

2.6.2. Chemotaxis assays

Above agar chemotaxis assays were performed as previously described [67]. Cells were grown in fresh HL5 medium 24 hours prior to harvesting and washing in phosphate buffer (12mM NaH_2PO_4 adjusted to pH 6.1 with KOH) and suspended at 1 x 10^8 cells/ml

for *Dictyostelium* or $2x10^7$ cells/ml for *Acanthamoeba* (*Acanthamoeba* are approximately 5 times the size of *Dictyostelium*) unless otherwise noted. Droplets (< 1µl) of cell suspensions were spotted on to nonnutrient agar plates (1.5% agar in phosphate buffer) and then 1µl of chemoattractant was spotted approximately 2 mm from the cell droplet. Images of the cells were recorded immediately after the plating of the cells and chemoattractant and recorded again 2.5 hour later. The agar surface near the cell droplet was scarred with a needle to allow the early and late images to be aligned so that the original cell droplet perimeter could be overlaid on the later image. Cell movement toward the chemoattractant source was determined by measuring the distance from the original cell droplet perimeter to the leading edge of migrating cells. Chemotaxis index (A/B) was defined as the number of cells outside the original cell perimeter that moved toward the chemoattractant (A) divided by the number of cells outside the original cell perimeter that moved away from the chemoattractant (B). Chemotaxis to bacterial cells was performed as that described for folate except 1 µl droplets of bacterial cell suspensions were used as the chemotactic stimulus. Prior to being used in the chemotaxis assays, bacterial cultures were grown overnight in shaking cultures at 22ºC (*K. aerogenes*) or 37ºC (*E. coli* and *P. aeruginosa*). Chemotaxing cells were analyzed using a dissecting microscope (Nikon SMZ2). Videos were created using time-lapse photography with 20s intervals between images for 33 min. ImageJ with MTrackJ plugin software was used to trace cell migration tracks and determine the migration distance for selected cells. Directionality values were determined using Chemotaxis and Migration Tool Version 1.01 plugin software.

2.6.3. G protein ortholog analysis

G protein sequences were identified using BLASTp searches using default parameters in the non-redundant protein sequences database (NCBI). Amoebae sequences were available primarily due to genome sequencing projects of *Dictyostelium discoideum*, *Acanthamoeba castellanii*, *Dictyostelium purpureum*, *Dictyostelium fasciculatum*, and *Polysphondylium pallidum* [43,68-70]. Initial searches were queried with *Dictyostelium discoideum* protein sequences but queries were also conducted using representative proteins from *Acanthamoeba castellanii*, mammals and yeast. Molecular phylogenetic analysis was conducted in MEGA7 using the maximum-likelihood method based on the JTT matrix-based model [71,72]. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1000 replicates) are shown next to the branches [73]. Only branches corresponding to partitions reproduced in more than 50% of the bootstrap replicates are labeled. Each tree is drawn to scale, with branched lengths measured in the substitutions per site. Addition BLAST searches were conducted in the AmoebaDB database (AmoebaDB and MicrosporidiaDB:functional genomic resources for Amoebozoa and Microsporidia species) using the *Acanthamoebae* data sets (Andrew Jackson, Liverpool, UK).

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2.8. Supplemental data

Fig. S2.1. Movie of *Dictyostelium* movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source

is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S1434461016300487?via%3Dihub#upi0005

Fig. S2.2. Movie of *Acanthamoeba* movement in the presence of folate. Time-lapse recording of *Acanthamoeba* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S1434461016300487?via%3Dihub#upi0005

Fig. S2.3. Movie of *Dictyostelium* movement in the presence of *K. aerogenes*. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The bacterial source is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S1434461016300487?via%3Dihub#upi0005

Fig. S2.4. Movie of *Acanthamoeba* movement in the presence of *K. aerogenes*. Time-lapse recording of *Acanthamoeba* movement over 33 min period with images collected every 20 s. The bacterial source is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S1434461016300487?via%3Dihub#upi0005

Fig. S2.5. Movie of *Acanthamoeba* movement near a droplet of *K. aerogenes*. Time-lapse recording of *Acanthamoeba* movement over 33 min period with images collected every 20 s. The bacterial source is oriented at the upper side of the image and the original droplet of *Acanthamoeba* was positioned at the lower side of the image.
https://www.sciencedirect.com/science/article/pii/S1434461016300487?via%3Dihub#upi0005

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CHAPTER III

DICTYOSTELIUM ERK2 IS AN ATYPICAL MAPK REQUIRED FOR CHEMOTAXIS

This chapter is slightly modified from the following published research article:

Schwebs, D.J., Pan, M., Adhikari, N., Kuburich, N. A., Jin, T., & Hadwiger, J. A. (2018). *Dictyostelium* Erk2 is an atypical MAPK required for chemotaxis. *Cellular signalling, 46*, 154-165.

3.1. Abstract

The *Dictyostelium* genome encodes only two MAPKs, Erk1 and Erk2, and both are expressed during growth and development. Reduced levels of Erk2 expression have been shown previously to restrict cAMP production during development but still allow for chemotactic movement. In this study the *erk2* gene was disrupted to eliminate Erk2 function. The absence of Erk2 resulted in a complete loss of folate and cAMP chemotaxis suggesting that this MAPK plays an integral role in the signaling mechanisms involved with this cellular response. However, folate stimulation of early chemotactic responses, such as Ras and PI3K activation and rapid actin filament formation, were not affected by the loss of Erk2 function. The *erk2*$^{-}$ cells had a severe defect in growth on bacterial lawns but assays of bacterial cell engulfment displayed only subtle changes in the rate of bacterial engulfment. Only cells with no MAPK function, *erk1*$^{-}$*erk2*$^{-}$ double mutants displayed a severe proliferation defect in axenic medium. Loss of Erk2 impaired the phosphorylation of Erk1 in secondary responses to folate stimulation indicating that Erk2

has a role in the regulation of Erk1 activation during chemotaxis. Loss of the only known *Dictyostelium* MAPK kinase, MekA, prevented the phosphorylation of Erk1 but not Erk2 in response to folate and cAMP confirming that Erk2 is not regulated by a conventional MAP2K. This lack of MAP2K phosphorylation of Erk2 and the sequence similarity of Erk2 to mammalian MAPK15 (Erk8) suggest that the *Dictyostelium* Erk2 belongs to a group of atypical MAPKs. MAPK activation has been observed in chemotactic responses in a wide range of organisms but this study demonstrates an essential role for MAPK function in chemotactic movement. This study also confirms that MAPKs provide critical contributions to cell proliferation.

3.2. Introduction

Mitogen activated protein kinases (MAPKs) are components of many eukaryotic signal transduction pathways [1-4]. These proteins generally function downstream of protein kinase cascades that include MAPK kinases (MAP2Ks) and MAPK kinase kinases (MAP3Ks). Once activated, MAPKs phosphorylate and regulate a wide variety of proteins throughout the cell. Mitogens, chemoattractants, and other extracellular signals can stimulate MAPK pathways and lead to changes in cell growth, movement, gene expression, and differentiation [3,5]. While the interactions and functions of some MAPKs have been well documented, many members of this regulatory protein family remain uncharacterized [6]. Sequence similarities and functional roles have provided the basis to organize the family of mammalian MAPKs into subfamilies such as the ERKs (extracellular signal regulated kinases), p38 MAPKs, pJNKs (c-Jun N-terminal kinases), and other smaller groups but not all of these groups are present in other eukaryotes [5]. Some MAPKs are known to have redundant functions (e.g., mammalian Erk1/Erk2) and

others can have common activators and substrates but promote different cellular responses (e.g., yeast Fus3 and Kss1 in regulating mating responses and filamentous growth, respectively) [4,7,8]. The signaling pathways that use MAPKs can be quite varied but the activation mechanism of most characterized MAPKs includes a dual phosphorylation of residues in a highly conserved motif (TXY) within a catalytic domain [5]. This activation is typically mediated by MAPK kinases (MAP2Ks; also known as Meks) that are capable of phosphorylating both serine/threonine and tyrosine residues [9]. However, a group of atypical MAPKs does not appear to be phosphorylated by conventional MAP2Ks [5,10,11]. MAPKs have been found in all eukaryotic kingdoms and appear to be present in all free-living eukaryotes suggesting these proteins regulate basic cellular processes in eukaryotes [12,13].

Simple eukaryotic organisms have been particularly useful for the characterization of MAPK function and specificity. These eukaryotes are typically amenable to both genetic and biochemical analysis and they tend to have relatively few MAPKs. The yeast *Saccharomyces cerevisiae* has 5 MAPKs and the soil amoeba *Dictyostelium discoideum* has only 2 MAPKs compared to the 13 MAPKs found in mammals [3,4]. MAPKs in yeast have been associated with responses to mating pheromones, starvation, osmotic stress, and cell wall stress [4]. The two MAPKs in *Dictyostelium*, designated as Erk1 and Erk2 (also referred to as ErkA and ErkB, respectively), play important roles in the developmental life cycle that is initiated by the loss of nutrients [3]. Starved *Dictyostelium* aggregate using a relayed intercellular cAMP signal and the multicellular mounds undergo morphogenesis to form a slug and then a fruiting body consisting of a spore mass on top of a stalk [14,15] . Cells lacking Erk1

aggregate into small mounds that have accelerated morphogenesis and the overexpression of Erk1 results in large aggregates that have delayed morphogenesis indicating that Erk1 function can inhibit developmental progression [16-18]. Genetic analysis of Erk2 function has been extensive but limited to the characterization of a leaky erk2 allele in which Erk2 expression is reduced but not eliminated [17,19-23]. A reduction of Erk2 expression results in cells with insufficient external cAMP signaling to allow cell aggregation in clonal populations but cells retain the ability to chemotax to cAMP [19,23,24]. In the presence of wild-type cells, the reduced Erk2 expression mutant can co-aggregate because of the cAMP provided by wild-type cells [19,22,23]. The deficiency in cAMP signaling of this mutant can also be suppressed by the loss of the cAMP-specific phosphodiesterase, RegA, allowing the double mutant to undergo and complete multicellular development [20].

Stimulation of *Dictyostelium* with the chemoattractants folate or cAMP results in a rapid phosphorylation of Erk2 that is followed by the phosphorylation of Erk1 as the level of phosphorylated Erk2 decreases [17,25]. Folate stimulation of Erk2 phosphorylation requires the folate receptor, Far1, and its coupled G protein, Gα4 [21,26]. However, cAMP stimulation of Erk2 phosphorylation only requires a cAMP receptor, cAR1 or cAR3, and appears to be independent of G protein function, at least the function of Gα2 and Gβ subunits [27,28]. The basis for this distinction remains to be determined and the proteins that transduce the signals from the receptor to the MAPK are not well characterized [29]. The *Dictyostelium* genome encodes only a single MAP2K, MekA (also known as Mek1), based on sequence similarity to characterized orthologs [16]. Cells lacking MekA form small aggregates with accelerated development, similar to phenotype observed for *erk1*$^{-}$

cells. Previous studies have also suggested that Erk1 but not Erk2 kinase activity is dependent on MekA function [16,27,30].

In this study the function of *Dictyostelium* Erk2 was investigated through the creation of an *erk2*$^-$ gene disruption mutant, resulting in a complete loss of Erk2 function. This *erk2*$^-$ mutant was analyzed for growth and developmental phenotypes including chemotaxis and phagocytosis. A double MAPK mutant, *erk1*$^-$*erk2*$^-$, was also created and analyzed. In addition, the dependence of Erk1 phosphorylation on Erk2 function was examined in response to chemotactic stimulation. The results of these analyses suggest that Erk2 is essential for chemotactic movement and stimulation of Erk1 phosphorylation. Erk2 function was also found to be important for early phagocytic responses and, together with Erk1, Erk2 contributes to axenic growth. Similarities of the *Dictyostelium* Erk2 with the mammalian MAPK15 (also known as Erk8) were investigated to assess the possible role of Erk2 as an atypical MAPK.

3.3. Material and methods

3.3.1. Strains and development.

All of the *Dictyostelium* strains used in this study were derived from the parent axenic strain KAx-3 except for the noted loci. Axenic strains have been derived from wild-type strains through mutations, including those at the *NF-1* locus [31,32]. The JH10 thymidine auxotrophic strain, disrupted at the *thyA* locus (also designated *thy1*), has been previously described [33]. The *erk1*$^-$, *erk1*$^-$*thyA*$^-$, and *mekA*$^-$ strains have been previously described [17,18]. Phenotypic comparisons of the MAPK mutants were done with KAx-3 cells due to the auxotrophic requirements of the JH10 strain. *Dictyostelium* were grown in HL-5 axenic medium (with or without thymidine supplement) or on lawns of *Klebsiella*

aerogenes on SM+/3 agar plates [34]. For the analysis of plaque growth rate cells were mixed with bacteria and plated at a low density on SM+/3 plates to allow the formation of plaques from single cells. DNA constructs and vectors were inserted into *Dictyostelium* using electroporation as previously described [35]. For developmental analysis cells were harvested from axenic medium by centrifugation and washed in phosphate buffer (12mM NaH_2PO_4 adjusted to pH 6.1 with KOH). Cells were plated on nonnutrient plates (1.5% agar in phosphate buffer) from suspensions of 5 $x10^7$ cells/ml or less. For chimeric development, clonal populations were mixed at indicated ratios prior to plating cells on nonnutrient plates. Fluorescent images were detected and recorded using fluorescence microscopy.

3.3.2. Recombinant DNA constructs and amplifications.

A genomic fragment containing the *thyA* gene excised with *Bam*HI was inserted into the unique *Bgl*II sites of an *erk2* cDNA to disrupt the *erk2* open reading frame. The *erk2::thyA* construct was excised at flanking sites with *Xho*I and *Xba*I and electroporated into $thyA^-$ or $erk1^-thyA^-$ cells to disrupt the *erk2* locus. Erk1 and Erk2 expression vectors utilizing the *act15* promoter were created using the pTX-GFP2 vector (replacing the GFP with MAPK sequence) as previously described [22]. The pTX-GFP2 vector was also used to label strains with GFP as previously described [36]. An Erk2 expression vector conferring blasticidin resistance was created by transferring a p*act15:GFP2:erk2 Spe*I fragment into a pBluescript SK- vector containing a blasticidin resistance gene at the *Pst*I site [37]. This vector was linearized at a unique *Sph*I site near the amino-terminal coding region of *erk2* and integrated into the *erk2: thyA* locus of $erk2^-$ cells through a single homologous recombination event. This knock-in construct regenerated a complete *erk2*

open reading frame downstream of the endogenous *erk2* promoter. Verification of integration events into the *erk2* locus was conducted using PCR amplification. PCR primer sequences and binding locations are described in supplementary figures (Fig. S1).

3.3.3. Chemotaxis assays

Above agar chemotaxis assays were performed as previously described [22]. Cells were grown in fresh axenic medium 24 h prior to harvesting, washing and suspension in phosphate buffer at 2 x 10^7 cells/ml for *Dictyostelium*. Droplets (< 1 µl) of cell suspensions were spotted on nonnutrient agar plates and then 1 µl of chemoattractant was spotted approximately 2 mm from the cell droplet. Images of the cells were recorded immediately after the plating of the cells and chemoattractant and recorded again 2.5 - 3 h later. The agar surface near the cell droplet was scarred with a needle to allow the early and late images to be aligned so that the original cell droplet perimeter could be overlaid on the later image. Cell movement toward the chemoattractant source was determined by measuring the distance from the original cell droplet perimeter to the leading edge of migrating cells. Chemotaxis was analyzed using a dissecting microscope (Nikon SMZ2). Videos were created using time-lapse photography with 20s intervals between images for 33 min. ImageJ with MTrackJ plugin software was used to trace cell migration tracks and determine the migration distance for selected cells.

3.3.4. Analysis of bacterial cell engulfment

Dictyostelium engulfment of bacteria in suspension cultures was conducted as previously described [26]. Axenic *Dictyostelium* cells were washed and resuspended at 1X10^6 cells/ml in phosphate buffer. Live *K. aerogenes* bacteria were labeled with pHrodo Red (Thermo Scientific) and incubated with *Dictyostelium* in phosphate buffer at a 100:1

ratio at 22°C in shaking cultures (150 rpm). At indicated times, *Dictyostelium* cells were centrifuged and suspended in buffer containing 50 mM Tris pH 8.8 and 150 mM NaCl to quench the fluorescence of non-engulfed bacteria. *Dictyostelium* and bacteria were distinguished through forward and side scatter (FSC and SSC) and the engulfment of bacteria was indicated by the level of fluorescence detected using FACSort flow cytometer (BD Bioscience) with Cell Quest software (v. 3.3). Data analysis was conducted using FlowJo (v. 10.0.8; Tree Star). Quantification of engulfed bacteria number per *Dictyostelium* cell was analyzed using confocal microscopy. *Dictyostelium* were plated in four well chambers (Lab-Tek) and incubated with pHrodo labeled *K. aerogenes* in phosphate buffer. After 15 min, phosphate buffer was replaced with basic buffer to stop engulfment and quench extracellular bacteria fluorescence for imaging.

3.3.5. Reporter protein translocation

Reporter protein translocation was measured as previously described [26]. Cells expressing PH_{CRAC}-GFP, RBD-GFP or LimEΔcoil-GFP were harvested, washed with phosphate buffer prior to plating in four well chambers (Lab-Tek). A Zeiss Laser Scanning Microscope 880 with a 60x, 1.3 NA Plan-Neofluar objective lens was used to acquire time-lapse images every 2 seconds. Cells were exposed to a final concentration of 100 μM folic acid to induce PH_{CRAC}-GFP, RBD-GFP or LimEΔcoil-GFP translocation from cytosol to plasma membrane. Confocal images were used to determine the temporal-spatial intensity changes of PH_{CRAC}-GFP or LimEΔcoil-GFP. Fluorescence intensity at the plasma membrane was measured over time and normalized to the first frame when folate was added. At least ten cells were quantified for each strain.

3.3.6. Immunoblot analysis of MAPKs

For analysis of MAPK abundance, cells were harvested from axenic medium, washed in phosphate buffer and lysed by mixing with SDS-PAGE loading buffer on ice. Immunoblot analysis of Erk2 protein was conducted using an affinity-purified Erk2 antiserum as the primary antibody. This antiserum was generated in rabbits using the ERK2 peptide ERKKQTNPTKPD (containing a cysteine residue at the amino terminus for attachment procedures) as an antigen and the peptide was also used for the affinity purification (Genscript). The analysis of MAPK phosphorylation was conducted as previously described [17]. Cells were grown in shaking cultures to mid log phase (~3 x 10^6 cells/ml) and then harvested by centrifugation. Cells were washed once in phosphate buffer and suspended at 1 x 10^8 cells/ml. Starved cells were shaken in a conical tube for 3-5 hours with pulses of 100nM cAMP every 15 min except for the 15 min prior to an assay. For analysis of cAMP stimulation, cells were stimulated with 100 nM cAMP and cell samples were collected and lysed at the indicated time by mixing with SDS-PAGE loading buffer on ice. Cell extracts were subjected to immunoblot analysis using a rabbit monoclonal antibody phospho-p42/44 MAPK (#4370, Cell Signaling Technology). For folate stimulation of MAPK phosphorylation, cell suspensions were shaken for 1 h in phosphate buffer prior to stimulation with 50 µM folate. A secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) and bioluminescence reaction was used for detection of the primary antibodies. In some blots the biotinylated mitochondrial 3-methylcrotonyl-CoA carboxylase α (MCCC1) was used as a gel loading control and this protein was detected using HRP-Streptavidin as previously described [38].

3.3.7. MAPK ortholog analysis

MAPK sequences were identified in sequence databases using UniProt and BLASTp searches using default parameters in the non-redundant protein sequences database (NCBI). Molecular phylogenetic analysis was conducted in MEGA7 using the Maximum Likelihood method based on the JTT matrix-based model [39,40]. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1000 replicates) are shown next to the branches [41]. Only branches corresponding to partitions reproduced in more than 50% of the bootstrap replicates are labeled. Each tree is drawn to scale, with branched lengths measured in the substitutions per site.

3.4. Results

3.4.1. Disruption of the *erk2* gene

Previous analyses of *Dictyostelium* Erk2 function have used *erk2* mutants that contain an insertion mutation near the *erk2* open reading frame [17,19-23]. This allele was originally created through restriction enzyme-mediated insertion (REMI) mutagenesis and the allele has been recapitulated in axenic strains through homologous recombination. This allele has a significant reduction of *erk2* expression that leads to inadequate cAMP production for the aggregation stage of development but only subtle changes in chemotactic responses to either cAMP or folate [24]. Although this allele results in developmental defects, activated Erk2 can still be detected [17]. For clarity this allele will now be referred to as $erk2^{RE}$ (reduced expression) to distinguish it from an $erk2^{-}$ allele described in this report that does not produce functional Erk2 protein. Given the apparent importance of Erk2 in *Dictyostelium* development we created and verified an $erk2^{-}$ strain that contains the *erk2* open reading frame disrupted with the auxotrophic marker gene

thyA (Fig. 3.1A and S3.1A). *D. discoideum* has only two MAPKs, Erk1 and Erk2, and so we also created a strain with no MAPKs by disrupting the *erk2* gene in a strain already containing a disrupted *erk1* gene. Both *erk2*$^-$ and *erk1*$^-$*erk2*$^-$ strains were transformed with Erk2 expression vectors for complementation and the *erk1*$^-$*erk2*$^-$ strain was also transformed with an Erk1 expression vector. Levels of Erk2 protein in mutants and complemented strains were verified by immunoblot analysis using antiserum generated against an Erk2 peptide (Fig.3.1C). Wild-type and complemented *erk2*$^-$ mutants displayed a band at approximately 42 kDa (predicted size of Erk2) and this band was absent in *erk2*$^-$ and *erk1*$^-$*erk2*$^-$ strains.

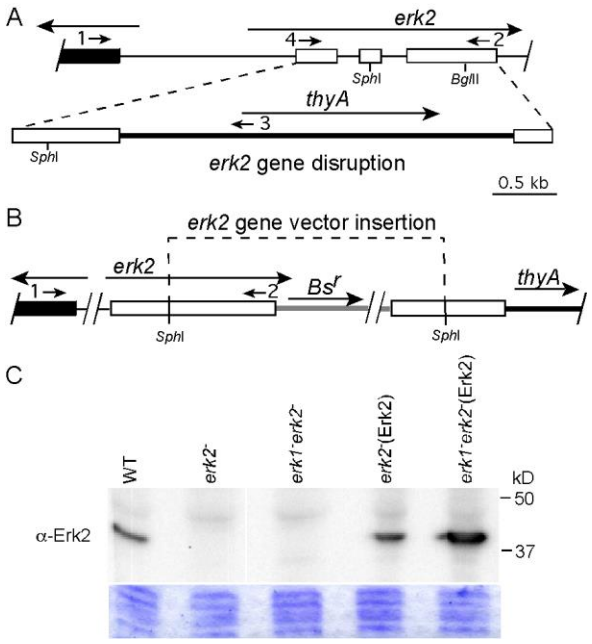

Fig. 3.1. Disruption and knock-in complementation of the *erk2* locus. A) Homologous recombination of the *erk2::thyA* fragment (see Materials and Methods for construction) with the *erk2* locus. The location of primer binding sites (arrows) used for PCR verification of recombination are shown. Open rectangles represent the *erk2* open reading frame, the closed rectangle represents the open reading from of an adjacent gene and the thick black line represents the *thyA* genomic fragment. B) Knock-in of an Erk2 expression vector with blasticidin resistance into the disrupted *erk2::thyA* locus at the *Sph*I site. Hashed lines represent sequences not shown

to reduce the size of image. Description of PCR products and primer sequences are described in Fig. S3.1. C) Immunoblot of Erk2 protein in wild-type (WT), *erk2*$^-$, and *erk1*$^-$*erk2*$^-$ strains and in mutant strains complemented with Erk2 expression vector (Erk2). Lysates of cells grown in axenic medium were analyzed for Erk2 protein by immunoblot analysis. Coomassie staining of the gel was used as a lane loading control.

3.4.2. *erk2*$^-$ cells have growth defects on bacterial lawns

When grown on bacterial lawns, cells with the *erk2* gene disruption displayed a slow plaque growth rate and no multicellular development compared to wild-type cells (Fig. 3.2A). Transformation of the *erk2*$^-$ cells with Erk2 expression vectors (both extrachromosomal and integrating) using the relatively constitutive *act15* promoter resulted in a rescue of the plaque growth rate and, in some transformants, a rescue of multicellular development was also observed. The lack of multicellular development in some transformants might be due to the heterologous overexpression of Erk2 because these vectors can also result in aggregation deficient phenotypes in wild-type cells. To express Erk2 from its endogenous promoter an Erk2 expression vector conferring blasticidin resistance was linearized within the *erk2* open reading frame and integrated into the *erk2: thyA* locus upstream of the gene disruption site (Fig. 3.1B, Fig. S3.1). This knock-in integration of an *erk2* vector resulted in cells with a single copy of a complete *erk2* open reading frame downstream of the endogenous *erk2* promoter. Erk2 expression from the endogenous promoter provided a more efficient rescue of both plaque growth rate and multicellular development. Cells with both MAPK gene disruptions, *erk1*$^-$*erk2*$^-$, also had a slow plaque growth phenotype but this phenotype was more extreme than that of cells with only the disruption of the *erk2* gene. Reduced plaque growth rates have also been observed with other *Dictyostelium* mutants, particularly those defective in responding to folate. Two mutants with defects in folate responses, *far1*$^-$ and *gα4*$^-$ strains,

displayed plaque growth rates slower than wild-type cells but faster than *erk2*$^{-}$ cells suggesting that Erk2 likely functions in cellular processes other than folate responses. To assess whether the slow plaque growth phenotypes of *erk2*$^{-}$ and *erk1*$^{-}$*erk2*$^{-}$ are due to general growth defects the mutants were analyzed for cell proliferation in shaking cultures of axenic medium. Interestingly, the *erk2*$^{-}$ mutants had proliferation characteristics similar to that of complemented cells and wild-type cells (Fig. 3.2B). However, *erk1*$^{-}$*erk2*$^{-}$ cell proliferation was much slower under these conditions but complementation of the *erk2*$^{-}$ allele in this strain rescued this defect suggesting that only a loss of both MAPKs has an impact on proliferation in axenic medium. The *erk1*$^{-}$*erk2*$^{-}$ cell proliferation defect was not the result of unusual cytokinesis mechanisms because the distribution of single and multinucleated cell particles was similar to the other strains (Fig. S3.2).

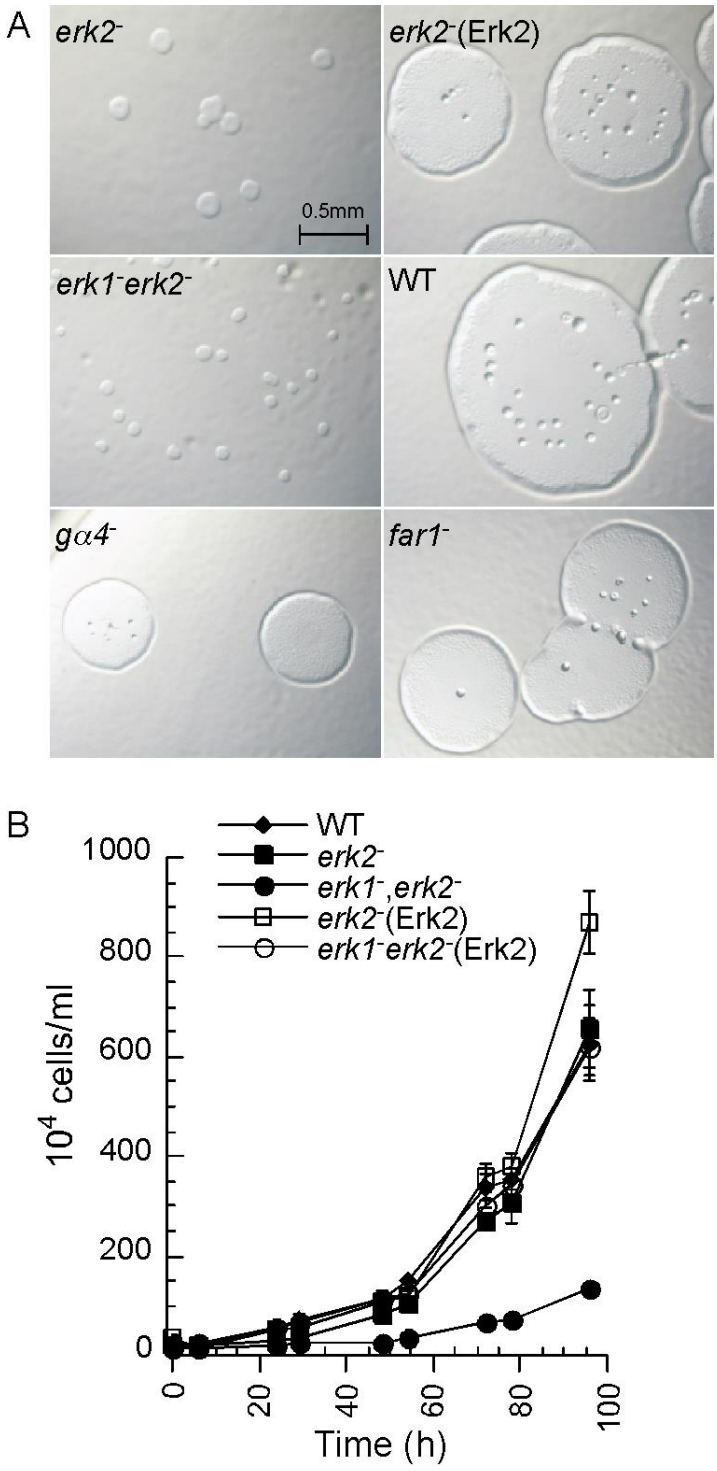

Fig. 3.2. *Dictyostelium* growth. A) Growth of MAPK mutants and wild-type cells on bacterial lawns. Individual strains were mixed with bacteria and plated on SM+/3 plates as described in the Materials and Methods section. Images of plaques were captured 5 days later. All images are the same magnification. B) Growth of MAPK mutants and wild-type cells in shaking cultures of

axenic medium. Wild-type (WT), *erk2*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, and the *erk2* mutant strains complemented Erk2 expression vectors (Erk2) were inoculated into shaking cultures of HL-5 axenic medium and cells concentrations were determined using a hemacytometer at the indicated times. Each data point represents 4 counts of at least 100 cells. Error bars represent standard deviation in multiple counts.

3.4.3. Loss of Erk2 impairs bacterial engulfment

Engulfment of bacteria by *Dictyostelium* is mediated in part by the folate receptor Far1 and stimulation of this receptor also activates Erk2 [26]. To determine the potential role of Erk2 in bacterial phagocytosis, we used a flow cytometry analysis to quantitatively compare the engulfment of pHrodo-labeled *K. aerogenes* by wild-type, *erk2*$^{-}$, and other mutant strains. Cells lacking Erk2 or Far1 displayed a similar delay in the initial engulfment in the phagocytosis of the fluorescently-labeled bacteria in suspension cultures compared to wild-type cells. The phenotypic similarity between *erk2*$^{-}$ and *far1*$^{-}$ cells suggests that Erk2 plays a role in Far1-mediated bacterial phagocytosis (Fig. 3.3A and B). This delayed uptake of bacteria was also consistently observed when cells were monitored by confocal microscopy (Fig. 3.3C and D). The number of engulfed bacteria in *erk2*$^{-}$ cells was less than wild-type cells. This defect was rescued by complementation with the Erk2 expression vector. After the initial delay, the rate of *erk2*$^{-}$ bacterial engulfment was similar to that of wild-type cells implying that other mechanisms might contribute to the slow plaque growth rate on bacterial cell lawns. The lower fluorescence intensity of pHrodo-bacteria in *erk2*$^{-}$ cells suggests a potential defect in phagosomal maturation.

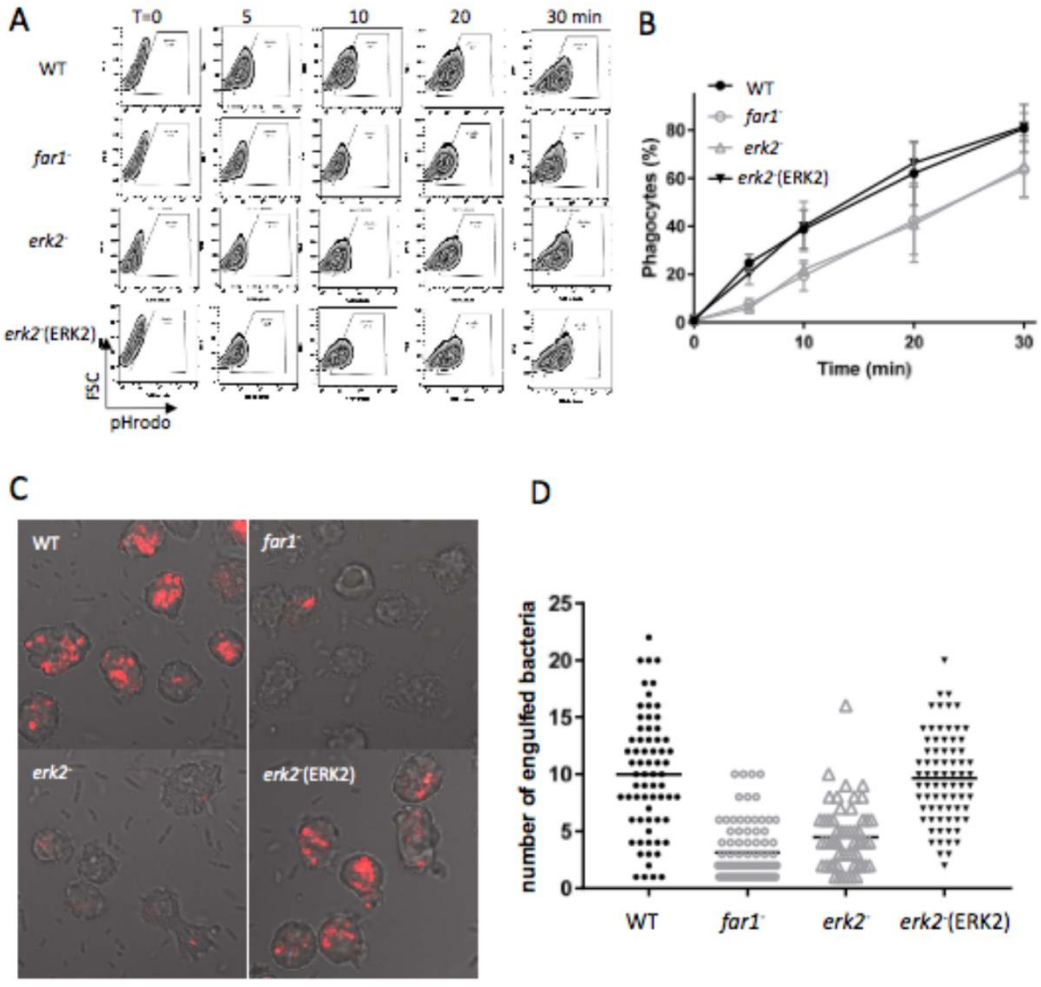

Fig. 3.3. Engulfment of bacteria. A) Wild-type (WT), *far1*$^{-}$, and *erk2*$^{-}$ strains and *erk2*$^{-}$ mutant complemented with Erk2 expression vector (Erk2) were mixed with pHrodo-labelled live bacteria and analyzed at indicated times for the percentage of pHrodo-positive cells. B) Graphical representation of data from (A). C) Images of engulfed bacteria in cells after 15 min. D) Quantitation of bacterial cell uptake into cells. The engulfed bacterial number in each cell was measured and plotted.

3.4.4. Erk2 is required for folate chemotaxis

Reduced plaque growth rates can potentially result from defects in the ability of cells to properly forage for bacteria at the perimeter of the plaque. *Dictyostelium* forage

for bacteria primarily using the folate receptor and downstream G proteins as a mechanism to facilitate chemotactic movement [42,43]. The *erk2*$^{-}$ cells displayed a defect in folate chemotaxis similar to that of *far1*$^{-}$ and *gα4*$^{-}$ mutants when analyzed in an above agar assay (Fig. 3.4A and S3.3). This defect is rescued by the presence of the *erk2* gene. In the absence of folate stimulation *erk2*$^{-}$ cells did not migrate as far as wild-type and the complemented *erk2*$^{-}$ cells suggesting Erk2 function contributes to cell motility, directionality, and/or other mechanisms (e.g., cell repulsion) associated with cell dispersal. The *erk1*$^{-}$*erk2*$^{-}$ cells also lacked chemotaxis to folate but these cells did not disperse from the initial cell droplet as much as the *erk2*$^{-}$ cells suggesting that the loss of both MAPKs has a detrimental impact on cell dispersal. Time-lapsed videos of *erk2*$^{-}$ cells showed migratory paths typical of random movement compared to the more directed movement paths observed for wild-type cells or complemented mutants in the presence of folate (Fig. 3.4B, C and S3.4- 3.8). The average path lengths for *erk2*$^{-}$ or *erk1*$^{-}$*erk2*$^{-}$ cells were comparable but substantially less than that of wild-type or complemented cells. This difference in individual cell path lengths suggests that chemotactic cell movement is compromised in the MAPK mutants. The reduced cell dispersal of the double MAPK mutant compared to the *erk2*$^{-}$ mutant was surprising given that *erk1*$^{-}$ cells do not have a defect in folate chemotaxis (Fig. S3.9). The basis of this cell dispersal defect is not known but the defect was also observed in cAMP chemotaxis assays (Fig. 3.6C) and during drug selection of transformants (Fig. S3.10). It is possible that both MAPKs might have overlapping contributions to cell movement. The requirement of Erk2 for folate chemotaxis indicates that this MAPK plays an important role in the foraging of *Dictyostelium* to bacteria.

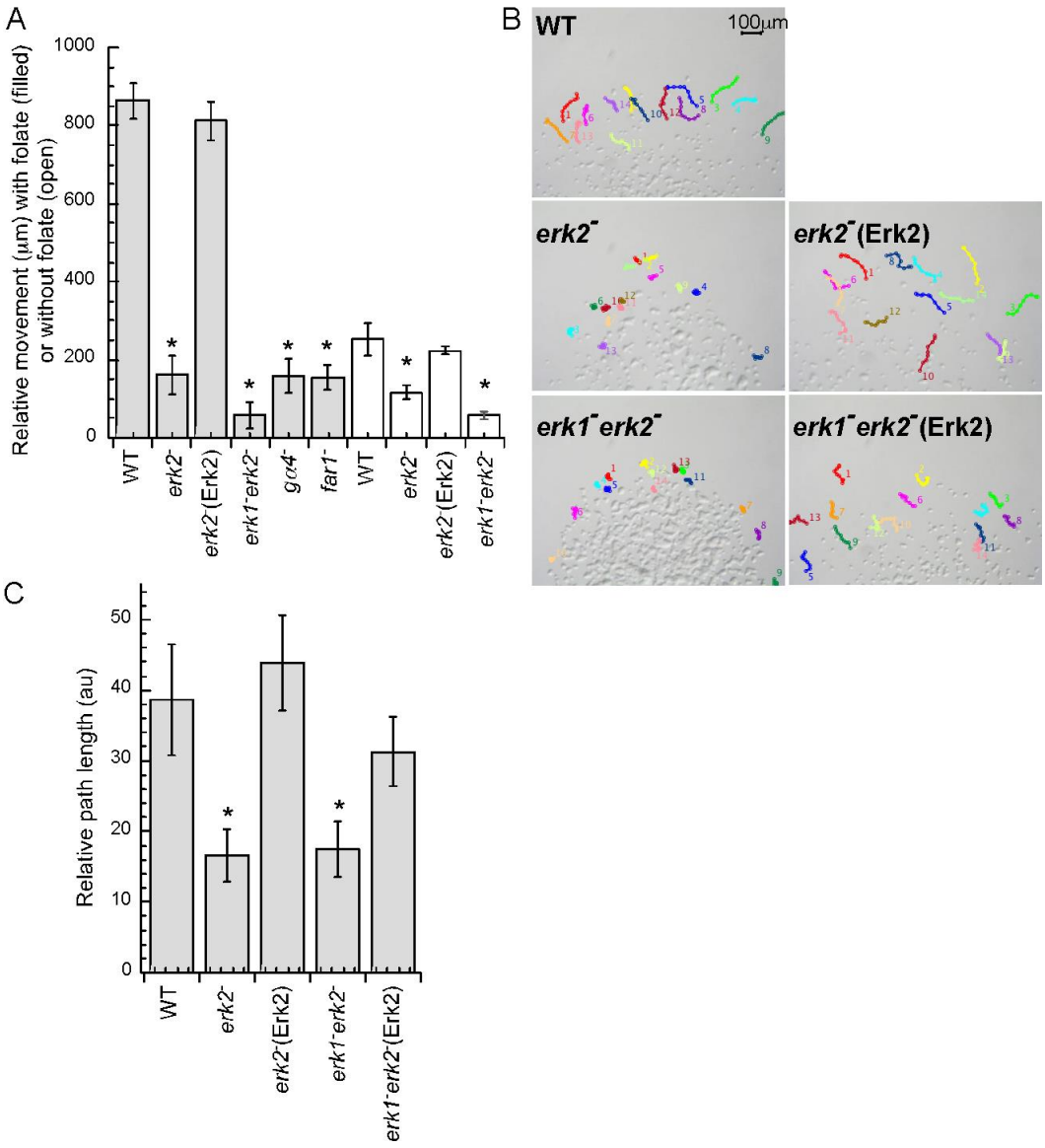

Fig. 3.4. Chemotaxis of MAPK mutants to folate. A) Above-agar chemotaxis assay images for wild-type (WT), *erk2*$^-$, and *erk1*$^-$*erk2*$^-$ strains and *erk2*$^-$ mutants complemented with Erk2 vector (Erk2) after 2.5 h exposure to droplets of 100 μM folate. A) Relative movement of wild-type (WT), *erk2*$^-$, *erk1*$^-$*erk2*$^-$, *gα4*$^-$ and *far1*$^-$ strains and MAPK mutants complemented with Erk2 expression vector (Erk2) toward folate (filled bars) and relative movement in the absence of folate (open bars). Values indicate maximum distance of cell migration toward the source of folate or migration in any direction in the absence of folate. Error bars represent the standard deviation of the error. B) Cell migration paths of select cells were mapped over a 30 min period using time-lapse photography as described in the Materials and Methods section. All images are the same magnification. C) Graphical representation of the average path lengths in arbitrary units

(a.u.) from (B). Error bars represent standard deviation. Student's unpaired *t*-test comparing to WT, P<0.0001 (*).

3.4.5. Loss of Erk2 does not affect folate detection & early signaling events

Chemoattractant sensing in *Dictyostelium* is mediated in part by the rapid activation of Ras proteins, phosphoinositide 3-kinases (PI3Ks), and actin polymerization [44,45]. Fluorescent reporters that bind to activated Ras (RBD-GFP), phosphorylated inositol lipids (PH_{CRAC}-GFP), and actin filaments (LimEΔcoil-GFP) can assess these cellular responses through the translocation of the reporter to the plasma membrane [46-48]. All of these responses typically begin within a few seconds of chemoattractant stimulation and prior to the activation of Erk2 suggesting these responses occur independently of Erk2 function. When expressed in *erk2*$^-$ cells, these reporters translocated to the membrane with kinetics and amplitudes similar to that observed for wild-type or complemented *erk2*$^-$ cells (Fig. 3.5A-C). This observation is also consistent with previous studies that suggest MAPK activation might occur in a parallel signaling pathway [49,50]. Therefore, the loss of Erk2 function does not significantly impact early chemotactic responses to folate.

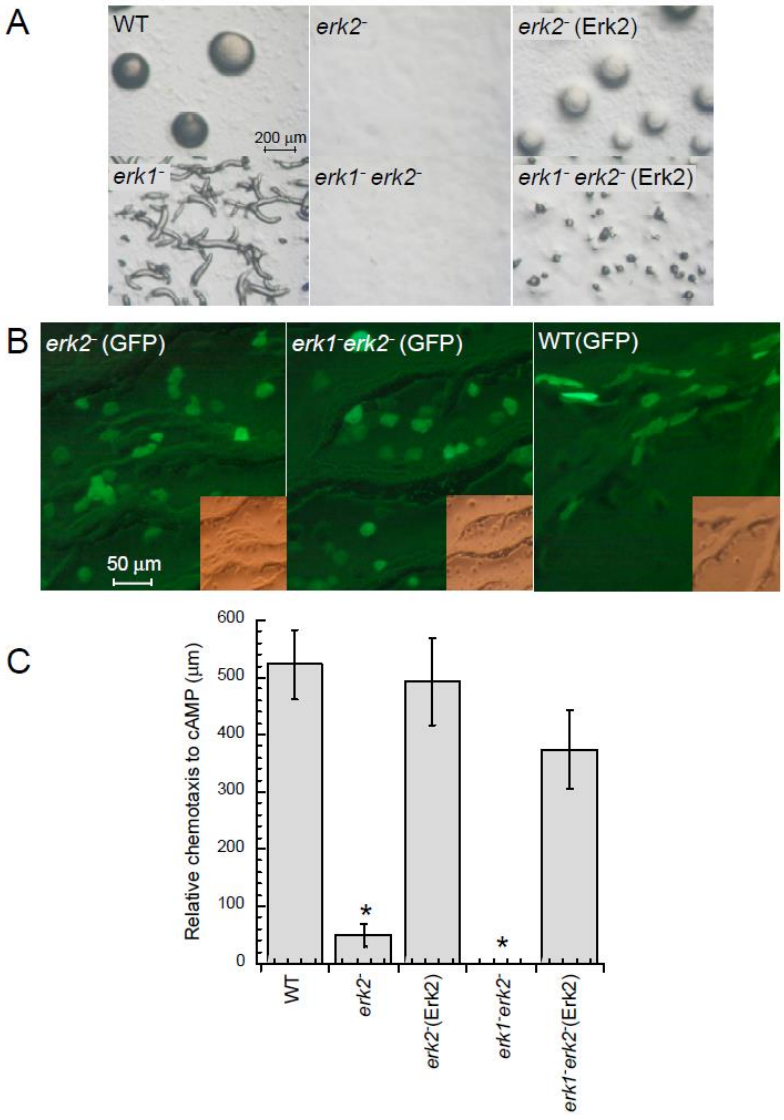

Fig. 3.5. Early chemotactic signaling in response to folate. Translocation of Ras, PI3K, and actin filament reporters in wild-type (WT), *erk2*$^-$ cells (*erk2*$^-$), and complemented *erk2*$^-$ cells (Erk2) in response to folate stimulation was assayed as described in the Materials and Methods section. A) Translocation of the Ras activation reporter RBD-GFP to the membrane. B) Translocation of the PI3K activation reporter PH_{CRAC}-GFP to the membrane. C) Translocation of the actin filament reporter LimEΔcoil-GFP to the membrane. Graphs indicate relative intensity of fluorescence at the membrane and 1 represents the intensity at the start of the response. Error bars represent standard deviation. All images are the same magnification and scale bar represents 5µm.

3.4.6. Erk2 is required for development and cAMP chemotaxis

The *erk2*$^{-}$ and *erk1*$^{-}$*erk2*$^{-}$ mutants failed to aggregate when synchronously starved on nonnutrient agar but the expression of Erk2 in these mutants restored multicellular development similar to that of wild-type and *erk1*$^{-}$ cells, respectively, including the small aggregate and accelerated development characteristic of *erk1*$^{-}$ development (Fig.3.6A). Earlier studies have shown that the aggregation defect of mutants with reduced Erk2 expression can be rescued by the presence of wild-type cells in a chimeric population because the *erk2*^{RE} mutants retain the ability to chemotaxis to cAMP [22,23]. In contrast, the *erk2*$^{-}$ or *erk1*$^{-}$*erk2*$^{-}$ cells do not co-aggregate with wild-type cells as indicated by lack of cell elongation and the absence of these cells in aggregation streams (Fig. 3.6B). This observation suggests that the mutants do not respond to wild-type cAMP signaling or produce an inhibitory mechanism to the cAMP-mediated aggregation of wild-type cells. The *erk2*$^{-}$ cells were also analyzed in above-agar cAMP chemotaxis assays. Cells lacking Erk2 were not capable of chemotaxing to cAMP but chemotaxis could be restored by complementation with the Erk2 expression vector (Fig. 3.6C). Both the lack of cAMP chemotaxis and the inability to co-aggregate with wild-type cells suggest that the failure of *erk2*$^{-}$ mutants to undergo multicellular development is due to a chemotaxis defect and not just a defect in cAMP production.

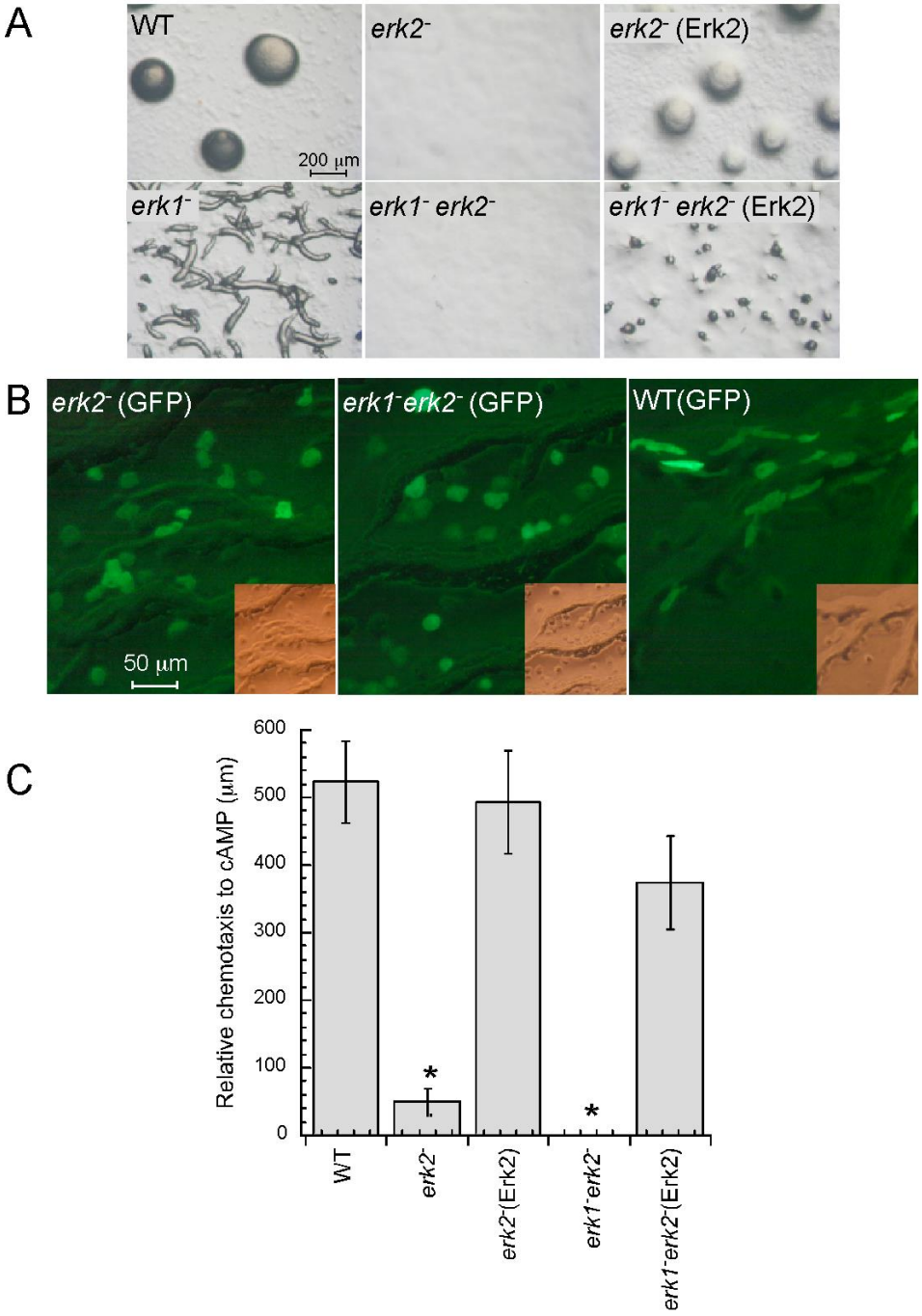

Fig. 3.6. Development and cAMP chemotaxis. A) Wild-type (WT), *erk2*$^-$, and *erk1*$^-$*erk2*$^-$ mutants and mutants complemented with Erk2 expression vector (Erk2) developed on nonnutrient plates for 13 hours. All images are the same magnification. B) A GFP vector was used to label *erk2*$^-$, *erk1*$^-$*erk2*$^-$, and wild-type (WT) cells. Labeled cells (GFP) were mixed in a 1:9 ratio with unlabeled wild-type cells and and cell droplets ($1x10^7$ cells/ml) plated for development on nonnutrient agar plates. Images of aggregation streams were taken at 12 hours. All images are the

same magnification. C) Above-agar cAMP chemotaxis assay. After 4 h of starvation in shaking phosphate buffer cells were plated on nonnutrient plates near droplets of 100 µM cAMP. Images of cells were taken at 0 h and 2.5 h and distance was measured of the leading edge of cells toward the source of cAMP. Migration distances under 100 µm are typical for random movement in the absence of exogenous cAMP. Error bars represent the standard deviation of the error. Student's unpaired *t*-test comparing to WT, P<0.0001 (*).

3.4.7. Loss of Erk2 impairs Erk1 activation in folate chemotactic response

We had previously shown that reduced levels of Erk2 in *erk2*^{RE} cells did not impact the phosphorylation of Erk1 in the secondary response to folate stimulation [17]. Given the importance of Erk2 in chemotaxis, the phosphorylation of Erk1 in response to folate was examined in the *erk2*$^{-}$ cells and found to be absent, indicating a dependence on Erk2 function (Fig. 3.7A). However, a low level of phosphorylated Erk1 could be detected in *erk2*$^{-}$ cells suggesting that Erk2 function is only required for the burst of phosphorylated Erk1 as a secondary response to chemotactic stimulation. Erk1 activity has been previously shown to be dependent on MekA, the only known MAP2K in *Dictyostelium*, but a requirement of MekA for the phopshorylation of Erk1 had not been demonstrated [16]. The stimulation of *mekA*$^{-}$ cells with folate or cAMP resulted in the phosphorylation of Erk2 but not Erk1 indicating that MekA only regulates Erk1 and not Erk2 (Fig. 3.7B). This result implies that the phosphorylation of Erk2 must be facilitated through a mechanism that does not require a conventional MAP2K.

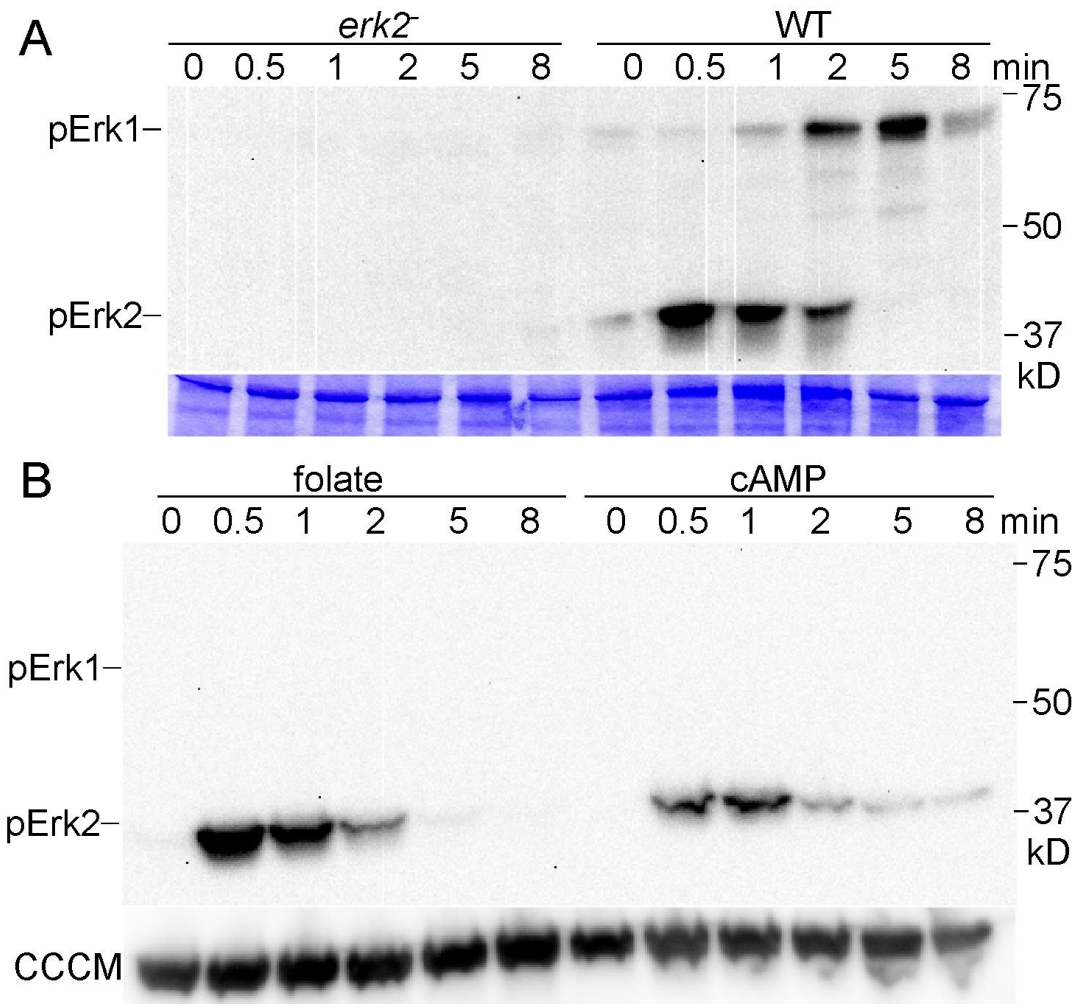

Fig. 3.7. Phosphorylation of MAPKs. A) After 50 µM folate stimulation *erk2*- and wild-type (WT) were lysed at times indicated and analyzed for the phosphorylation of MAPKs by immunoblots using phospho-MAPK specific antibodies (upper panel). Coomassie blue stained gel as loading control (lower panel). B) Phosphorylation of MAPKs in *mekA*- cells in response to folate or cAMP. Cells were stimulated with either 50 µM folate or 100 nM cAMP and then analyzed for phosphorylation of the MAPKs as described in (A) (upper panel). Detection of CCCM using HRP-streptavidin as a loading control (lower panel).

3.4.8. Erk2 sequence is related to atypical MAPKs

A phylogenetic analysis of the *Dictyostelium* MAPKs with other eukaryotic MAPKs suggests that the *Dictyostelium* Erk1 belongs to a group of MAPKs that is found in a wide variety of eukaryotes (Fig. 3.8). This group contains prototypical MAPKs in

yeast (e.g., Fus2) and mammals (e.g., ERK1/2) that have been characterized extensively. In contrast, the *Dictyostelium* Erk2 shares more sequence similarity to a group of MAPKs that includes the mammalian MAPK15 (also referred to as Erk8). This group of kinases has been previously referred to as atypical MAPKs because typical MAP2Ks have not been identified as the activators of these MAPKs [5,10,11]. This atypical regulation is consistent with the *Dictyostelium* Erk2 belonging to this group of MAPKs. Orthologs of the *Dictyostelium* Erk2 exists in other amoebae and in animals where cell movement plays important roles but not in fungi where cell movement is absent. The fungal MAPKs (e.g., *Aspergillus nidulans*, *An*MAPK) that are most closely related to the *Dictyostelium* Erk2 belong to other MAPK groups suggesting the evolution of organisms without cell movement did not require this group of atypical MAPKs.

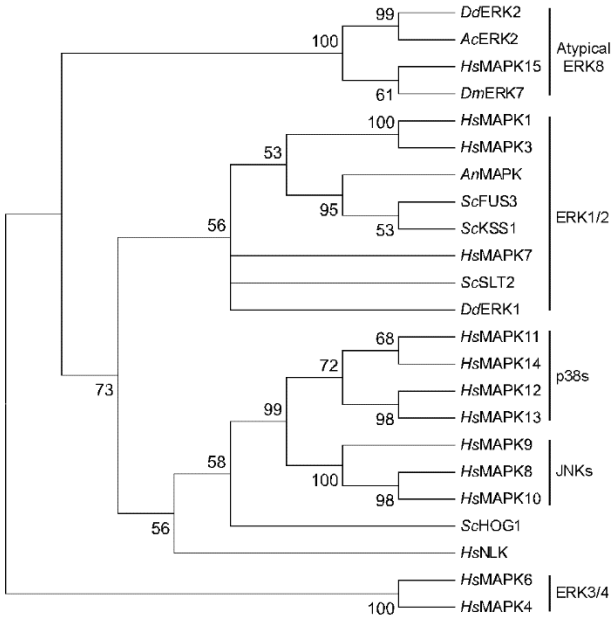

Fig. 3.8. Phylogenetic analysis of MAPKs. All known MAPKs in human (*Hs*), yeast/*Saccharomyces cerevisiae* (Sc), and *Dictyostelium discoideum* (*Dd*) were used to construct the phylogenetic tree using MEGA7 as described in the Materials and Methods. Selected MAPKs with similarity to atypical human MAPK15 (Erk8) from *Drosophila melanogaster* (*Dm*) and

Acanthamoeba castellanii (*Ac*) were also included in the tree. A BLAST search of fungal genomes using the *Dictyostelium* Erk2 protein as the query yielded only MAPKs with similarities to the human Erk1/2 group such as the one representative MAPK included from *Aspergillus nidulans* (*An*).

3.5. Discussion

This study of *erk2* gene disruption mutants has revealed the essential role of the *Dictyostelium* Erk2 in chemotaxis to folate and cAMP whereas previous studies of *erk2*^{RE} mutants had implied only a subtle role in chemotaxis (Fig. 3.9). While required for chemotaxis, Erk2 function does not impact early chemotactic responses such as Ras and PI3K activation and early actin filament assembly. Previous studies have shown levels of Erk2 activation in *rasC*$^{-}$ mutants to be the same as wild-type cells in response to cAMP and only reduced by half in response to folate suggesting Erk2 regulation occurs through a parallel signaling pathway [49,50]. The requirement of Erk2 function for two different chemotactic responses suggests that this MAPK plays an integral role in general chemotactic responses and could possibly be important for other cell fates that involve chemotactic movement. The rapid activation of Erk2 in response to chemoattractants argues that Erk2 function is necessary for cell movement in response to chemoattractants rather than being a general requirement for all cell movement. However, *erk1*$^{-}$*erk2*$^{-}$ mutants show a strong defect in cell dispersal suggesting both MAPKs have overlapping contributions to cell movement in the absence of an exogenous stimulus. The role of Erk2 function in chemotactic responses is clearly different than that of Erk1 function. In an earlier study, *erk1*$^{-}$ cells have been described as impaired with respect to cAMP responses but these cells can aggregate and complete all other phases of development in clonal

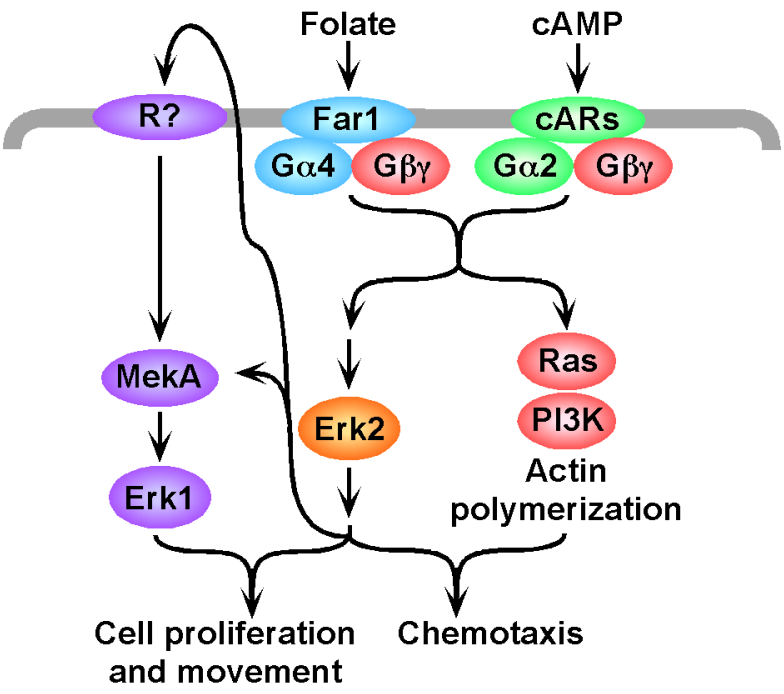

Fig. 3.9. Model of Erk2 mediated signaling pathways. Multiple chemoattractant stimulated pathways lead to the activation of Erk2 and downstream cellular responses such as chemotaxis and Erk1 activation. Early chemotactic responses such as Ras and PI3K activation and actin filament formation are not dependent on Erk2 function. Like mammalian atypical MAPKs, the activation of Erk2 does not require the only known MAP2K in *Dictyostelium*. Folate but not cAMP responses require G protein function for Erk2 activation.

populations [17,18]. However, *erk1*$^-$ cells typically form smaller aggregates with precocious development suggesting that developmental signaling is aberrant. Folate chemotaxis and foraging capabilities of *erk1*$^-$ cells are comparable to that of wild-type cells (Fig. S3.9). The phenotypic differences between *erk1*$^-$ and *erk2*$^-$ cells in foraging and multicellular development suggest that the two *Dictyostelium* MAPKs regulate different cellular processes even though they appear to have overlapping contributions to cell movement.

How Erk2 mediates chemotaxis independent of early chemotactic responses remains a major question because little is known about the regulators and targets of atypical MAPKs. Genetic evidence suggests Erk2 is a negative regulator of the cAMP-

specific phosphodiesterase, RegA, and therefore Erk2 function could indirectly lead to increased cAMP-dependent protein kinase (PKA) activity [20]. However, the loss or over-expression of RegA does not eliminate chemotaxis, suggesting other downstream regulatory proteins exist. Epp2, a protein phosphorylated in an Erk2-dependent manner, is important but not essential for cAMP chemotaxis and cAMP production [51]. The primary structure Epp2 has so far not provided clues as to the function of this protein. Typical MAPKs are known to phosphorylate and regulate other protein kinases and transcription factors but such downstream regulatory proteins have not yet been reported in *Dictyostelium* [5]. Erk2 function could potentially regulate the expression of genes that facilitate chemotaxis but such function would not address the role of Erk2 activation during chemotaxis.

MAPKs in other organisms have been associated with the regulation of cell proliferation and so MAPK pathways have been a focus for understanding and treating cancerous growth [1,52-54]. The association of MAPK function with cell growth and proliferation in mammalian systems has been largely based on the activation of MAPKs downstream of receptor tyrosine kinases and Ras proteins that drive these processes [5,52,55-59]. The compromised proliferation of the *Dictyostelium* double MAPK mutant indicates that the MAPKs are important but not essential for proliferation. Some synergy may exist between the *erk1*$^{-}$ and *erk2*$^{-}$ gene disruptions because no proliferation defects have been noted for strains carrying one or the other mutant alleles implying Erk1 and Erk2 signaling pathways might have some overlap in the regulation of cell proliferation. While the basis of the *erk1*$^{-}$*erk2*$^{-}$ proliferation defect remains to be determined, this proliferation phenotype supports early assertions that MAPK signal transduction might be a good

target for inhibiting cell proliferation. *Dictyostelium* growth and proliferation are typically intertwined with finding nutrient sources but the cell proliferation defect of *erk1*$^{-}$*erk2*$^{-}$ mutants in axenic suspension cultures occurs in the absence of cell migration. However, it is possible that nutrient uptake in *Dictyostelium* suspensions could include cellular processes related to those important for cell migration.

The roles of Erk2 and Erk1 function in *Dictyostelium* development are quite different and possibly oppositional. The loss of Erk2 function blocks development at the aggregation stage and the loss of Erk1 function can accelerate developmental progression [17]. Therefore, it is interesting that both MAPKs become activated in response to cAMP and folate stimulation. The rapid phosphorylation of Erk2 and then later phosphorylation of Erk1, as Erk2 becomes dephosphorylated, indicates a temporal distinction in the regulation of these MAPKs. The timing of Erk1 phosphorylation correlates with the adaptation to the stimulus and therefore Erk1 activation could be associated with a mechanism to down regulate the initial chemotactic signal. The mechanism by which Erk2 function regulates the phosphorylation of Erk1 is unclear but it requires the activation of MekA and possibly intercellular signaling, as suggested by a previous study [17]. Interestingly, the timing of Erk1 phosphorylation in response to chemotactic signals in *Dictyostelium* is similar to that of mammalian Erk1/Erk2 phosphorylation in mammalian neutrophils after chemotactic stimulation with fMLP, in that the phosphorylated form persists for over 5 minutes [60-62]. If *Dictyostelium* and mammalian MAPK orthologs play analogous roles in chemotaxis then it is possible that mammalian Erk1/Erk2 could be involved with an adaptive secondary response to chemoattractants and the mammalian MAPK15 might have a role in mediating the initial chemotactic signaling. Studies of the

mammalian MAPK15 regulation have often focused on relatively slow or long term responses (10 minutes to hours after stimulation) rather than rapid responses (within a couple minutes), like the rapid phosphorylation of Erk2 in *Dictyostelium*, and so possible chemotactic regulation of MAPK15 activity might have been overlooked [63-65]. *Dictyostelium* and mammals share many similarities in chemotactic responses including G protein-mediated signaling and a rapid rise in cAMP suggesting similarities could possibly extend to MAPK function and regulation [45,66,67].

The sequence similarity of the *Dictyostelium* Erk2 with the mammalian MAPK15 and inability of these MAPKs to be activated by typical MAP2Ks suggest that these MAPKs might share related functions and regulation [5,10,11]. Thus far no gene disruptions have been created in the animal orthologs but recently mutations within the kinase domain of a nematode (*Caenorhabditis elegans*) ortholog have been shown to interfere with the formation of motile cilium formation [68]. RNA interference and kinase inhibitor analyses suggest that orthologs in trypanosomes (*Trypanosoma brucei*) are important for proliferation [69,70]. The human MAPK15 has been found widespread in tissue distribution and throughout development and studies using RNA interference suggest this MAPK can regulate proliferation in a variety of cell types [11,71-73]. The corresponding ortholog in flies, Erk7, regulates insulin-like peptide secretion and perhaps this production of a secreted hormone has some analogy with the intercellular signaling associated with Erk1 activation in *Dictyostelium* [74]. Understanding the activation kinetics of mammalian MAPK15 and other orthologs has been hampered due to the limited characterization of possible endogenous extracellular signals that activate these pathways [64,65]. Therefore, defining the regulation and function of the *Dictyostelium* Erk2 MAPK in response to

known endogenous signals is likely to provide a useful model for characterizing the regulation and function of this group of atypical MAPKs.

3.6. Supplemental Data

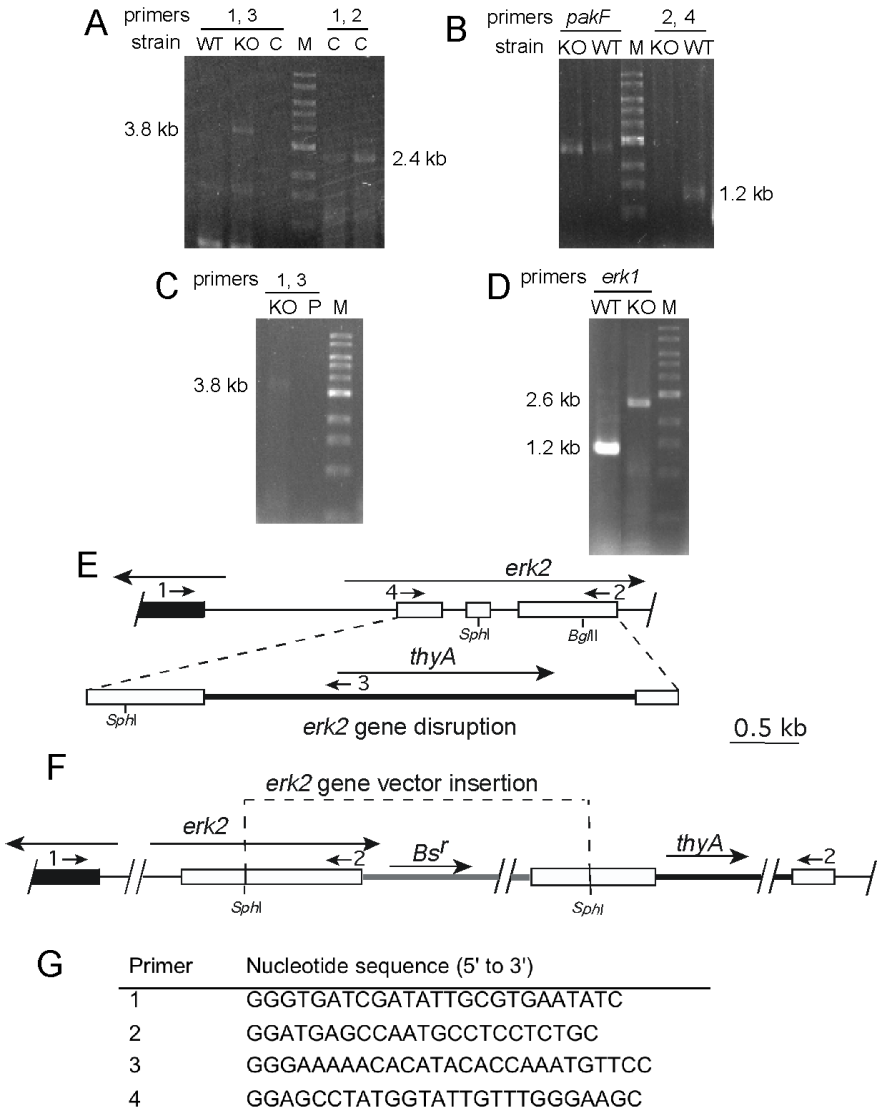

Fig. S3.1. Verification of genomic insertions. (*A*) PCR amplification of genomic DNA from JH10 (WT), *erk2*$^-$ (KO), and *erk2*$^-$ complemented with Erk2 vector knock-in (KI). PCR with primers 1 and 3 generates a 3.8 kb band in only the *erk2*$^-$ strain. PCR with primers 1 and 2 generates a 2.4 kb band only in the complemented *erk2*$^-$ strains. (*B*) PCR amplification of genomic DNA from *erk2*$^-$ (KO) and JH10 (WT) genomic DNA using control primers to another locus (*PakF*) generates fragments in both strains but primers 2 and 4 only generate a fragments in

only the JH10 strain and not the *erk2*$^{-}$ strain. C. PCR amplification of genomic DNA from *erk1*$^{-}$ *erk2*$^{-}$ (KO) and parental strain *erk1*$^{-}$*thyA*$^{-}$ (P) using primers 1 and 3 produces a 3.8 kb band in only the *erk1*$^{-}$*erk2*$^{-}$ strain. D. PCR amplification of genomic DNA from JH10 (WT) and *erk1*$^{-}$*erk2*$^{-}$ (KO) genomic DNA using *erk1* specific primers produces a fragment 1.4 kb greater in size for the *erk1*$^{-}$*erk2*$^{-}$ strain confirming the disruption of the *erk1* locus with the blasticidin resistance marker.

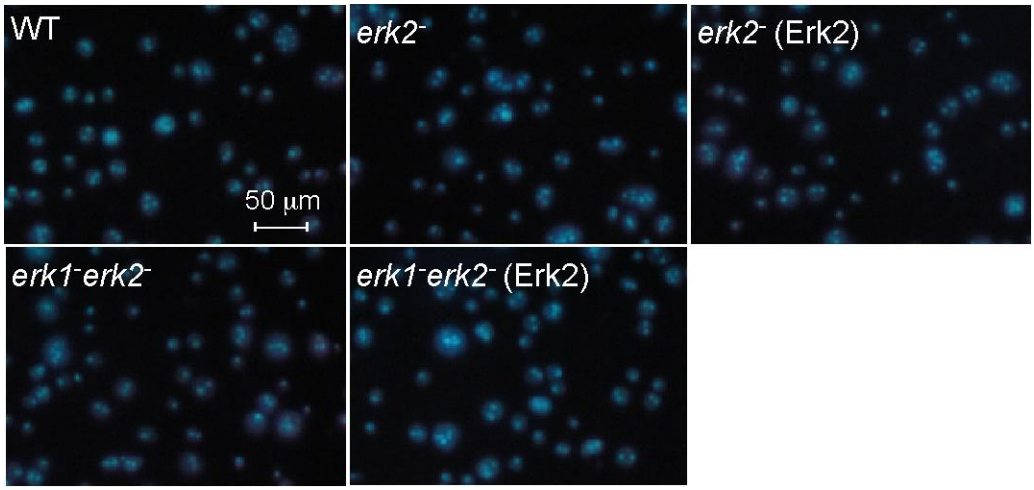

Fig. S3.2. Nuclei staining of axenic shaking cultures. Wild-type (WT), *erk2*$^{-}$, *erk1*$^{-}$*erk2*$^{-}$, and the *erk2*$^{-}$ mutant strains complemented Erk2 expression vectors (Erk2) were inoculated into shaking cultures of HL-5 axenic medium for 3 days and then fixed and stained with Hoechst dye to detect nuclei. All images are the same magnification.

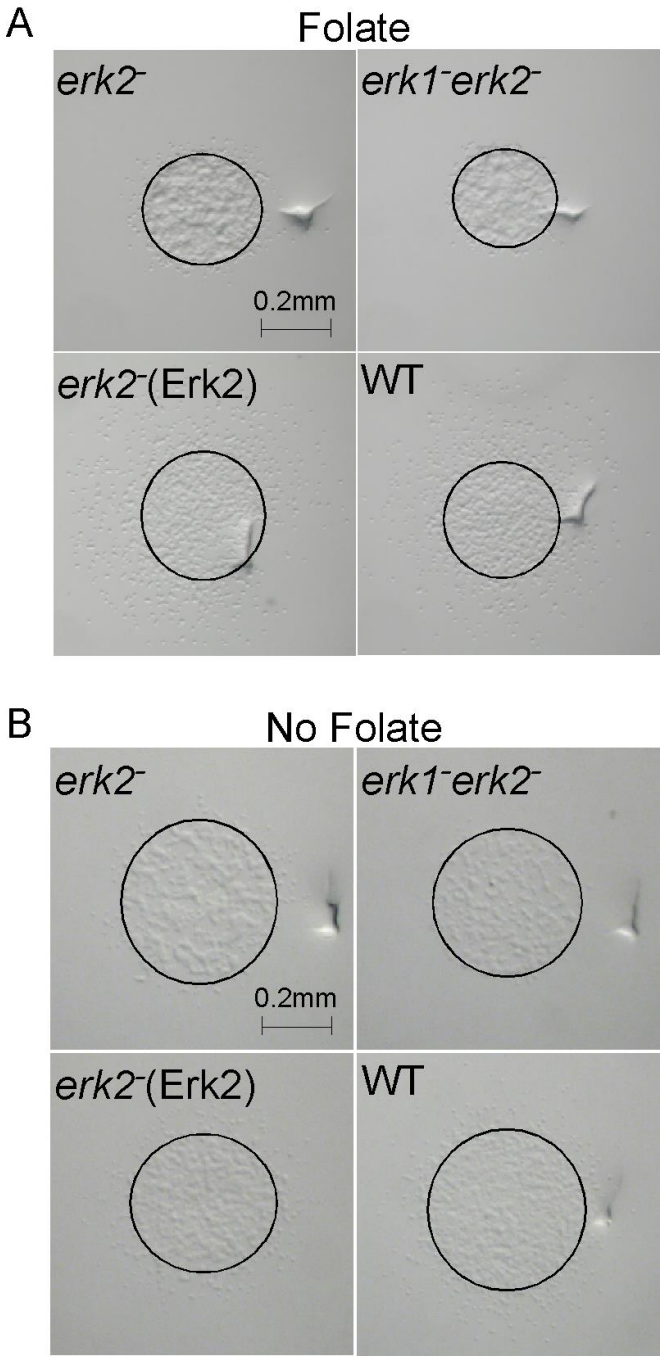

Fig. S3.3. Chemotaxis of MAPK mutants to folate. A) Above-agar chemotaxis assay images for wild-type (WT), *erk2*$^-$, and *erk1*$^-$*erk2*$^-$ strains and *erk2*$^-$ mutants complemented with Erk2 vector (Erk2) after 2.5 hour exposure to droplets of 100 μM folate. Circle indicates original perimeter of cell droplet and the source of folate is located on the top of each image. B) Same assay as (A) except in the absence of folate exposure. All images are the same magnification

Fig. S3.4. Movie of wild-type cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S0898656818300706?via%3Dihub

Fig. S3.5. Movie of *erk2*$^-$ cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.
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Fig. S3.6. Movie of *erk2*$^-$ mutant complemented with Erk2 expression vector cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S0898656818300706?via%3Dihub

Fig. S3.7. Movie of *erk1*$^-$*erk2*$^-$ cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image.
https://www.sciencedirect.com/science/article/pii/S0898656818300706?via%3Dihub

Fig. S3.8. Movie of *erk1*$^-$*erk2*$^-$ mutant complemented with Erk2 expression vector cell movement in the presence of folate. Time-lapse recording of *Dictyostelium* movement over 33 min period with images collected every 20 s. The folate source is oriented at the upper side of the image. https://www.sciencedirect.com/science/article/pii/S0898656818300706?via%3Dihub

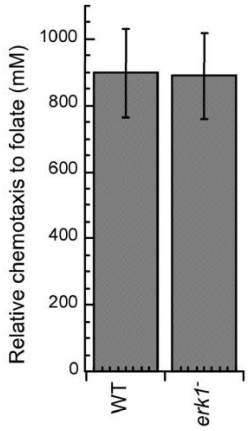

Fig. S3.9. Wild-type (WT) and *erk1*$^{-}$ cell chemotaxis to folate. Folate chemotaxis assays were performed as described in the Methods. Quantitation of wild-type and *erk1*$^{-}$ cell chemotaxis was measured as described for other strains in Fig. 3.4.

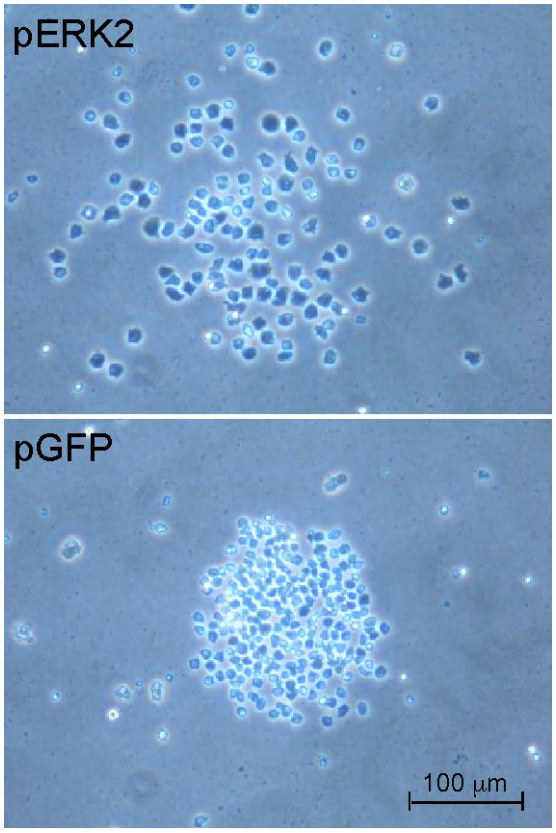

Fig. S3.10. Images of typical *erk1*$^{-}$*erk2*$^{-}$ colonies. The *erk1*$^{-}$*erk2*$^{-}$ cells were electroporated with different expression vectors conferring G418 drug resistance and grown in axenic medium. After several days' images were taken of the drug resistant colonies expressing Erk2 or GFP.

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CHAPTER IV

MAPK REGULATION OF THE REGA PHOSPHODIESTERASE IN *DICTYOSTELIUM*

4.1. Introduction

Phosphodiesterases degrade and regulate the levels of cyclic nucleotides, such as cAMP or cGMP. Therefore, phosphodiesterases play critical roles in many signal transduction pathways. In mammalian systems, eight different genes that typically express several isoforms encode cAMP phosphodiesterases. These isoforms of phosphodiesterases differ with each other in respect to regulation, expression, and intracellular localization. The specificity of isoforms makes these phosphodiesterases critical therapeutic targets in different clinical conditions[1]. This complexity of phosphodiesterase expression presents significant challenges in understanding the function and regulation of specific phosphodiesterases in signaling pathways and designing specific phosphodiesterase inhibitors [2].

In contrast to mammalian systems, the soil amoeba *Dictyostelium discoideum* has only four cAMP phosphodiesterase genes and none of these produce multiple isoforms. Among them, phosphodiesterase RegA is the primary regulator of intracellular cAMP during the developmental life cycle. The loss of the RegA results in accelerated development and precocious spore formation that severely alters the final developmental

morphology. In addition, RegA functions are associated with proper chemotactic movement as well [3–6].

The regulation of intracellular cAMP levels by RegA directly impacts the role of the cAMP-dependent protein kinase (PKA) during development [7–9]. Multiple adenylyl cyclase mediate the production of cAMP during various stages of development, but RegA phosphodiesterase activity reduces the intracellular cAMP levels [10]. RegA protein levels increase during aggregation and remain at a high level until the slug stage [11]. RegA function delays PKA mediated spore formation and loss of RegA results in elevated cAMP levels and precocious spore formation [3, 5]. In this regard, the regulation of RegA plays an important role in the timing of cellular differentiation during the development of *Dictyostelium.*

The phosphorylation of different residues within a phosphodiesterase can up or down regulate phosphodiesterase function [12, 13]. Phosphodiesterase are regulated by upstream protein kinases such as MAP kinases, and by downstream protein kinases such as PKA through negative feedback mechanisms. In mammals, multiple isoforms of PDE4D can be phosphorylated by the MAP kinase Erk2 and a MAPK phosphorylation motif (PXS/TP) has been identified near the C-terminus [14]. Near this phosphorylation site, multiple putative MAPK docking sites include two docking site motifs (D-motifs) with a consensus sequence of (K/R)2-X4-6I/L-X-I/L and another common motif (FXF) [15,16]. Alteration of the downstream D-motif or the FXF motifs resulted in a reduced physical interaction of Erk2 with the PDE4D3 isoform. The mutation also prevented Erk2 down regulation of PDE4D3 in response to epidermal growth factor. Erk2 phosphorylation of PDE4D3 was also attenuated *in vitro* [17]. These MAPK docking sites

are similar to those found on other proteins that can be regulated by MAPKs in a wide range of eukaryotes [18]. These studies were conducted in a cell line transfected with phosphodiesterase expression vectors or conducted *in vitro*, and so the impact of the docking site alterations on cell fate was not examined. The regions of MAPKs that interact with docking sites on other proteins are not well defined but one region suspected to interact with the positively charged regions of docking sites has been referred to as the CD (common docking) region. The CD region of Erk1 (HPYFQSLHDPSDEPI) has two aspartic acid residues (underlined) that are highly conserved with other typical MAPKs. In the analogous region of Erk2 (HPFVTQFHNPAEEPH) these residues correspond to an asparagine and glutamic acid residue. The analogous residues in the human atypical MAPK are cysteine and aspartic acid, respectively, suggesting that atypical MAPKs have some distinctions in this region compared to typical MAPKs. Such distinctions might contribute to the recognition of different docking sites on other proteins [19]. These challenges in investigating phosphodiesterase function can be solved through the genetic analysis of MAPK regulation of phosphodiesterase function in model organisms where the expression of mutant proteins can be regulated through endogenous promoters. The model organism *Dictyostelium* is a simpler system to study for MAP kinase interaction owing to the presence of only two MAP kinases, Erk1 and Erk2.

Earlier studies in *Dictyostelium* have indicated that the atypical MAPK Erk2 is a negative regulator of RegA. The previous characterization of a leaky *erk2*$^{-}$ strain, Erk2^{RE} (reduced expression) indicated this mutant could not aggregate due to insufficient cAMP production [20]. The aggregation of this mutant can be rescued by the loss of RegA function due to the increase in cAMP levels [21]. A putative MAPK phosphorylation site on RegA

(T676) can be mutated to an alanine residue and this prevents Erk2 from downregulating RegA function. Comparison of RegA with mammalian PDE4 shows that both contain putative MAPK docking sites near the C-terminus suggesting that MAPK docking and phosphorylation of phosphodiesterases might be similar in mammals and *Dictyostelium*. The role of a putative MAPK docking site in RegA investigated in this study through genetic alteration of this region. Comparisons of strains with this altered RegA with those containing the wild-type RegA conducted concerning development progression. In the present study, the interactions of Erk2 with the D-motif mutated and wild-type RegA were examined.

4.2. Methods

4.2.1. Strains and mutants

Laboratory grown *Dictyostelium discoideum* KAx3 strain was used as wild-type control strain and as the background strain for making mutants. To make *regA* disruption mutant, a DNA construct made of blasticidin resistance gene flanked by sequences homologous to ends of *Dictyostelium regA* gene was used to disrupt the *regA* gene. The resultant clones were obtained by selection under low concentration of blasticidin. The clones were verified for successful transformation by PCR and phenotype study. For expressing complementary *regA*/*regA*$^{D-}$ under endogenous *regA* promoter, thymidine auxotrophic double mutant (*regA*$^{-}$ *thy1*$^{-}$) was made (Fig. 4.1). The *regA/regA*$^{D-}$ gene integration was confirmed by PCR (Fig. S4.1).

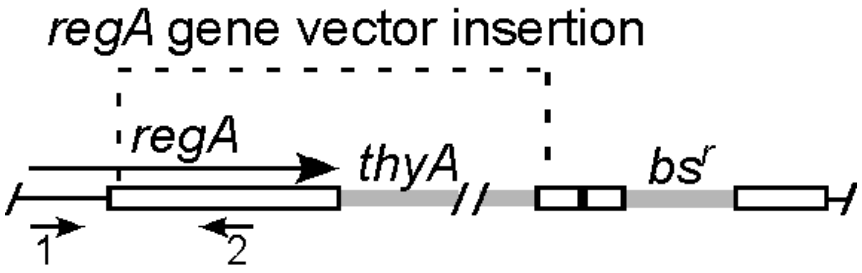

Fig. 4.1. Insertion of complementary *regA/regA*$^{D-}$ gene under endogenous *regA* promoter. Complementary *regA/regA*$^{D-}$gene were inserted at disrupted *regA* locus in thymidine auxotrophic *regA*$^{-}$ *Dictyostelium* cells. Blasticidin resistance construct used for partial disruption of the *regA* gene in thymidine auxotrophic *Dictyostelium* cells. Another construct with an open reading frame of the *regA* gene and consisting of thymidine gene was inserted into disrupted *regA* location to express it under *regA* promoter.

4.2.2. Growth and culture conditions

Cells were grown in HL5 media at room temperature in Petri dishes. Fresh media was changed every 3-4 days to keep the cells from starvation. For shaking culture, growing cells were grown in a glass flask at room temperature overnight until the cell density reaches around 5* 10^6 cells/ml before harvesting.

4.2.3. Cloning and mutagenesis

The cDNA of *Dictyostelium discoideum regA* gene was mutated at the putative MAP Kinase docking motif site in the catalytic domain by PCR based site-directed mutagenesis. The PCR product was cloned into topo vector and verified by DNA sequencing. The verified gene was cloned into the *Dictyostelium* expression vector.

4.2.4. Transformation and selection of clones

Transformation of the *Dictyostelium* cells were carried out by electroporation [22]. The transformed cells were selected under low level of G418 drug. The surviving selected clones were picked onto bacterial lawn Ka plate. The chosen clones, based on

phenotype, were transferred to microwells. Successfully transformed auxotrophic thymidine cells were selected in media without any thymidine added.

4.2.5. Phenotype study and chimera study

Cells were grown in HL-5 medium overnight, in shaking culture at room temperature until the cell density reached reaches 10^6- 10^7 cells/mL. The cells were collected by centrifugation, washed and suspended in phosphate buffer. The cells were plated on nutrient deficient phosphate agar plate at 5* 10^7 cells/mL for development. For chimera study, the cells expressing GFP vectors were suspended at 10^7 cells/mL and mixed with KAx3 wild-type cells at same density in the ratio of 1:10 or higher ratio and allowed for development on phosphate plate.

4.2.6. Immunoprecipitation and western blot

For immunoprecipitation, the cells were grown overnight at Room temperature in HL5 medium until they reach mid-long phase of growth ~5* 10^6 cells/mL. The cells were harvested, washed and suspended at 5* 10^7 cells/mL in phosphate buffer. The cells were starved for three hours and stimulated with 100µM cAMP at every15 minutes interval for the next 3 hours. The cells were collected in ice-cold phosphate buffer at zero, 40 and 70 seconds interval after final cAMP stimulation. The cell pellets were freeze-cracked in buffer with 1% Triton and cocktail of protease inhibitors. The cell supernatant obtained after centrifugation was treated overnight with anti-RegA monoclonal antibodies and protein–A beads in a shaker at 4-degree centigrade. The bead pellets were washed and processed for western blot. The sample was run in 10% SDS gel, transferred to PVDF membrane and treated with rabbit anti-Erk2 primary antibody overnight. The HRP-conjugated anti-rabbit monoclonal antibody was used for the detection of protein bands.

4.3. Results

4.3.1. Putative MAPK docking sites in *Dictyostelium* RegA

An earlier study has suggested that the Erk2 MAPK probably down regulates RegA function through the phosphorylation of the RegA T676 residue. Similar studies have indicated that MAPK binding and phosphorylation occurs in the same region of some mammalian phosphodiesterase. To examine the possibility that MAPKs can also bind RegA, a search for putative MAPK docking sites was conducted on RegA. MAPK docking sites in other organisms have consisted of a K/R (1-2) X (4-6) I/LXI/L motif (D-motif) and in some cases an additional FXF motif. RegA has three potential D-motifs, but only one (residues 588-596) is located near the C-terminus where the potential MAPK phosphorylation site (T676) is determined (Fig. 4.2). Alignment of *Dictyostelium* RegA with mammalian PDE4D indicates the C-terminal putative D-motif is conserved in its consensus sequence in both species and other Dictyostelids (Fig. 4.3). A previously characterized D-motif in PDE4D, associated with mammalian Erk2 interactions *in vivo* and *in vitro*, is located 25 residues closer to the C-terminus does not exist in RegA [17,18,23]. The predicted MAPK phosphorylation site of RegA also has a similar motif in PDE4, but earlier studies have indicated that MAPKs phosphorylate PDE4D at a site 58 residues closer to the C-terminus. An FXF motif was not found near the C-terminus of RegA but is present near the C-terminus of PDE4D. The residues of the putative RegA D-motif or the mammalian PDE4 D-motifs did not include any of the residues highly conserved among phosphodiesterases involved with metal or AMP binding suggesting that these motifs are associated with the phosphodiesterase catalytic domain (Fig.S4.1).

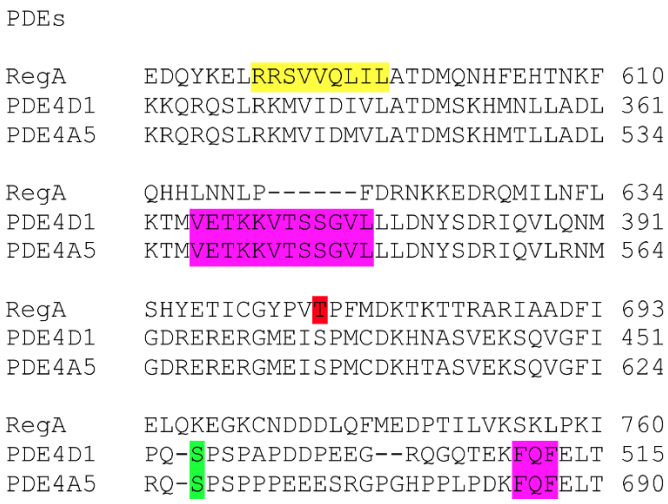

Fig. 4.2. Sequence alignment of catalytic domain of *Dictyostelium* phosphodiesterase RegA with mammalian phosphodiesterases. Mammalian phosphodiesterases has two MAPK docking region, KIM and FQF (both pink) with MAPK phosphorylation amino acid straddled between them (green). *Dictyostelium* RegA has a MAPK D-motif consensus sequence (yellow) similar to KIM docking motif. A MAP kinase consensus docking motif is characterized by positively charged amino acids followed by spacer and hydrophobic amino acids (++XXXXHXH). The threonine (red) in RegA is possibly phosphorylated by MAPK.

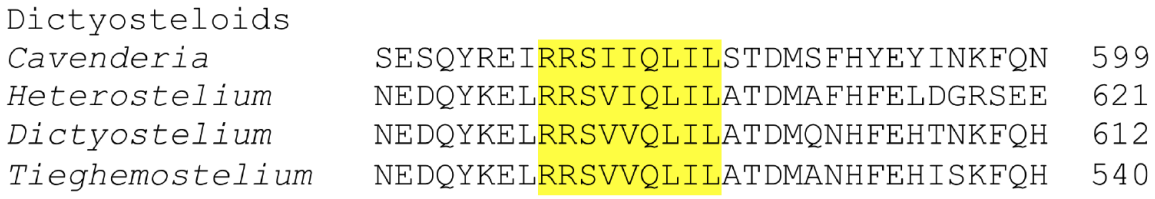

Fig. 4.3. Comparison of RegA sequences between different species of Dictyostelids. Putative MAPK D-motif in *Dictyostelium* RegA is highly conserved across phosphodiesterases.

4.3.2. Over-expression of *regA* with alter D-motif delays development

To investigate the potential role of the C-terminal D-motif in RegA, site-directed mutagenesis was used to replace the arginine and leucine residues with alanine residues. This *regA*$^{D-}$ allele was inserted into both extrachromosomal and integrating expression vectors that used the constitutively active *act15* promoter. The presence of these vectors in *regA*$^{-}$ cells delayed developmental progression similar to that observed for *regA*$^{-}$ cells expressing the wild-type allele from the same expression vectors (Fig. 4.4). The delay in

development is consistent with overexpression of the *regA*$^{D-}$ and wild-type *regA* gene causing an excess of RegA function and lower cAMP levels as previously described. The extent of developmental delay correlated with the level of *regA* overexpression. The overexpression of *regA*$^{D-}$ also corrected the precocious fruiting body formation that occurred in background *regA*$^{-}$ cells (Fig. 4.5). Attempts to identify clones with low copy numbers of integrating vectors by using low levels of G418 drug selection presented were not successful.

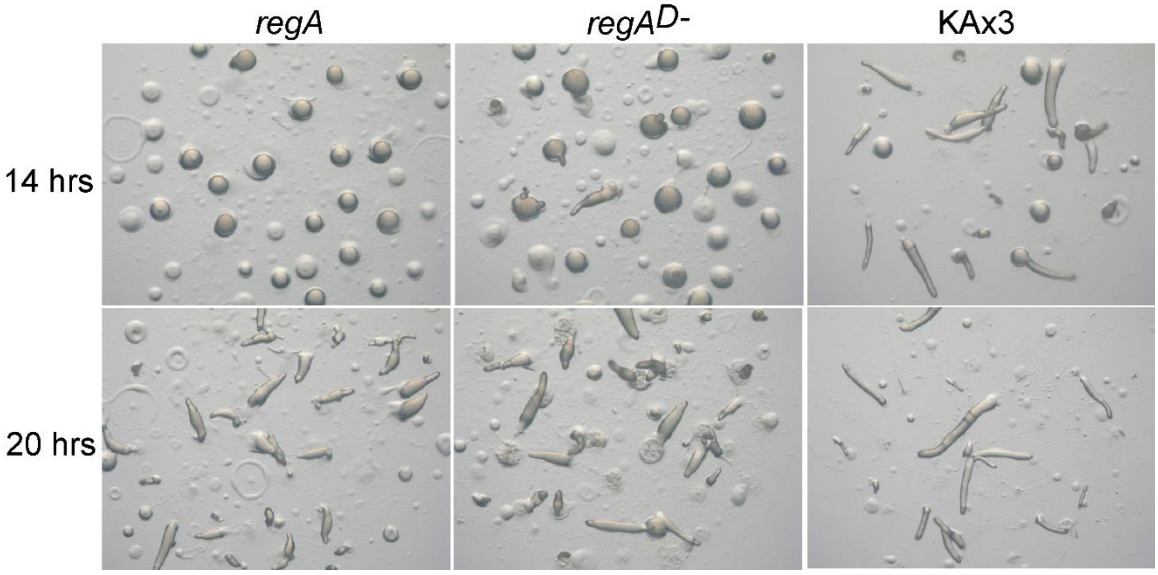

Fig. 4.4. Developmental phenotype of high copy *regA/regA*$^{D-}$ cells. The high copy regA/*regA*$^{D-}$ cells shows delay in development transition from mound to slug stage in comparison to control KAx3 wild type cells. The cells were plated on phosphate agar plate at 5 x 10^7 cells/ml density.

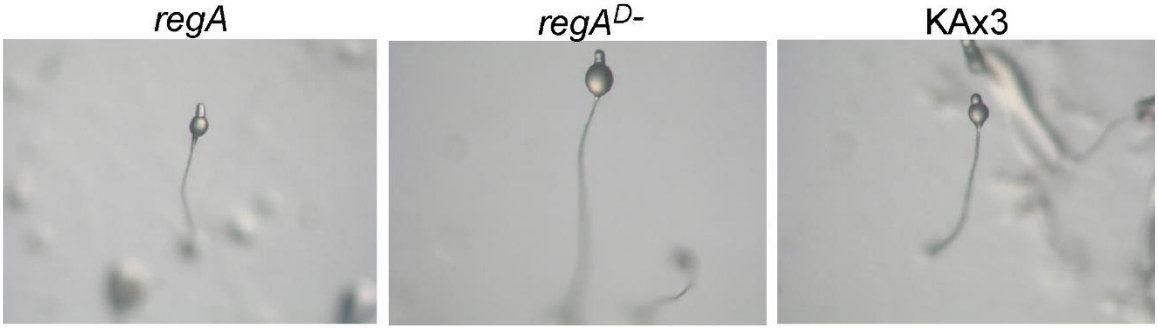

Fig. 4.5. Fruiting body structure of high copy *regA/regA^{D-}* cells. Overexpression of *regA^{D-}* gene in *regA$^-$* background also rescued the precocious fruiting body seen in *regA$^-$* cells. The cells were plated on phosphate agar plate at 5 x 10^7 cells/ml density, and allowed for development. The fruiting body structure is formed around 24-hour time of development.

4.3.3. Expression of *regA^{D-}* from the endogenous *regA* promoter did not rescue the phenotypic defect of *regA$^-$* cells

To reduce the overexpression of the mutant and wild-type RegA proteins, the open reading frames encoding these proteins were inserted into vectors that contained the *thyA* gene and then integrated into the disrupted *regA* locus of *regA$^-$thyA$^-$* double mutants (Fig. 4.1). The single crossover integration allows a single copy of the mutant and wild-type alleles to be expressed from the endogenous *regA* promoter. The reduced expression of the *regA^{D-}* and wild-type *regA* in these strains compared to the parental strain is likely due to the absence of designated transcription termination sequence. However, it seems that the levels of these proteins were sufficient for the RegA protein to provide wild-type morphological development (Fig. 4.6). All subsequent analysis of RegA^{D-} was conducted in these gene knock-in strains and phenotypes were compared to the control strain with the wild-type allele knock-in. The control strain exhibited a slight acceleration of development compared to the parental strain due to the reduced RegA expression. However, the *regA^{D-}* expressing strains displayed small aggregates, precocious spore formation, and aberrant fruiting body morphology similar to *regA$^-$* strains (Fig. 4.7). This phenotype suggests that RegA^{d-} cannot provide sufficient RegA function at the lower expression level. The alteration of the putative D-motif is unlikely to cause a loss of RegA stability because the level of the mutant protein is comparable to the wild-type RegA protein.

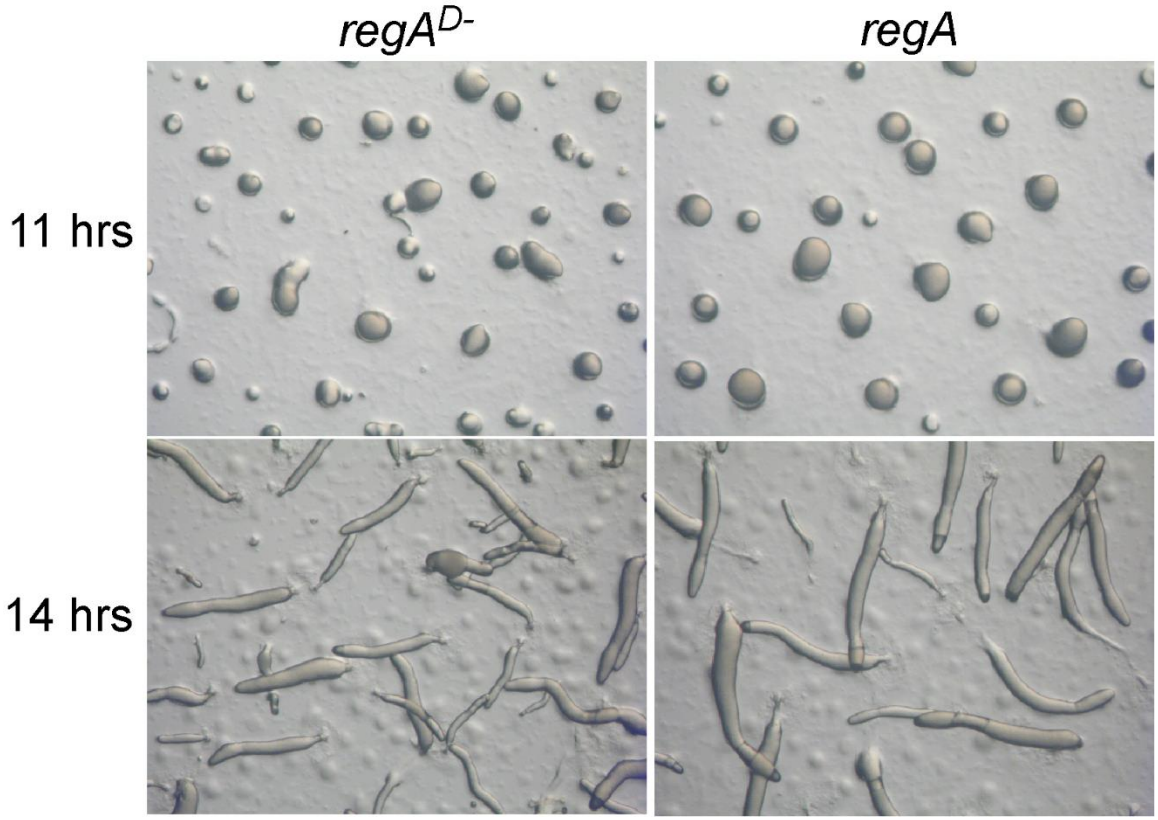

Fig. 4.6. Late stage development phenotype of cells expressing *regA/regA*$^{D-}$ under endogenous *regA* promoter. Development phenotype of *regA/regA*$^{D-}$ cells with complementary *regA/regA*$^{D-}$ genes expressed under endogenous *regA* promoter at *regA* locus. Both of these cells types show similar development rate.

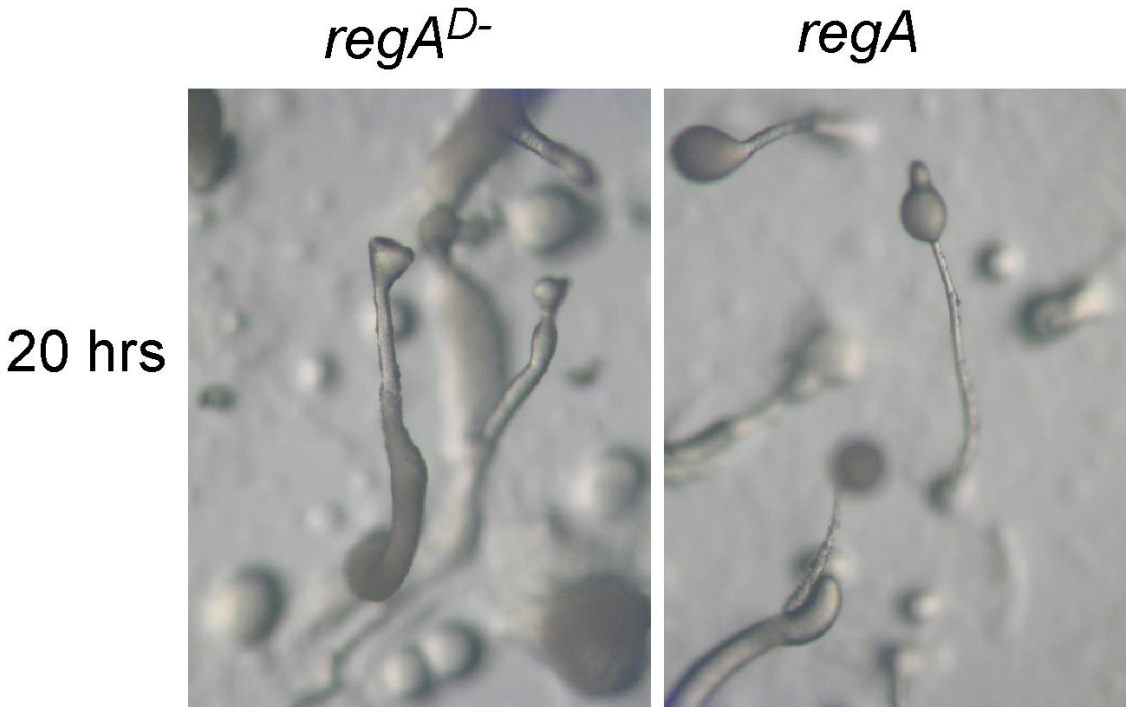

Fig. 4.7. Fruiting body of *regA/regA*$^{D-}$ cells. Expression of *regA*$^{D-}$ gene at *regA* locus cannot rescue the aberrant morphology of *regA*$^{-}$ background cell. The *regA* cells rescue the abnormal fruiting body morphology given by background *regA*$^{-}$ cells. The fruiting bodies are formed early by both type of cell at 20 hour of development.

4.3.4. *regA*$^{D-}$ cells display a bias in their distribution in chimeric aggregates

The aberrant development of *regA*$^{D-}$ expressing cells suggests that these cells might have alterations in cellular differentiation or cell distribution. During the developmental, cells differentiate into prespore and prestalk cells that become spatially localized to the central/posterior and anterior regions, respectively. Prestalk-like cells, referred to as anterior-like cells, are distributed around the core of prespore cells in the central/posterior region. To assess whether the D-motif alteration impacts cell distribution in chimeric organisms, the *regA*$^{D-}$ cells were tagged with GFP expression vector and then mixed with unlabeled control cells. The *regA*$^{D-}$ cells were present throughout the aggregate but the distribution was biased to the anterior region whereas GFP labeled control cells were evenly distributed throughout the chimeric aggregates.

This pattern of *regA*$^{D-}$ cells resembles the distribution of cells expressing phospho-ablative *regA*^{T676A} allele (Kuburich et al., unpublished data). The uneven distribution of the *regA*$^{D-}$ cells in the chimera did not closely match the biased distribution of GFP-labeled *regA*$^{-}$ cells in wild-type cell chimeras (Fig. 4.8).

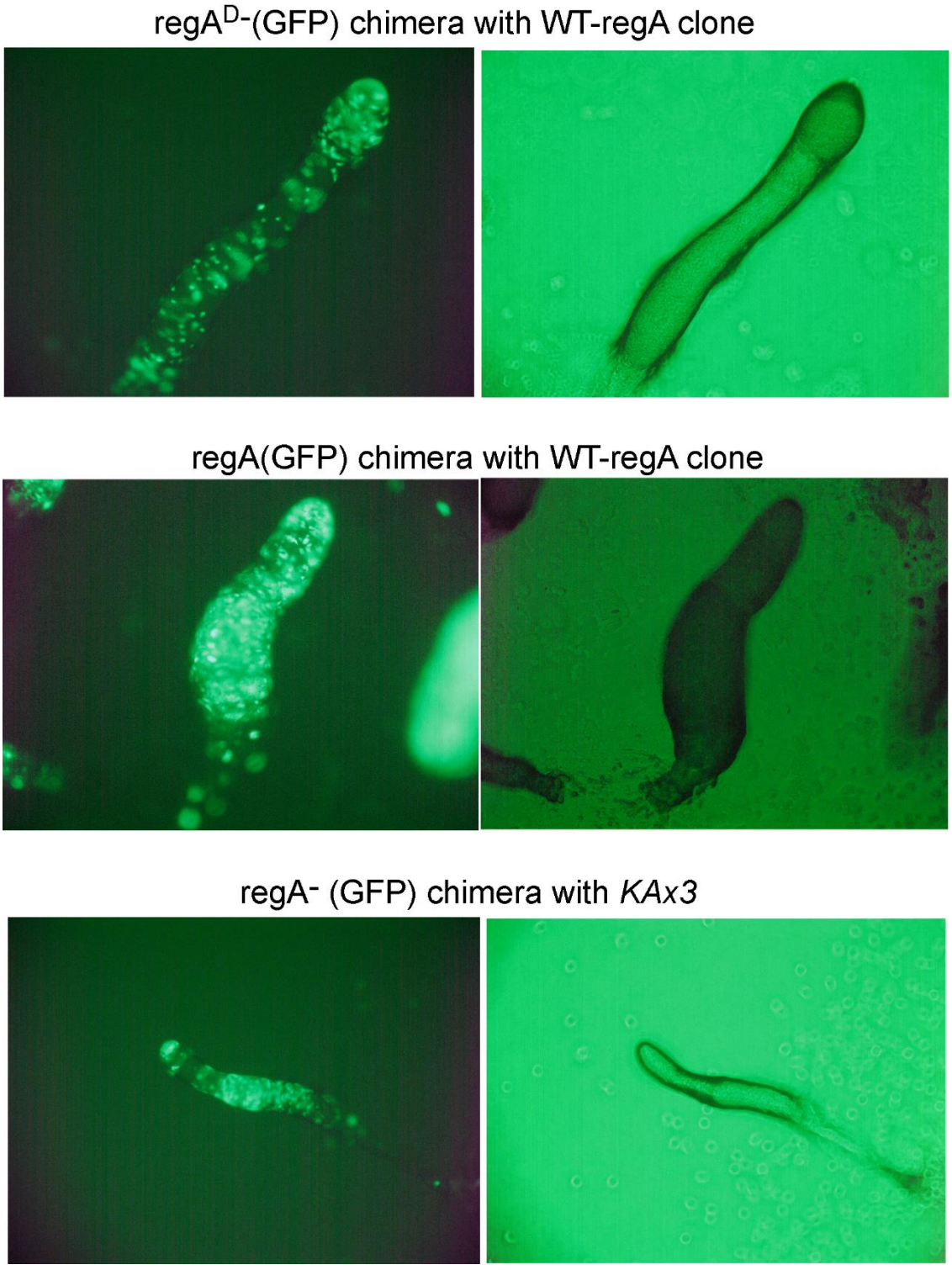

Fig. 4.8. Chimera study of GFP tagged *regA/regA^{D-}* cells. GFP tagged *regA/regA^{D-}* cells with are in chimera wild type *regA* complemented cells to see the cell fate of these cells during development. Cells expressing complementary *regA/regA^{D-}* genes at *regA* locus were tagged with

GFP vector. These cells were mixed with untagged *regA* complemented cells in the ration of 1:10 (GFP-tagged: untagged cells) and allowed for development. GFP tagged *regA*$^{-}$ cells were mixed with KAx3 wild type control cells. *GFP-regA*$^{D-}$ cells show biasness towards the prestalk region of the slug.

4.3.5. Putative D-motif is not essential for Erk2 and RegA interactions

Investigating possibility that Erk2 might interact with the C-terminal putative D-motif of RegA, immunoprecipitates of RegA protein complex were assessed for the presence of Erk2. Cells expressing *regA*$^{D-}$ were starved and lysed after cAMP stimulation and the extracts were immunoprecipitated with antiserum that recognizes a peptide corresponding to the sequence near the RegA C-terminus. Immunoblots of the immunoprecipitates displayed Erk2-specific bands from both *regA*$^{D-}$ and control cells indicating that Erk2 can associate with RegA proteins *in vivo*, consistent with Erk2 regulation of RegA. Unexpectedly, the intensity of the Erk2 bands was significantly higher in extracts from the *regA*$^{D-}$ mutant compared to the wild-type control cells (Fig. 4.9). This result suggests that the RegA putative D-motif is not essential for Erk2 association and that the alterations to this motif increased Erk2 association. This difference in Erk2 association is not due to an increasing level of Erk2 protein in *regA*$^{D-}$ cells as indicated by the comparable levels of Erk2 in both *regA*$^{D-}$ and control cell extracts. The presence of an Erk2 specific slower migrating band was more apparent in the RegA^{D-} immunoprecipitate and cell extract lanes compared to those lanes of the control cells. Reprobing of blots with anti-phosphoMAPK antibody indicated that the higher band did not correspond to the activated form of Erk2.

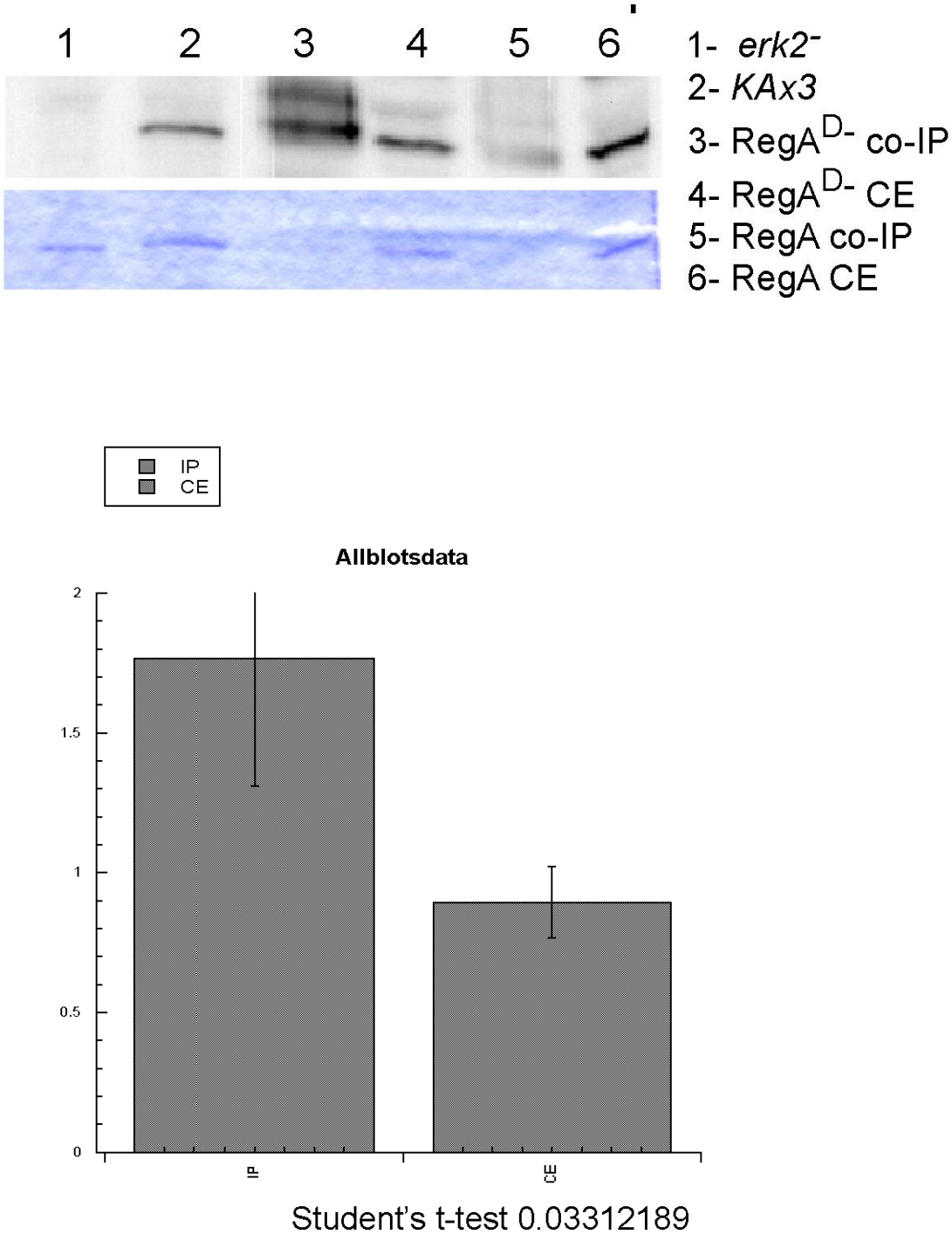

Fig. 4.9. Co-immunoprecipitation of Erk2 with RegA/RegA^{D-} protein. Immunoprecipitate sample obtained by RegA immunoprecipitation using anti-RegA antibody was treated with anti-Erk2 antibody in immunoblot. Sample from *regA*$^{D-}$ cells show higher Erk2 protein band intensity (lane 3) than *regA* complemented cells (lane 5). Lane 4 and 6 are fraction of corresponding *regA*$^{D-}$*/regA* crude extract samples not subjected to immunoprecipitation. The quantitation of ERK2

band intensity ratio ($RegA^{D-}$:RegA) of co-IP sample is significantly higher than in crude samples indicating significant. The quantitation is an average of three different immunoblots.

4.3.6. Erk1 genetic epistasis test shows RegA and ERK1 are likely to function in same pathway

The enhanced interaction of Erk2 with $RegA^{D-}$ compared to RegA suggests that the putative D-motif could potentially reduce interactions with Erk2. One possible explanation for this observation is that the D-motif allows for other proteins to compete with Erk2 for interactions with RegA. Although RegA is down regulated by Erk2 function the putative D-motif might allow for the binding for the other *Dictyostelium* MAPK, Erk1. Loss of RegA or Erk1 results in accelerated development suggesting that Erk1 could be a positive regulator of RegA. To test for epistasis relationships between Erk1 and RegA mutations a *regA*$^-$*erk1*$^-$ double mutant was created and analyzed for developmental phenotypes. When starved the *regA*$^-$*erk1*$^-$ cells formed smaller aggregates than *erk1*$^-$ cells and these aggregates were accelerated in developmental morphogenesis, like that observed for *erk1*$^-$ or *regA*$^-$ cells. The final fruiting body morphology was aberrant, resembling *regA*$^-$ structures but much smaller. These developmental phenotypes suggest that the double mutant displays phenotypes representative of both *erk1*$^-$ and *regA*$^-$ mutations. Overexpression of either *erk1* or *regA* can delay developmental progression and so an extrachromosomal vector expressing *regA* from the *act15* promoter was transformed into *erk1*$^-$ cells. The overexpression of *regA* resulted in larger aggregates and delayed developmental progression compared to *erk1*$^-$ with only endogenous *regA* expression (Fig. 4.10). Likewise, the overexpression of *erk1* in *regA*$^-$ cells delayed development compared to *regA*$^-$ cells with only endogenous *erk1* expression but aggregation size did not appear to noticeably different (Fig. 4.11). These phenotypes

suggest that overexpression phenotypes of Erk1 and RegA are not dependent on the presence of the other protein and that these proteins do not exclusively function in the same signaling pathway.

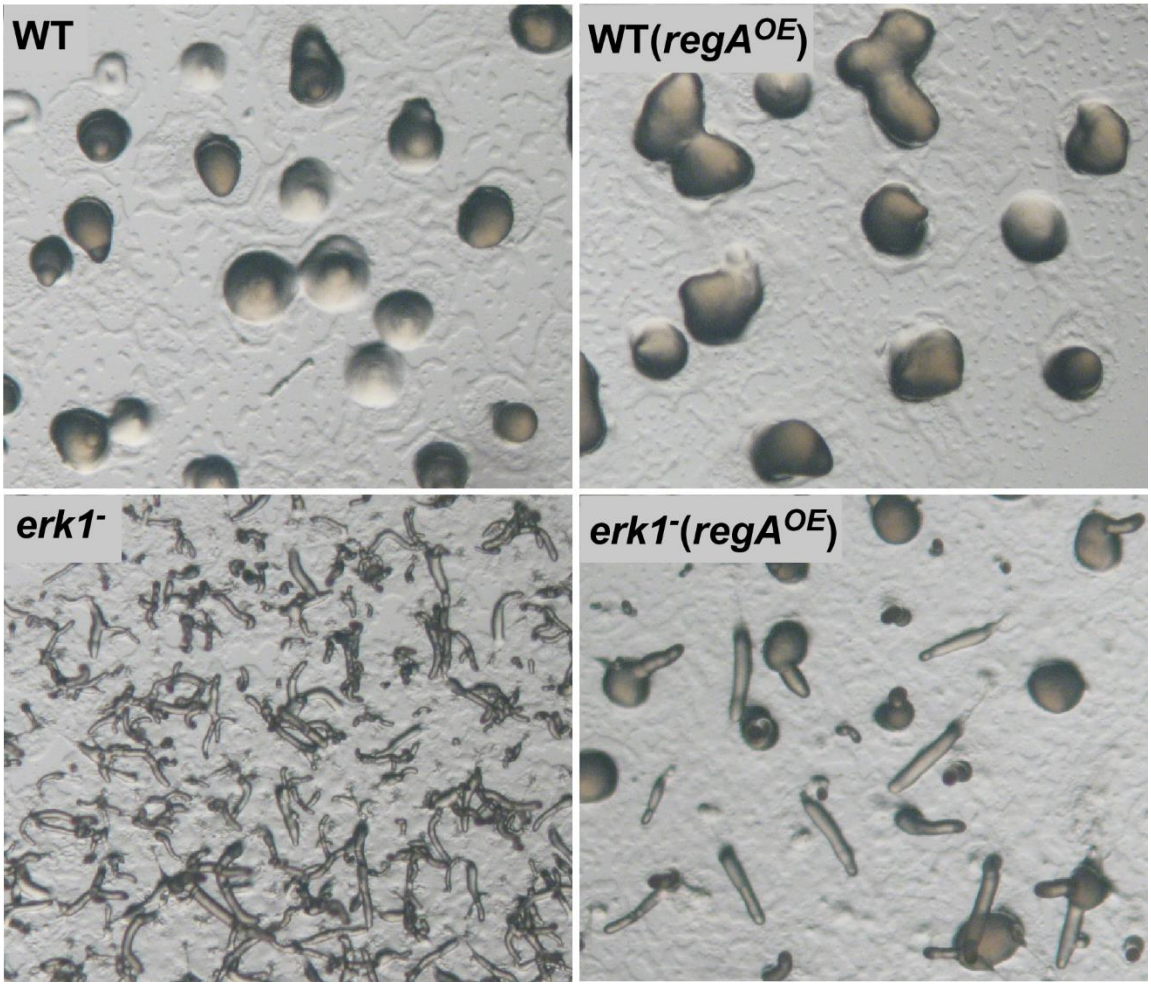

Fig. 4.10. Genetic epistasis test to show relation between *erk1* and *regA*. Phenotype study of WT and *erk1*- cells with or without *regA* overexpression at 12 hours of development. Overexpression of *regA* in *erk1*- cells slowed down the developmental rate with many cells still at the mound stage (lower panels).

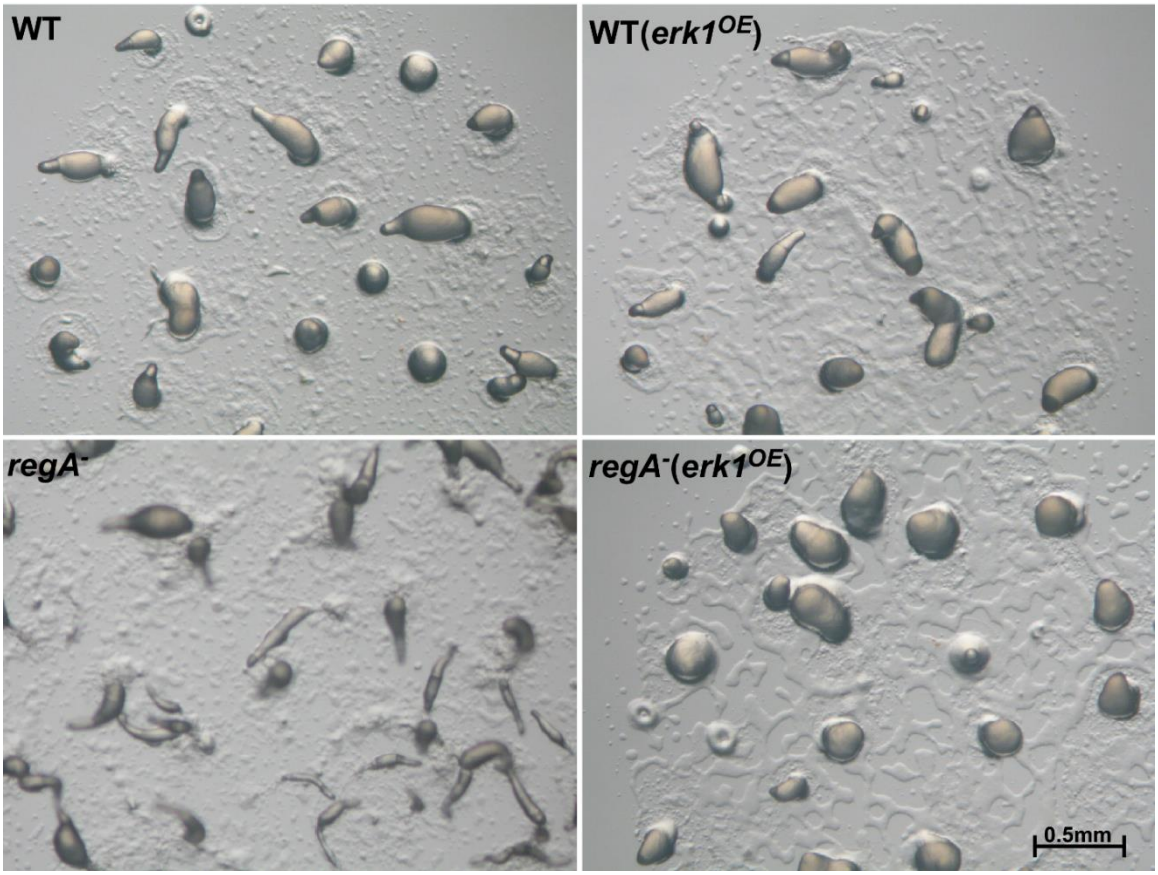

Fig. 4.11. Genetic epistasis test to show relation between erk1 and regA. Development morphology of WT and *regA*$^{-}$ cells with or without *erk1* overexpression at 15 hours. Overexpression of *erk1* in *regA*$^{-}$ cells caused slowing down of developmental rate (lower panels) indicating positive relation between *regA* and *erk1*.

4.4. Discussion

While earlier studies have implicated Erk2 as a negative regulator of RegA, the presence of a putative D-motif near the predicted phosphorylation site could potentially serve as an Erk2 docking site to allow for the phosphorylation and down-regulation of RegA. However, alteration of this D-motif unexpectedly resulted in phenotypes consistent with reduced RegA function rather than up-regulated RegA function that would be expected for a loss of Erk2 interaction. The analysis of wild-type RegA and RegA^{D-} interactions with Erk2 from the co-immunoprecipitation revealed that the D-motif is not essential for Erk2 interactions with RegA. Alterations of the D-motif actually

increased interactions with Erk2 and this observation is consistent with the RegA^{D-} having reduced RegA function, perhaps through Erk2 phosphorylation of the T676 residue. The alterations in the mutated D-motif could potentially create a better docking site for Erk2, but this seems unlikely given that similar changes in mammalian phosphodiesterase D-motifs reduce MAPK interactions. Many other alternative mechanisms could potentially be involved with this regulation, but one attractive possibility is that the D-motif serves as a docking site for Erk1, the only other MAPK in *Dictyostelium* (Fig. 4.12). The presence of Erk1 at the D-motif could block Erk2 access to RegA through some form of steric hindrance. Support for this model is based on several criteria that relate to differences in the two *Dictyostelium* MAPKs. First, loss of Erk1 results in accelerated development that is consistent with Erk1 serving as a positive regulator of RegA function and a negative regulator of cAMP and PKA activity. Second, the activation of Erk1 occurs approximately 2-3 min after the stimulation of cAMP receptors, and this timing corresponds to a drop in cAMP levels[24, 25] Such a role for Erk1 is consistent with this MAPK acting as part of the adaptation response rather than the activation process. Third, the recent discovery of another phosphorylation residue (S142) has an adjacent proline residue that suggests a MAPK could phosphorylate this site and genetic analysis indicates the phosphorylation of this site increases RegA function (Kuburich et al. unpublished data). Fourth, phospho-ablative mutation of the T676 residue generates a phenotype similar to the alteration of the D-motif, including bias in cell distribution during multicellular stages.

The alterations of the D-motif do not produce a *regA*$^{-}$ phenotype when the protein is over-expressed and the high levels of RegA^{D-} might titrate out Erk2 and this could lead

to the inability of Erk2 to negatively regulate some portion of $RegA^{D-}$. Developmental progression is sensitive to the stoichiometry of RegA because overexpression of *regA* leads to developmental delays and the reduced expression of *regA* increases the rate of development. The modified residues of the D-motif are not among the highly conserved residues of the conserved catalytic domain of phosphodiesterase in general, and therefore the alterations of amino acid are not expected to destroy catalytic activity.

The overexpression of RegA in *erk1*$^-$ cells leads to a delay in development suggesting RegA function is downstream of Erk1 function and RegA can mask the accelerated growth of *erk1*$^-$ cells. However, the ability of Erk1 overexpression to delay the precocious development of *regA*$^-$ cells suggests that Erk1 might be involved in other pathways that can mask the elevated cAMP levels and PKA activity in *regA*$^-$ cells.

Erk1 and Erk2 are both MAPKs that can be activated through the phosphorylation of a conserved motif TEY, but sequence comparisons indicate Erk1 is a typical MAPK and Erk2 belongs to a small group of atypical MAPKs. These atypical MAPKs are distinguished by their activation not being facilitated by conventional MAPK kinases that activate other classes of MAPKs [26, 27]. Atypical MAPKs have been demonstrated to phosphorylate different residues in common substrates compared to other MAPKs. The motifs surrounding target residues is unknown but the T676 residue predicted to be phosphorylated by Erk2 has prolines at the -3 and +1 positions resembling motifs typical for MAPK targets. Docking motifs have not been defined for atypical MAPKs and so it is possible that Erk2 does not recognize the C-terminal D-motif in RegA. However, the D-motifs of other MAPKs are highly conserved throughout eukaryotic organisms and so it seems unlikely that the D-motif for atypical MAPKs would be radically different. If

atypical MAPK docking sites are similar to other MAPK motifs then it is possible that Erk1 could compete with Erk2 for binding to D-motifs such as the one in the C-terminal of RegA. It has not been established whether D-motifs are preferentially bound by inactive or active MAPKs because D-motifs exist on both activators (e.g., MAP2Ks) and substrates. Preferences for binding activated MAPKs could allow RegA to bind different MAPKs depending on the stage of signal reception because Erk2 is initially activated and then becomes inactive as Erk1 becomes activated.

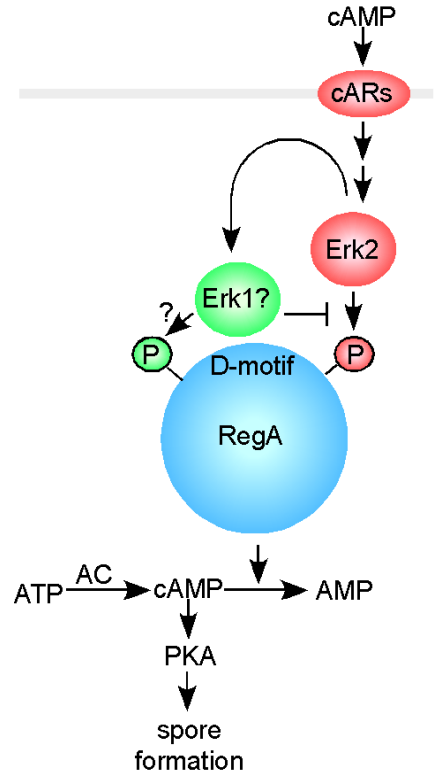

Fig. 4.12. Proposed model for regulation of RegA by Erk1 and Erk2 respectively. Erk1 is activated as a secondary response to Erk2 activation. We propose that activated Erk2 downregulates the RegA first. Later when activated Erk2 subsides, Erk1 is activated and positively regulates RegA.

4.5. Supplementary data

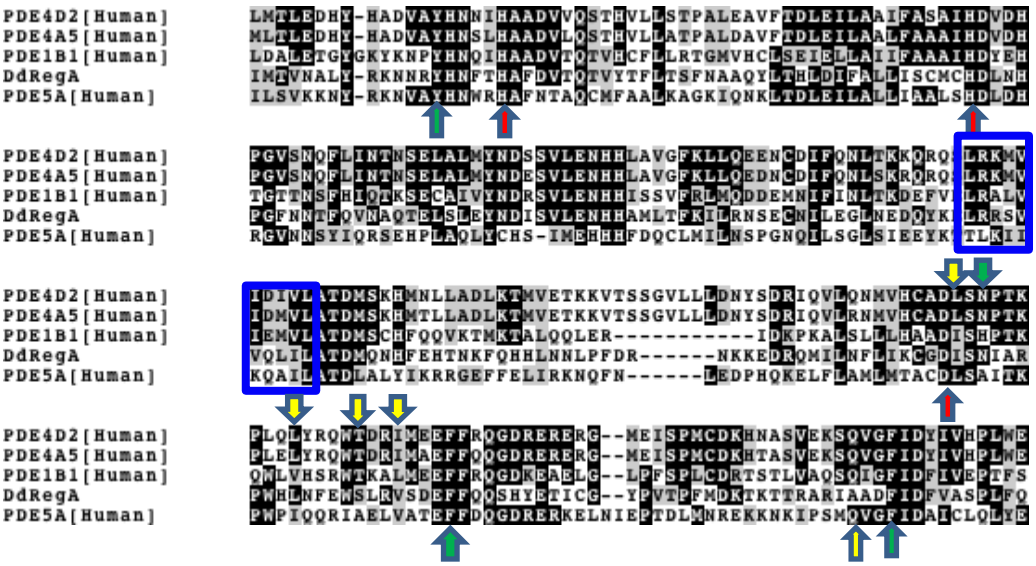

Fig. S4.1. Alignment of catalytic domain RegA phosphodiesterase with different mammalian phosphodiesterases. The sequences in RegA D-motif (encircled in blue rectangle) mutation does not include the highly conserved amino acid residues involved in catalytic activity. Amino acid pointed by Red arrows are metal binding sites, amino acid indicated by yellow and green arrow are AMP binding sites. Alteration of MAP docking site does not include important amino acid residue required for RegA function.

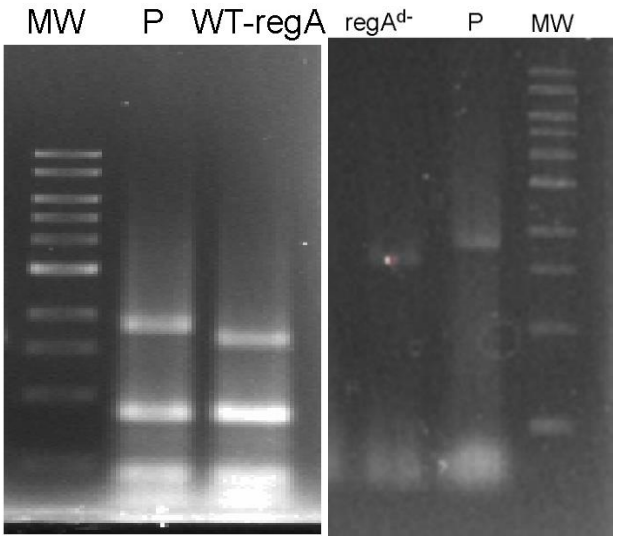

Fig. S4.2. PCR verification of *regA/regA^{D-}* gene integration into *regA* locus. Cells expressing complementary *regA*/*regA^{D-}* genes at *regA* locus in *regA^{-}thy^{-}* background. The integration of *regA*/*regA^{D-}* into *regA* locus is successful as indicated by smaller size of PCR bands than the positive control bands (P) due to absence of introns.

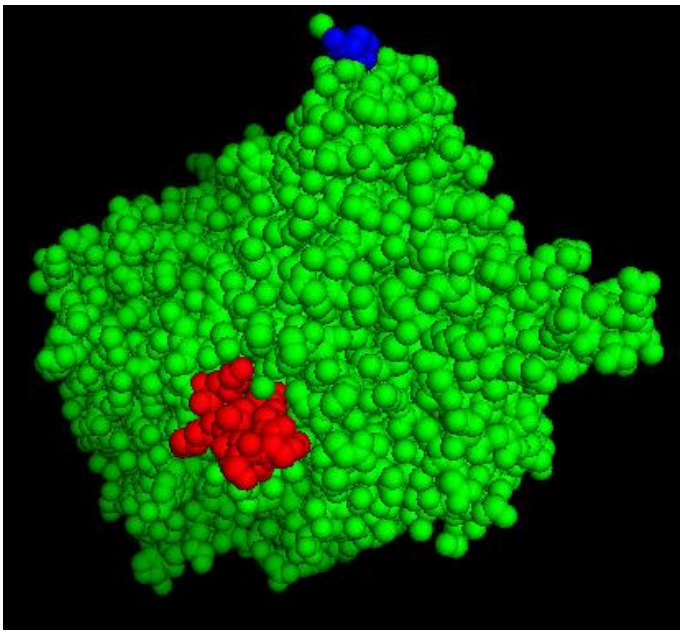

Fig. S4.3. Model of catalytic domain of RegA phosphodiesterase. Area indicated with Red color is the putative MAP kinase docking motif site, while the area in blue color is the region near to the possible MAP kinase phosphorylation site (T676). Homology modeling was done using Swiss-model ExPASy web server.

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CHAPTER V

STUDY OF PUTATIVE MAPK D-MOTIF IN *DICTYOSTELIUM* Gα2 PROTEIN

5.1. Introduction

Heterotrimeric G proteins relay the extracellular stimuli to an intracellular environment in response to the activation of G protein coupled receptors (GPCRs) present on a cell surface. GPCRs and G proteins have important physiological roles as they receive different intercellular and environmental stimuli and relay the signal to the inside of a cell for the response. The source of stimuli includes hormones, neurotransmitters, sounds, light, and different other signals. Three different subunits, α, β and γ make the heterotrimeric G protein. The human genome encodes 21Gα, 6 Gβ and 12 Gγ protein subunits creating a diversity that is important in determining the specificity of the cellular response. Among the three subunits, Gα transitions between inactive GDP bound and active GTP-bound forms in response to the changes occurring in GPCR, which dissociates the heterotrimeric subunits. As a result, the subunits interact with downstream proteins initiating protein-signaling events [1, 2].

In a heterotrimeric G protein, the Gα subunit seems critical for binding G protein complex to GPCR, release, and activation of Gβγ subunit. The Gβγ subunit interacts with

several downstream effectors like ion channels, phospholipases, adenylyl cyclase, receptor kinases, phosphatidyl-3 kinases, and MAP kinase cascades [3, 4]. Several effectors that interact with Gα subunits have been reported. These effectors include kinases, adenylyl cyclases, phospholipases, ion channels, protein phosphatases, and many others. The interaction between Gα and effectors can be both stimulatory as well as inhibitory [5].

Soil amoeba *Dictyostelium discoideum* has 12 different genes encoding Gα subunits and single genes encoding the Gβ and Gγ subunits [6, 7]. Some of these Gα proteins are associated with chemotaxis and spore cell development. Gα4 subunit is required for folate chemotaxis while Gα2 is necessary for cAMP chemotaxis during early aggregation stage of developmental life cycle [8, 9]. The Gα5 subunit has an inhibitory role in folate chemotaxis. In addition to its role in the kinetics of tip morphogenesis. Another subunit, Gα9, also displays inhibitory roles in different intercellular signal response [6, 10–13]. Mutational and overexpression studies have confirmed these functions of Gα subunits. The protein interaction network of the Gα mentioned above proteins, related to their role, is not entirely understood.

The study of this Gα protein revealed that a particular region within these Gα proteins, docking motif (D-motif), is essential for interaction with MAP kinase proteins. These D-motifs might have some physiological roles. Phenotypes studies indicated a change in D-motif or loss of Erk1 function reduced the lethality associated with Gα5 subunit overexpression, suggesting that Gα5 D-motif region is possibly a requirement for Erk1 function [14]. A study of Gα4 indicated that in vivo interactions occur between Gα4 and another MAP kinase protein Erk2. Alteration of D-motif in Gα4 caused loss of the interaction between these two proteins and resulted in a defect in culmination during

development [10]. The interaction between Gα proteins and MAP kinase proteins have confirmed in other organisms including budding yeasts.

In response to mating pheromone yeast *Saccharomyces cerevisiae* Gα protein, Gpa1, binds with the MAPK, Fus3, influencing the chemotrophic response to pheromone. This interaction contributes to the adaptation response to the pheromone by downregulating Fus3. The interaction also promotes cell polarization and gradient tracking. Both active and inactive Fus3 can bind directly to Gpa1 subunit through a MAPK docking site [15, 16]. The *Dictyostelium* Gα2 subunit might be analogous to the yeast Gpa1 because it is required to sense chemical gradients. The Gα2 subunit is necessary for cAMP-mediated chemotaxis during the aggregation process. The Gα2 subunit also has a putative MAP kinase docking near the N-terminal end. *Dictyostelium discoideum* has two MAP kinase proteins, Erk1 and Erk2 [17]. The presence of a putative D-motif point suggests that MAPKs might contribute to Gα2-mediated signaling. We hypothesize that the Gα2 subunit plays a role in the adaptation response after cAMP stimulation during aggregation.

Several point mutations, on both GTPase and helical domains, in *Dictyostelium* Gα2 protein caused loss of Gα2 function. Some of those mutated amino acids were highly conserved. However, previous studies did not include the putative MAP kinase domain at N-terminal end [18]. To study the role of the putative D-motif region in Gα2, we altered the D-motif and examined the resulting phenotype.

5.2. Methodology

5.2.1. Strains and mutants

Dictyostelium discoideum laboratory strain KAx3 is used as the background strain for making mutants and as a wild-type control for our experiments. *gα2* gene knock out mutant strains were made in thymidine axenic strains by using a thymidine construct via homologous recombination. The resulting strains were studied for a phenotype.

5.2.2. Phenotype study

The cells were grown in HL5 medium overnight at room temperature in a shaker incubator until it reached mid-long phase ~ $3* 10^6$ cells/mL. The cells were harvested and placed on nutrient deficient phosphate agar plate at $5* 10^8$ cells/mL density. The development of phenotype was observed for 24 hours after plating onto the agar plate.

5.2.3. Cloning and mutagenesis

Complementary *gα2* gene was mutagenized at the putative D-motif site by using PCR based site-directed mutagenesis (Gene tailor site-directed mutagenesis system by Invitrogen). Forward primer and reverse primers used were 5'-CAACCAATACTGATGCTGCAGCATCTATTGAAAAAGAAAG-3' and 3'-CCTTCCTCTTCGTTGGTTATGACTACGAC-5' respectively. The mutagenized g*α2* gene ($g\alpha2^{D-}$) sequence obtained from PCR were cloned into topo TA cloning vector (Invitrogen) and *Dictyostelium* expression vectors pDXA-GFP2 and bluescript-based plasmid vector under a heterologous *act15* promoter.

5.2.4. Transformation of *Dictyostelium* cells

Gα2 and $G\alpha2^{D-}$ subunit expression plasmids were transformed into $g\alpha2^{-}$ strains by electroporation [19]. Transformed cells were selected by low level of G418 drug or Blasticidin; survived clones were picked up in a microwell plate and studied for phenotype assay and chemotaxis.

5.2.5. cAMP chemotaxis

Actively growing cells were starved for 4-7 hours in phosphate buffer at the density of 10^7 cells/mL. The starved cells were harvested at different interval of time, washed and suspended in phosphate buffer at the same concentration. Small droplets of the cells were plated on phosphate plate, to which small droplets of varying concentration of cAMP were placed about 2 mm away. Pictures of the cell droplets were taken immediately after placing cAMP droplet and after three hours. The motility of cells towards or away from cAMP source was measured by analyzing the picture (Canvas software).

5.2.6. Western blot for Gα2/ $G\alpha2^{D-}$ subunit levels

The cells were grown overnight in HL-5 medium until they reach mid-long phase. The cells were harvested, washed and resuspended in phosphate buffer at $6*10^7$ cells/mL. Cells were plated on whatman filter paper size-50, for development for up to 7 hours. The cells were harvested, lysed with SDS-loading buffer, boiled and ran on 10% SDS PAGE gel. The protein was transferred to PVDF membrane and treated with rabbit anti-Gα2 rabbit serum overnight at 4-degree centigrade. HRP conjugated mouse anti-rabbit polyclonal antibodies used for the detection of Gα2 chemiluminescence protein band.

5.3. Results

5.3.1. *Dictyostelium* Gα2 protein has a putative MAP kinase D-motif

Earlier studies from our lab have shown that several *Dictyostelium* Gα proteins have putative MAP kinase D-motifs [K/R]1-3 - X1-6 –[L/I] – X –[L/I]. The *Dictyostelium* Gα2 protein has a putative MAP kinase docking sequence at its amino-terminal end [14]. The putative D-motif in the Gα2 subunit spans from residue 12 to 21.

The region comprises a positively charged amino acid, lysine, and few hydrophobic residues, isoleucine, and leucine (Fig. 5.1). Other Gα subunits have a D-motif in this same general region. To test the role of the Gα2 D-motif the positively charged residue and the three large hydrophobic residues were substituted with alanine residues. The mutated *gα2* gene was designated as *gα2*$^{D-}$ and the encoded protein is designated as Gα2^{D-}.

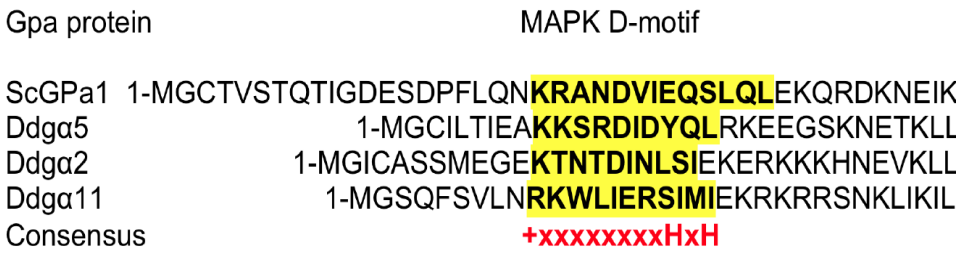

Fig. 5.1. Protein sequence alignment of different *Dictyostelium* Gα subunits and *Saccharomyces* Gpa1. The amino-terminal ends of these proteins have consensus MAPK docking motif. A MAPK docking motif has positively charged core of amino acid, some hydrophobic amino acids downstream of positively charged core, and spacer between the hydrophobic amino acids.

5.3.2. Expression of *gα2* or *gα2*$^{D-}$ genes from high copy number vectors impacts the progression of development

gα2^{-} cells are aggregation deficient and therefore cannot undergo the multicellular stages of development. Expression of the Gα2 or Gα2^{D-} subunit from the constitutively active *act15* promoter in high copy extrachromosomal vectors in *gα2*$^{-}$ background can rescue aggregation. The early development stages were similar to the parental KAx3 controls. The cells started streaming towards aggregation centers after 5-6 hours of starvation, and compact mounds were observed around 10 hours. However, the transition from mounds to slugs and the culmination stage were delayed in comparison to the parental KAx3 cells (Fig. 5.2 and 5.3). Only a small portion of the Gα2 expressing clones

produced fruiting bodies. The analysis of Gα2 subunit levels demonstrated that clones with the high copy number Gα2 and Gα2^{D-} vectors produced comparable levels of Gα2 subunits after four hour of starvation as did the parental KAx3 strain (Fig. 5. 4). The level of Gα2 subunit expression remained constant and comparable to the parental strain during early stages of development (Fig. 5.5).

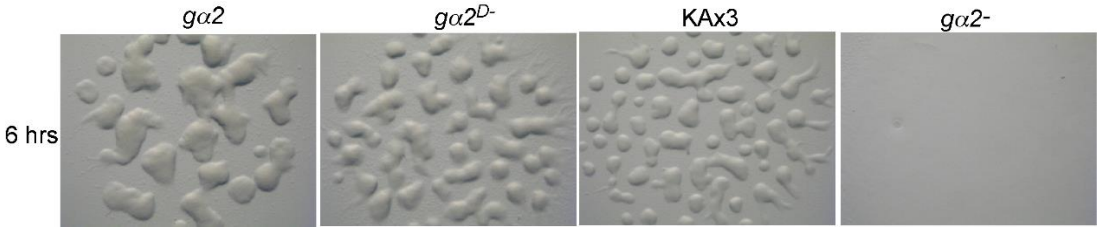

Fig. 5.2. Early stage developmental phenotypes of cells expressing the Gα2 and Gα2^{D-} subunits from high copy number vectors. Control KAx3 and *gα2*$^{-}$ cells with the Gα2, Gα2^{D-}, or no subunit (*gα2*$^{-}$) high copy number expression vectors were plated on nonnutrient plates and images were taken at 6 hour of development. All images are at the same magnification.

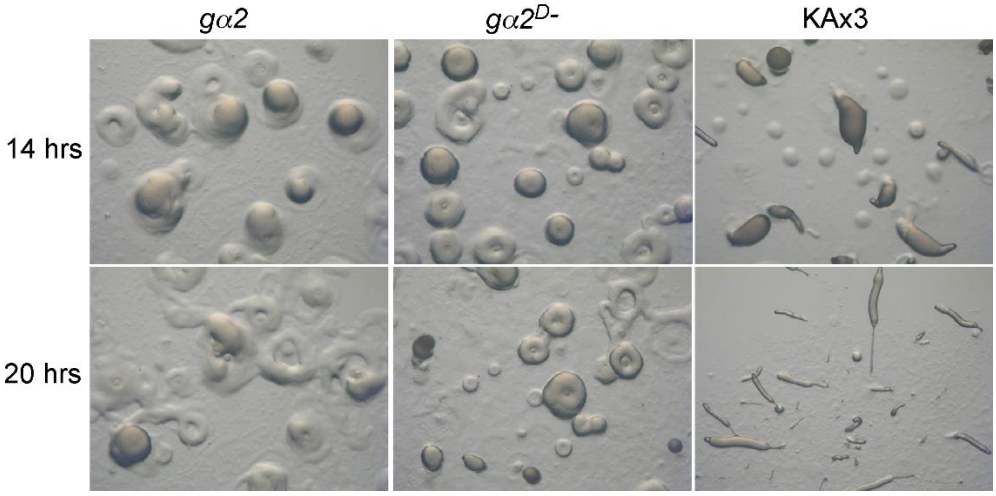

Fig. 5.3. Late stage developmental phenotypes of cells expressing the Gα2 and Gα2^{D-} subunits from high copy number vectors. Parental KAx3 and *gα2*$^{-}$ cells with the Gα2 or Gα2^{D-} subunit high copy number expression vectors were plated on nonnutrient plates and images were taken at 14 and 20 hour of development. All images are at the same magnification.

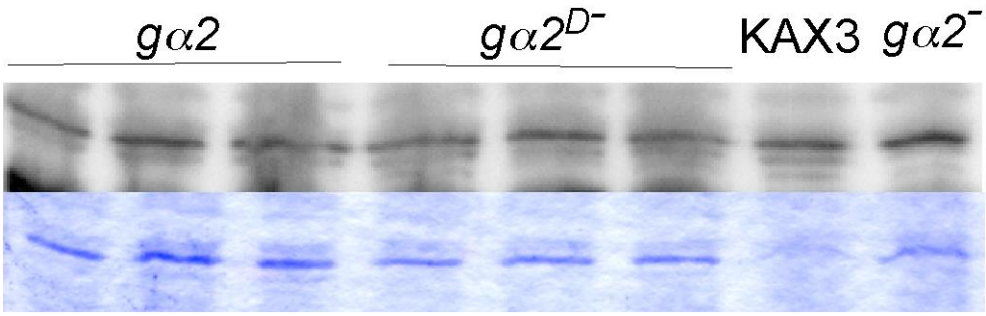

Fig. 5.4. Detection of Gα2 and Gα2^{D-} subunits expressed from high copy number expression vectors. Parental KAx3 and *gα2*$^{-}$ cells with the Gα2 or Gα2^{D-} subunit high copy number expression vectors were starved in phosphate buffer for 4 hours. Three different clones expressing the Gα2 or Gα2^{D-} were analyzed. Cells were harvested and extracts examined by immunoblot analysis using antiserum that recognizes a Gα2-specific peptide near the amino terminus (upper panel). Coomassie staining of the gel was used for a lane loading control (lower panel).

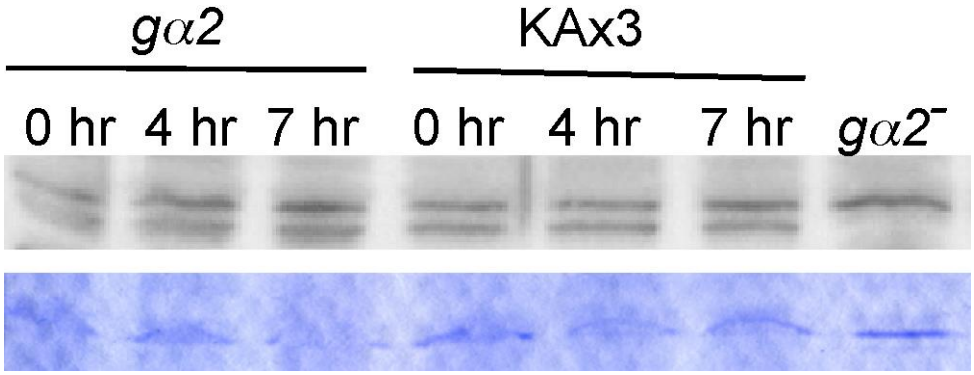

Fig. 5.5. Detection of Gα2 subunit at a different times of starvation. Parental KAx3 cells or *gα2*$^{-}$ cells expressing the Gα2 subunit from a high copy number vector were starved in shaking culture for the times indicated and then extracts were analyzed for Gα2 subunit as described in Figure 5.4. Extracts from *gα2*$^{-}$ cells were used as a control. Coomassie staining of the gel was used for a lane loading control (lower panel).

To determine if the delay or block in multicellular development was due to a lack of Gα2 function or excessive Gα2 function the high copy number vectors were transformed into the parental strain KAx3 that contains a functional *gα2* locus. Expression of the Gα2 or Gα2^{D-} subunit in the parental strain resulted in the delayed progression from mounds to slugs, but the aggregates were capable of completing fruiting body development after 24 hours of starvation (Fig. 5.6). These phenotypes are different

from the those observed when the same Gα2 or Gα2^{D-} subunit expression high copy number vectors were present in *gα2*$^{-}$ cells because the delay in development was approximately only a couple hours.

Fig. 5.6. Developmental phenotypes of parental KAx3 cells expressing the Gα2 and Gα2^{D-} subunits from high copy number vectors. KAx3 cells with or without the *gα2* or *gα2*$^{D-}$ high copy number expression vectors were plated on nonnutrient agar plates and images were taken at 14 hours of development. All images are at the same magnification.

5.3.3. Expression of the Gα2^{D-} protein from a low copy number vector does not rescue aggregation

The *gα2*$^{-}$ cells were also transformed with a low copy (blasticidin selection) integrating vector that expressed the Gα2 or Gα2^{D-} subunit. Clones expressing the Gα2 subunit rescued aggregation and subsequent development without any noticeable delays compared to the parent strain. This observation suggests that the developmental delays associated with the high copy number expression vectors are likely due to excessive Gα2 subunit levels. All clones with the low copy number vector expressing the Gα2^{D-} subunit were not capable aggregation indicating that the Gα2^{D-} subunit can only rescue aggregation when expressed from a high copy number vector (Fig. 5.7).

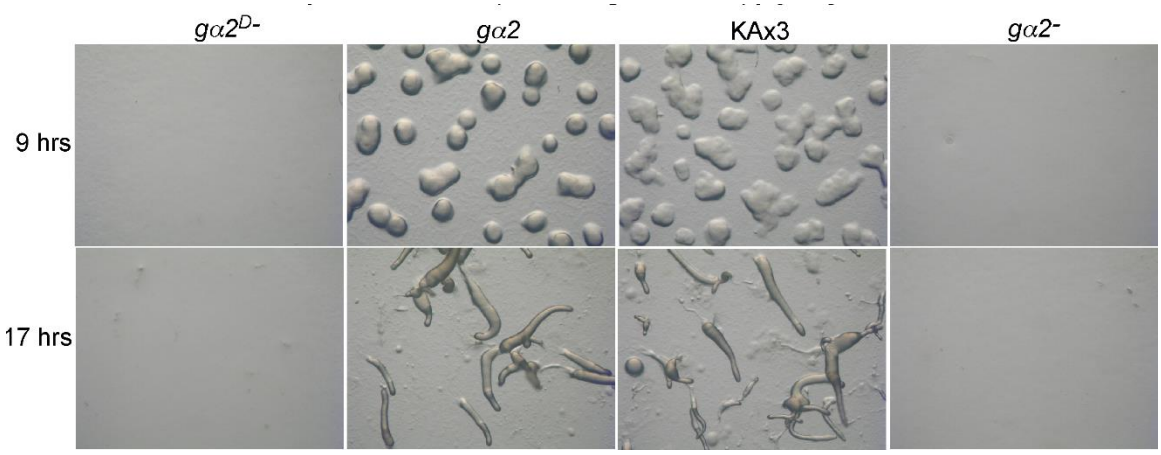

Fig. 5.7. Developmental phenotypes of cells expressing the Gα2 and Gα2^{D-} subunits from low copy number vectors. Parental KAx3 and *gα2*$^{-}$ cells with the Gα2, Gα2^{D-}, or no subunit (*gα2*$^{-}$) low copy number expression vectors were plated on nonnutrient agar plates and images were taken at 9 and 17 hour of development. All images are at the same magnification.

5.3.4. Presence of wild-type cell signaling does not rescue aggregation of cells expressing the Gα2^{D-} subunit from a low copy number vector

The non-aggregating phenotypes of cells expressing the Gα2^{D-} subunit could possibly result from the inability to respond to or produce extracellular cAMP. Previous studies have shown that some aggregation defective mutants (e.g., mutants with reduced Erk2 expression) do not aggregate as a clonal population due to insufficient extracellular cAMP signaling [20]. However, other aggregation deficient mutants (e.g., *gα2*$^{-}$, or *gβ*$^{-}$ null mutants) do not aggregate because they do not show chemotaxis to cAMP [21]. To determine whether cells with the Gα2^{D-} subunit can respond to wild-type cAMP signaling during aggregation, these cells were labeled with a GFP expression vector and mixed with the wild-type parental strain. The starvation of this chimeric population resulted in aggregates, but most cells expressing the Gα2^{D-} subunit were not included in the aggregates, suggesting that these cells do not respond to extracellular cAMP signaling. In some aggregates a small number of Gα2^{D-} subunit expressing cell or *gα2*$^{-}$ cells are

observed as part of the aggregate and this is likely due to these cells being carried along with the wild-type cells. In contrast to the Gα2^{D-} subunits cells, cells expressing the Gα2 subunit robustly participated in the formation of chimeric aggregates with the wild-type parental strain (Fig. 5.8).

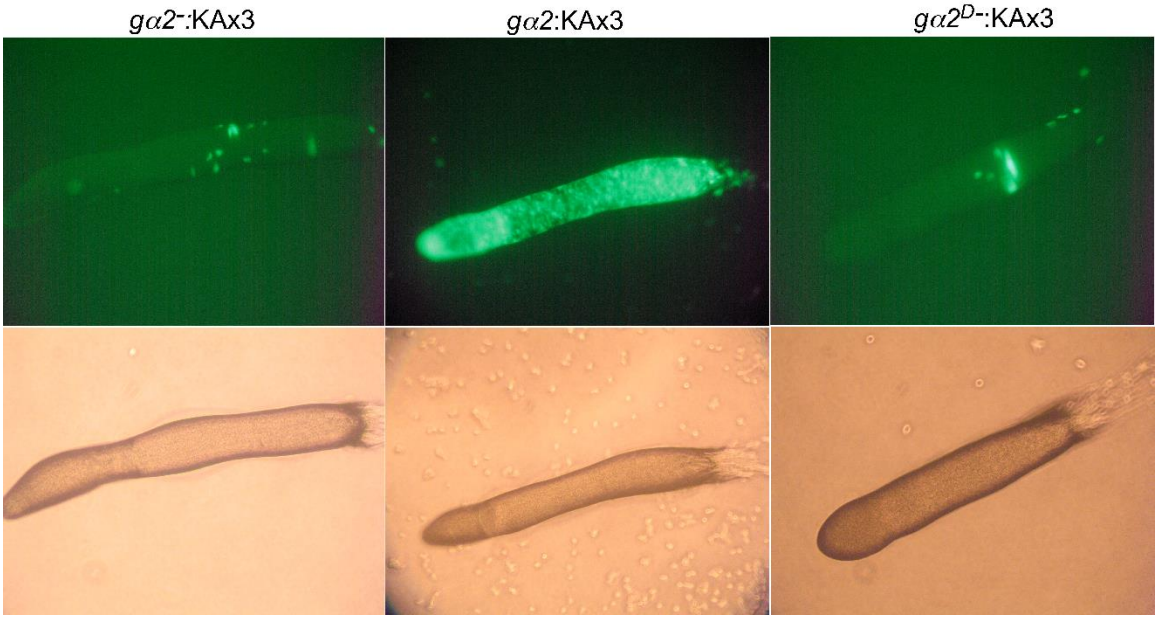

Fig. 5.8. Development of chimeric populations and cell fate. The chimeric populations consist of KAx3 cells and GFP-tagged *gα2*$^{-}$ cells expressing the Gα2 or Gα2^{D-} subunits from low copy number vectors. KAx3 cells were mixed at a ratio of 10:1 with GFP-tagged *gα2*$^{-}$ cells expressing the Gα2, Gα2^{D-}, or no subunit from low copy number vectors and plated on nonnutrient agar and fluorescent images were taken at 16 hour of development. Anterior of slugs located on the left and all images have the same magnification. Brightfield images (lower panels).

5.3.5. Low-copy *gα2*$^{D-}$ cells exhibit increased cell movement

To examine cAMP chemotactic responses, cells with the low copy number vectors expressing the Gα2 and Gα2^{D-} subunits were subjected to above agar cAMP chemotaxis assays. After 4 hour of starvation both Gα2 and Gα2^{D-} subunit expressing cells displayed cell movement in the presence of cAMP in comparison to *gα2*$^{-}$ cells (Fig. 5.9). However, only the Gα2 subunit expressing cells displayed a bias in their movement

up a gradient of cAMP. In control assays without an exogenous cAMP gradient the Gα2^{D-} subunit expressing cells displayed comparable movement as in the presence of cAMP but the Gα2 subunit expressing cells displayed less random movement in the absence of the exogenous cAMP (data not shown). These results suggest that the low-copy *gα2^{D-}* cells have robust cell movement but this movement is not directed by exogenous cAMP stimulation.

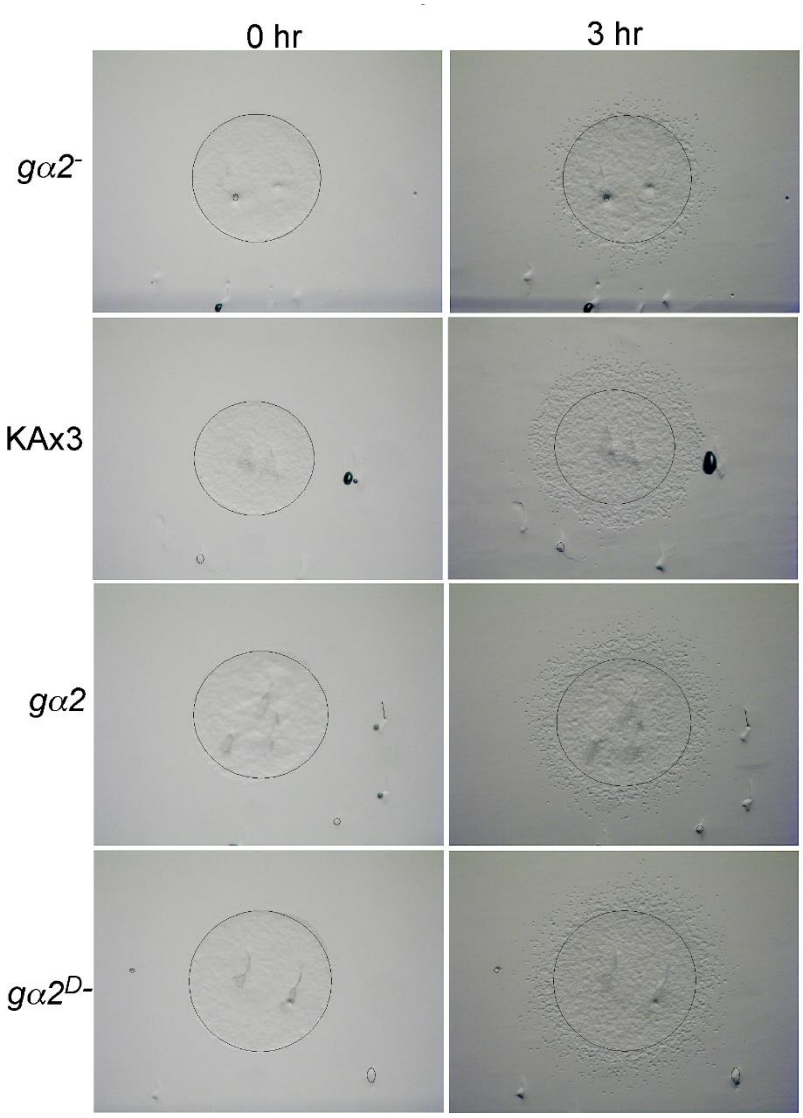

Fig. 5.9. Above agar cAMP chemotaxis assays. Parental KAx3 and *gα2^{-}* cells expressing the Gα2, Gα2^{D-}, or no subunit from low copy number vectors were starved for 4 hours in shaking culture and plated on nutrient deficient phosphate plates with droplets of 100µM cAMP placed

near 2 mm. Source of cAMP is located at the upper side of all images. Images were taken at 0 and 3 hours after the addition of cAMP.

5.4. Discussion

Both active and inactive Fus3 MAP kinase binds to the Gα subunit of the G protein involved with the mating response of the yeast *Saccharomyces cerevisiae*. However, the absence of the D-motif on the Gpa1 subunit reduces adaption to pheromone stimulation and impairs gradient tracking of chemotropism [16]. If *Dictyostelium* cAMP chemotaxis during developmental aggregate formation uses a similar signaling mechanism, then alteration of the Gα2 putative D-motif might be expected to interfere with adaptation processes and gradient perception. The ability of high levels of the Gα2 and $G\alpha2^{D-}$ subunits to rescue aggregation ability of $g\alpha2^{-}$ cells indicates that heterologous expression from the *act15* promoter provides sufficient Gα2 function at this stage of development. The developmental delay associated with mound to slug transition with a high copy but not low copy Gα2 subunit expression vectors suggests that excessive Gα2 function impairs some process with this stage of development.

Interestingly, early developmental expression of Gα2 from high copy number vectors does not indicate excessive Gα2 protein levels. This finding is surprising given that many genes expressed from the same *act15* promoter typically have much higher protein levels compared to the parental strain. Others have suggested that a translational or post-translational regulatory mechanism might limit production of the Gα2 subunit (B. Gundersen, personal communication). The endogenous *gα2* transcript levels rapidly decline before the transition into slugs, and so it is possible that expression from the constitutive *act15* promoter on high copy vectors produces excessive Gα2 subunit at this stage [22]. Excessive Gα2 subunit after aggregate formation might potentially impact

signaling the cAMP receptor Car2 that mediates the transition of mounds to slugs. Gα2 has been shown to interact with all four cAMP receptors (Car1 - Car4) but perhaps Gα2 only functionally couples with Car1 and Car3 that mediate the aggregation process. Other related Gα subunits such as Gα1 and Gα5 subunits might mediate signals through the Car2 and Car4 receptors that function in later stages of development.

High copy number vectors expressing the $G\alpha2^{D-}$ subunit, but not the low copy number vectors, rescued the aggregation deficiency of *gα2*$^{-}$ cells. These observations suggest that low-level expression of the $G\alpha2^{D-}$ subunit does not provide sufficient Gα2 function. The lack of Gα2 function could result from the decreased stability of the $G\alpha2^{D-}$ subunit. However, the level of $G\alpha2^{D-}$ and Gα2 were similar when expressed from high copy number vectors. Alternatively, the interaction of the $G\alpha2^{D-}$ subunit with other proteins, such as MAPKs, might be reduced due to the alteration of the D-motif and that overexpression of the $G\alpha2^{D-}$ subunit might increase such interactions. In support of this latter possibility, the low level of $G\alpha2^{D-}$ subunit expression increased cell movement on agar compared to *gα2*$^{-}$ cells suggesting the aggregation defect of *gα2*$^{D-}$ cells is not due to the absence of Gα2 function. The increased cell movement associated with the $G\alpha2^{D-}$ subunit in the lack of exogenous cAMP compared to Gα2 expressing cells, or wild-type parental cells, suggests the $G\alpha2^{D-}$ subunit might not be capable of down regulating cell movement. Such a deficiency might keep cells from regaining sensitivity for the chemotactic signal, and therefore the cells might remain to be incapable of reassessing the direction of a chemotactic gradient. In support of this idea, preliminary analysis of an Erk2-specific translocation reporter suggests that Erk2 phosphorylation of the transcription factor GtaC remains constitutive in cells with the low copy vector

expression of the Gα2^{D-} subunit. Erk2 is activated during the first 2 minutes of cell stimulation with a chemoattractant, the period that coincides with rapid cell movement. After this short period, Erk2 becomes inactive in wild-type cells, and the other MAPK Erk1 becomes active during the adaptation response. This latter period is associated with random pseudopod extensions without net chemotactic movement. Therefore, it is possible that the Gα2^{D-} subunit is compromised with Erk1 interactions that might facilitate the adaptation response. In addition, Erk2 is required for cAMP chemotaxis, but Erk1 is not [23].

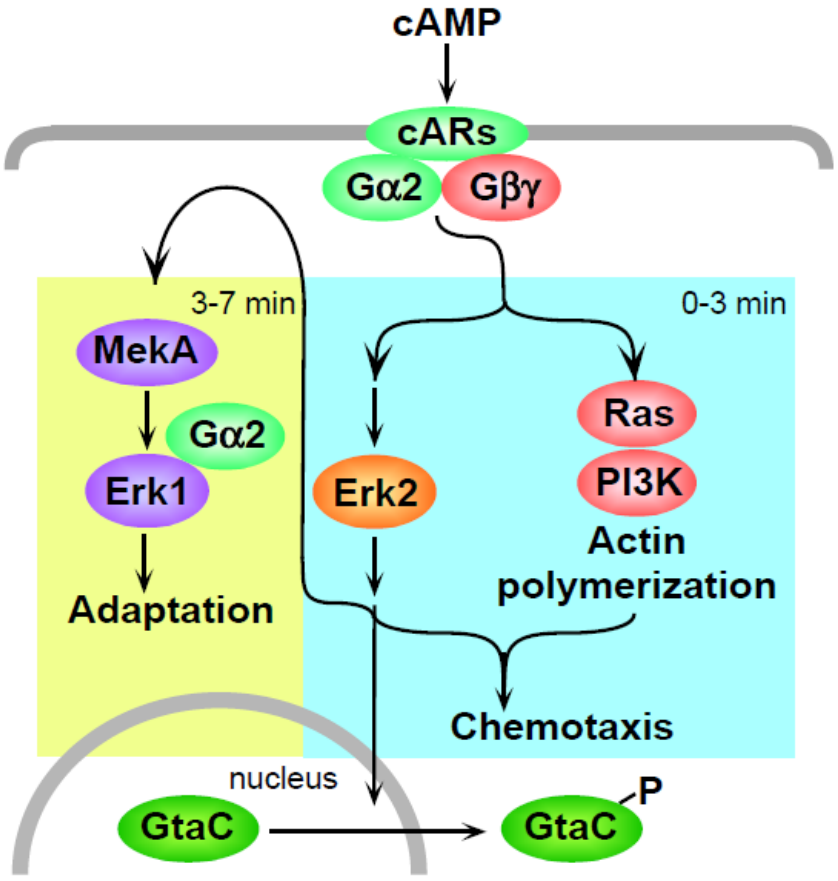

Fig. 5.10. A model representing possible role of *Dictyostelium* Gα2 protein in chemotaxis. Gα2 probably interacts with one of the MAPK protein via its D-motif for cAMP adaptation response.

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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

The focus of this research was to investigate the role of some important proteins in intracellular cell signaling during chemotaxis and development of *Dictyostelium discoideum*. *Dictyostelium discoideum* is a non-pathogenic amoeba used as a model organism to study chemotaxis and phagocytosis, processes also common to human neutrophils. *Dictyostelium* is also model system for understanding some aspects of developmental biology [1, 2].

Dictyostelium also serves a model to study pathogenic amoeba. In chapter two, we compared the chemotaxis between *Dictyostelium* and pathogenic *Acanthamoeba* during foraging. We found that their foraging strategies are different. *Acanthamoeba* shows robust random motility. The gradient dependent directional chemotaxis to folate and bacterial sources seems to be absent suggesting that they deploy random motility and dispersal as a foraging strategy. However, we used just a few types of bacteria and chemoattractants in our experiments. Therefore, a different foraging strategy may still be present in *Acanthamoeba*. The lack of a chemotactic foraging strategy in *Acanthamoeba* probably arises from this organism not being a social amoeba, like *Dictyostelium*. *Dictyostelium* exhibit strong chemotaxis to cAMP, allowing cells to form multicellular

aggregates during starvation. *Acanthamoeba* lacks such a chemotactic process because it develops as solitary amoeba. The possible absence of a Gβ subunit in the *Acanthamoeba* genome supports this difference. *Dictyostelium* Gβ disruption mutants do not show chemotaxis [3]. The non-responsiveness of *Acanthamoeba* towards chemoattractants may be a consequence of not having a Gβ subunit. However, the presence of RACK orthologs and other WD-repeat-containing proteins might allow other G protein-mediated signaling to occur in *Acanthamoeba*. Alternatively, incomplete sequencing or errors might have prevented to identification of a Gβ subunit in *Acanthamoeba*. The lack of molecular tools to explore *Acanthamoeba* genetics also limits the understanding of the differences between *Dictyostelium* and *Acanthamoeba*.

Dictyostelium MAP kinases, Erk1 and Erk2, have several biological roles. Both of these MAP kinases share 37% structural similarity. Erk2 is essential for cAMP and folate-mediated responses. Lowering of Erk2 expression causes the inability of *Dictyostelium* cells to aggregate and develop [4]. Previous Erk2 mutational studies were based on a leaky *erk2* mutation that reduced but did not eliminate Erk2 function. These cells cannot aggregate, as they could not produce sufficient cAMP. These mutant cells displayed chemotactic movement, therefore, could be rescued by the presence of cAMP produced from wild-type cells. Our study included a complete disruption of the *erk2* gene. The disruption of the *erk2* gene leads to the total loss of chemotaxis to folate and cAMP. Conventional MAP kinase pathways involve a cascade of kinases, consisting of a three-tiered set of kinases. Phosphorylation by upstream kinases activates the downstream kinases to transmit signals from surface receptors[5]. Atypical MAP kinases are not activated by conventional upstream kinases. Some atypical MAP kinases, unlike

typical MAPKs can activate themselves by auto-phosphorylation in heterologous systems [6]. Only one MAPK kinase (MAP2K), MEK1, is found in *Dictyostelium* [7, 8]. Our study revealed that MEK1 regulated Erk1 but not the Erk2 activation. Protein alignment studies revealed that *Dictyostelium* Erk2 is closely related to the atypical MAPK Erk8 in humans. Our study also showed that phosphorylation of Erk1 is dependent on Erk2 activation in response to folate stimulation.

We studied the regulation of the cAMP-specific phosphodiesterase RegA by MAP kinases. MAP kinases often interact with substrates by binding a D-motif site and then phosphorylate threonine/serine residues [9, 10]. Our study revealed a putative MAP kinase D-motif in RegA phosphodiesterase. Previous genetic epistasis tests with a leaky *erk2*$^{-}$ mutant have revealed that Erk2 might be down regulating the RegA function. The same study showed that alteration of specific threonine residue, T676, changed the levels of cAMP significantly [11]. These data suggest that Erk2 down regulates RegA function. However, we cannot rule out that Erk1 might be interacting with RegA as well. The alteration of D-motif in the *regA* gene, *regA*$^{D-}$, expressed at physiological levels did not rescue the aberrant fruiting body morphology of *regA*$^{-}$ cells. In contrast, high-level expression of *regA*$^{D-}$ rescued the fruiting body phenotype. These results suggest that alterations in the D-motif possibly affect interactions between MAPKs and RegA. However, the co-immunoprecipitation study showed that mutation in D-motif does not reduce affinity between Erk2 and RegA. Several *Dictyostelium* mutants, including *regA* disruption mutant, show high PKA activity. The high PKA activity is said to be responsible for early sporulation leading to fruiting body phenotype defects in *regA*$^{-}$ cells[12]. Phenotypic similarities between *regA*$^{D-}$ and *regA*$^{-}$ cells indicate the possibility of

high PKA and cAMP activity in cells expressing *regA*$^{D-}$ at physiological levels. Further experiments are needed to measure the PKA activity and cAMP levels in these cells. The *regA*$^{D-}$ cells, expressing altered *regA* gene at a physiological level, were preferentially localized in the pstAO region of chimeric slugs with wild-type *regA* complemented cells. The pstAO region is represented by the *ecmAO* marker gene expression driven by high PKA activity [12, 13]. The localization of *regA*$^{D-}$ cells in the pstAO region is consistent with high PKA activity in these cells.

Fast development and aberrant fruiting body formation of *regA*$^{D-}$ cells suggests the possibility that RegA^{D-} is a nonfunctional protein. However, the rescue of proper development in high copy clones argues against this idea. It is also possible that the alteration of a putative D-motif site in the catalytic domain might have altered the catalytic function of RegA. Sequence alignment studies show that putative D-motif lies near to highly conserved AMP and metal binding residues. Therefore, changes in the D-motif site might affect the RegA catalytic activity. High RegA levels in high copy *regA*$^{D-}$ cells might compensate such reduction in RegA function by the overexpression of RegA^{D-} protein. The D-motif site alteration does not include the important amino acid residues involved in catalytic function. Potential instability of the RegA^{D-} protein is also another factor that might explain the phenotypes. Immunoblot studies show the presence of significant levels of RegA^{D-} protein in *regA*$^{D-}$ cells, comparable to wild type *regA* complemented cells, indicating that the alterations do not affect the stability of the mutant protein. The precocious fruiting body phenotype of *regA*$^{D-}$ cells indicates a possibility of high PKA activity due to low RegA function. RegA can form a complex with FbxA-CulA for proteasome-mediated degradation after 8 hours of development. This RegA

degradation accompanies the increase in cAMP and PKA activity for post aggregative gene expression [14]. A high turnover rate of $RegA^{D-}$ at physiological levels might possibly result in precocious spore development. *Dictyostelium* Erk2 is an atypical MAP kinase protein. These atypical MAP kinases are regulated differently as compared to typical MAP kinases[15]. Atypical MAP kinases might interact with substrates in a different way, perhaps not requiring conventional D-motifs. Co-immunoprecipitation results from our study show that the putative D-motif is not essential for *Dictyostelium* Erk2 and RegA interaction. Other regions of RegA might possibly be involved with Erk2 interactions.

We propose that both Erk1 and Erk2 regulate RegA function. Genetic evidence shows that Erk2 is down regulating the RegA activity. The D-motif is not essential for Erk2 regulation of RegA activity. Genetic epistasis tests support the possibility of Erk1 and RegA can function in the same pathway. Erk1 activation is a secondary response to Erk2 activation. Therefore, competitive regulation of RegA by Erk2 and Erk1 is a possibility. Co-immunoprecipitation experiment for Erk1-RegA interaction should be carried out in future experiments to address such a possibility. In our study, the outcome of Erk2 regulation of RegA is based on phenotype studies. New approaches to measure the real-time changes in intracellular cAMP level and cAMP signaling should be developed to further strengthen the findings of our study. Unpublished mass spectrometry and phenotype study data from our laboratory shows the presence of a phosphorylated serine residue (S142) that is capable of up regulating RegA function. Erk1 activation, as a secondary response, corresponds to the lowering of cAMP levels in cells. These data indicate that Erk1 might be activating RegA by phosphorylation of the S142. Phospho-

ablative and phospho-mimetic mutational studies of S142 support this role of S142 in the regulation of RegA function.

The *Dictyostelium* Gα2 protein is another potential regulator of MAP kinases. Our study of the Gα2 protein sequence shows a putative MAP kinase D-motif site near the N-terminus. Such a putative MAP kinase D-motif has been found in other Gα proteins required for MAP kinase signaling [16]. Unlike MAP kinase substrates, the Gα2 protein does not contain a typical MAP kinase phosphorylation target motif. The Gα2 protein is required for cAMP-mediated aggregation during early developmental stages. Heterologous *gα2* expression can cause abnormal stalk formation during late development [17]. A similar role for Gα subunit function can be found in other organisms, including *Saccharomyces cerevisiae*. In pheromone stimulated yeasts, it was found that a Gα protein, Gpa1, played an essential role in the adaptation response. The MAP kinase protein, Fus3, interacts with the Gpa1 protein resulting in a change of mating response as well as chemotropism towards the pheromone [18]. It was discovered that both activated and inactive Fus3 can interact with Gpa1 in such an adaptation response[19]. cAMP-mediated aggregation in *Dictyostelium discoideum* has some similarities to yeast pheromone responses. The Gα2 protein, that plays a critical role in cAMP-mediated aggregation, might also be responsible for adaptation responses to cAMP.

The presence of a putative MAP kinase D-motif site points towards the possible interaction between a MAP kinase and the Gα2 subunit and a possible role in a cAMP-mediated chemotaxis adaptation response. Therefore, if a Gα2-MAP kinase interaction is critical for the adaptation response, then change in the MAP kinase D-motif might prevent proper adaptation. Two different types of aggregation phenotypes found when the

MAP kinase D-motif altered protein, Gα2^{D-}, is expressed in *gα2* gene disrupted cells (*gα2*$^{-}$). The expression of the Gα2^{D-} at low levels could not correct the deficient aggregation phenotype of *gα2*$^{-}$ cells. The higher-level expression of Gα2^{D-} rescued the defect in the aggregation process. The expression of wild-type Gα2 was able to restore aggregation at all levels of expression. The D-motif alteration might lower the affinity of Gα2^{D-} with MAPKs. Loss of function caused by an interaction affinity change might be rescued in high copy *gα2*$^{D-}$ cells because of Gα2^{D-} overproduction. The inability of low copy *gα2*$^{D-}$ cells to recover the early stages of cAMP-mediated aggregation indicates loss of a chemotaxis gradient tracking function. This finding is supported by the lack of *gα2*$^{D-}$ cell participation in aggregation when mixed with wild type *gα2* cells in a chimera study. Above agar chemotaxis assays show that both *gα2*$^{D-}$ and *gα2* cells have comparable migration response to extracellular cAMP indicating no change in chemokinesis function due to the D-motif alteration.

In yeasts, active and inactive MAP kinase Fus3 interacts with Gpa1. A similar mechanism of interaction might exist between *Dictyostelium* Gα2 and MAP kinases. Our preliminary data show that the cells expressing Gα2^{D-} protein have defects in the shuttling of a transcription factor, GtaC, from the cytoplasm to the nucleus. The GtaC shuttling from the nucleus to the cytoplasm is dependent on MAP kinase Erk2 activation and perhaps the interaction between Erk1 and Gα2 might be required for the return of GtaC to the nucleus. Future studies using co-immunoprecipitation will help establish possible MAP kinase-Gα2 interactions for the cAMP mediated chemotaxis response.

The studies of the MAP kinase D-motifs in both the RegA phosphodiesterase and the Gα2 subunit show that D-motifs are important for the function of these proteins.

Alterations in the D-motifs result in a reduction of function but the overexpression of the mutant proteins can rescue function.

6.1. References for chapter VI

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