

FUNCTION AND SPECIFICITY IN SIGNALING
PATHWAY OF TWO MAP KINASES ERK1 AND ERK2
IN *DICTYOSTELIUM*

By

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NOMENCLATURE

cAMP	cyclic adenosine monophosphate
cAR	cAMP receptor
CD	common docking site
cGMP	cyclic guanine monophosphate
D-motif	MAPK docking site
ERK	extracellular signal-regulated kinase
G protein	guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
MAPK	mitogen-activated protein kinase
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
STAT	signal transducer and activator of transcription

CHAPTER I

GENERAL INTRODUCTION

MAPK signaling pathways in eukaryotic cells

Mitogen-activated protein kinases (MAPKs) are kinases that respond to several extracellular stimuli such as hormones, chemoattractants, cytokines and stresses. Consequently, MAPKs play important roles in basic cellular activities including cell growth, differentiation, proliferation and survival. MAPK pathways are known to associate with several human diseases ranging from birth defects such as Noonan syndrome to later onset diseases such as cancer, cardiovascular diseases and obesity (Bost et al., 2005; Lawrence et al., 2008). Extracellular signal regulated kinases (ERKs) are a subgroup of MAPKs and these kinases have been characterized in many different eukaryotes. ERKs contain a conserved motif (TEY) allowing a dual-phosphorylation to occur in both the threonine and the tyrosine residues (Chen et al., 2001). ERKs often function downstream of G protein-coupled receptors (GPCRs). In mammals, ERKs can also be activated by receptor tyrosine kinases (RTKs) (Fig.1).

GPCRs are seven membrane-spanning domain receptors with the ability to bind extracellular ligands and intracellular guanine nucleotide-binding proteins (G proteins) composed of $G\alpha$, $G\beta$ and $G\gamma$ subunits. The $G\alpha$ subunit functions as a molecular switch. When binding to extracellular ligands, GPCRs cause an exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) in the $G\alpha$ subunits resulting in the dissociation of the $G\alpha$ subunit and the $G\beta\gamma$ dimer

from the receptor. The disassociated G proteins then expose their internal interaction sites allowing them to associate with downstream signaling proteins.

RTKs are transmembrane proteins that contain an extracellular domain that binds to ligands and an intracellular kinase domain. When binding to ligands, RTKs associate together to form dimers. The proximity of the two receptors in each dimer leads to autophosphorylation of the tyrosine residue in the cytoplasmic domains. These phosphorylated domains subsequently activate downstream effectors by phosphorylation. Both GPCRs and RTKs can activate ERK pathways through small monomeric G proteins (such as RAS) and MAP4Ks (such as PAKs) that are anchored to plasma membrane. These proteins, in turn, activate MAPK cascades typically consisting of MAP3Ks (RAF), MAP2Ks (MEKs) and MAPKs through phosphorylation (Raman et al., 2007) (Fig.1). The kinases in the MAPK cascade as well as other signaling proteins that function in MAPK pathways might be tethered together through scaffolding proteins (SCAs) such as STE5 in yeast (Chen and Thorner, 2007). The scaffolding proteins help to transduce signals and retain specific interactions among these proteins (Fig.1).

MAPKs might activate or inactivate a wide range of cytoplasmic and nuclear substrates at serine/threonine residues in the motif (P-X-S/T-P) (Songyang et al., 1996). The notable MAPK substrates include transcription factors, protein kinases (such as mammalian ribosomal S6 kinases) and phosphodiesterases that regulate cAMP and cGMP levels. In addition to the MAPK phosphorylation sites, the substrates might contain a MAPK docking site (R/K1-3-spacer2-6-L/I-X-L/I) commonly referred to as a D- motif that mediates the docking to MAPKs. The D-motif is also presents in upstream signaling proteins (such as MAP2Ks, phosphatases) or other proteins that play roles in MAPK pathways (Tanoue and Nishida, 2003). Another motif that also contributes to MAPK-substrate interactions is the DEF site (FXF/YP) (Sheridan et al., 2008). Although there is much information known about the roles of MAPKs, defining the function and pathway specificity of each

MAPK in mammals has been challenging due to the presence of many MAPKs (at least six subgroups) and the crosstalk among MAPK pathways.

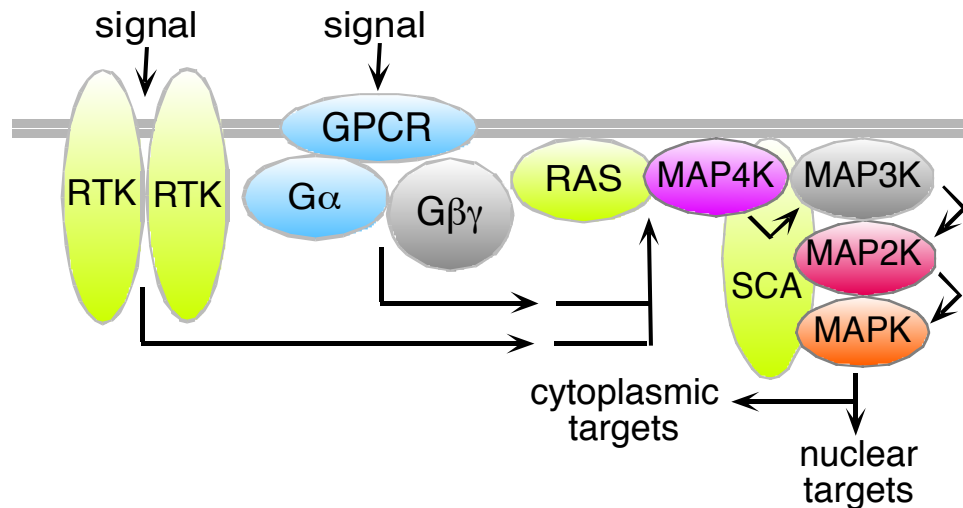


Fig. 1: The MAPK pathways in eukaryotic cells (Hadwiger and Nguyen, 2011).

***Dictyostelium discoideum* as a model organism**

Dictyostelia is a group of soil amoebae with over 120 known species and divided into four groups. *Dictyostelium discoideum* belongs to the group (group four) that is the most diverse group and members of this group use cAMP as a chemoattractant for aggregation during starvation (Schaap, 2011). This is an unusual role for cAMP because it typically functions as an intracellular signal in eukaryotes. After aggregation, *Dictyostelium* differentiates into prestalk cells and prespore cells that finally develop into stalks and spores respectively. Therefore, this organism has been used as a valuable model to study the fundamental processes in cell biology such as chemotaxis, differentiation, development, phagocytosis and signal transduction. Recently, this model has been also used to understand mechanisms of human diseases because its genome contains several orthologues to human genes that are associated with pathogenesis (Williams et al., 2006). Besides, *Dictyostelium* is amenable to both genetic and biochemical analysis. It can be transformed with recombinant DNA

vectors to express or knockout genes. The organism can be also cultured with bacterial or in axenic medium, allowing it to be subjected to various biochemical tests.

Developmental life cycle of *Dictyostelium*

Dictyostelium grows as individual amoebae but when the food source becomes exhausted, the genes for cAMP synthesis and detection are induced. These genes encode cAMP receptors (cARs - GPCRs that detect cAMP), adenylyl cyclase A (ACA, adenylyl cyclase for aggregation), phosphodiesterases (extracellular or intracellular PDEs, including RegA), cAMP-dependent protein kinase A (PKA) and other proteins required for chemotactic responses (guanylyl cyclase, phospholipase C, phosphoinositide 3-kinase) (Schaap, 2011). Several hours after starvation, a few cells begin to secrete cAMP and generate a cAMP gradient. The cAMP receptors (cARs) in neighboring cells detect this cAMP gradient resulting in activation of the proteins in chemotactic pathways and ACA. The responsive cells therefore can chemotax to the source of cAMP as well as synthesize cAMP. The newly synthesized cAMP accumulates in the cytoplasm to activate PKA, a protein kinase that regulates the proteins necessary for aggregation. cAMP is also secreted outside the cell to relay the cAMP signal to other cells. High extracellular cAMP concentration promotes expression and release of phosphodiesterases (PDEs) leading to the degradation of extracellular cAMP. Intracellular cAMP in the cytoplasm is also negatively regulated by another phosphodiesterase RegA (Fig. 2). Therefore, secretion and synthesis of cAMP are oscillatory rather than continuous (Maeda et al., 2004). This ensures a directional movement of cells to aggregation centers. After a couple of hours, cells rapidly aggregate into groups of about 100,000 cells and develop into multicellular mounds (Fig. 3).

In addition to the important role in aggregation, cAMP is also essential for many developmental processes including cell differentiation and cell sorting. In the late stages of

aggregation, cells begin to differentiate into prespore and prestalk cells. cAMP activation of PKA is required for prespore differentiation (van Es et al., 1996). The developing prespore cells produce

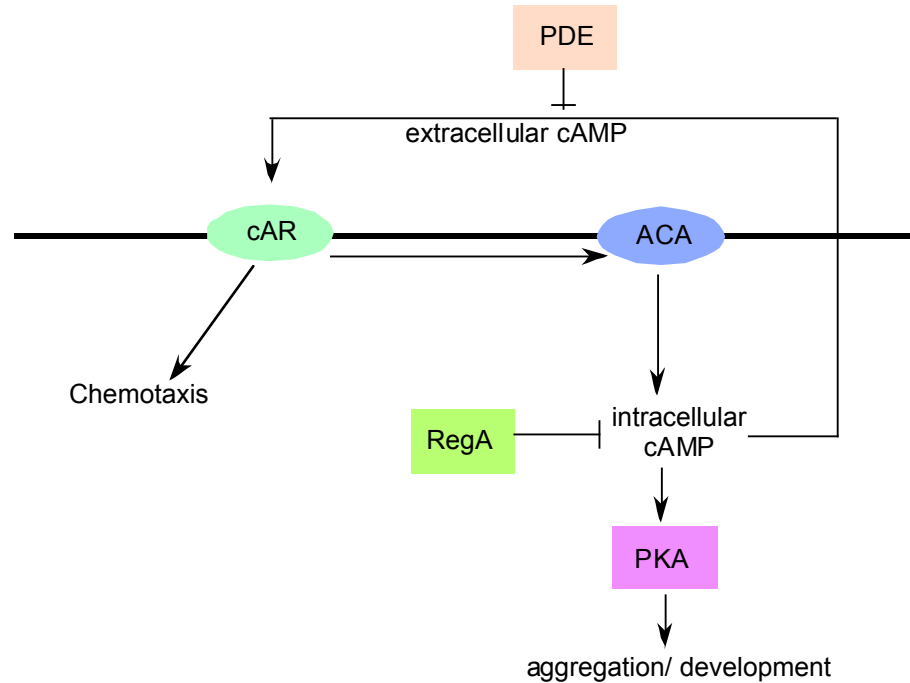


Fig. 2: The biochemical network in generating cAMP pulses that is essential for chemotaxis of cells during aggregation.

differentiation-inducing factor-1(DIF-1) to induce the differentiation of prestalk cells (Williams, 2006). At the mound stage, cells begin sorting with many prestalk cells moving to the top of the mound. The top of the mound has a high concentration of cAMP that attracts prestalk cells (Siegert and Weijer, 1995). The prestalk cells eventually form the anterior region, while the prespore cells, that are less sensitive to this source of cAMP, form the central and posterior regions. The mounds elongate to form a finger-like structure and then fall to the substratum as migratory slugs that consist of a prestalk anterior region at the front one third and a prespore region at the rear two thirds. The prespore cells are a homologous population but the prestalk cells are divided into at least five subgroups including prestalk A (pstA), O (pstO), AB (pstAB), B (pstB) and anterior-like cells (ALC) (Williams, 2006). PstA cells occupy the extreme anterior region of the slug and these cells contribute

to the upper cup of the fruiting body, the final developmental structure. PstO cells occupy the rear of the prestalk region and ultimately develop into the stalk that supports the spore mass. PstAB and pstB

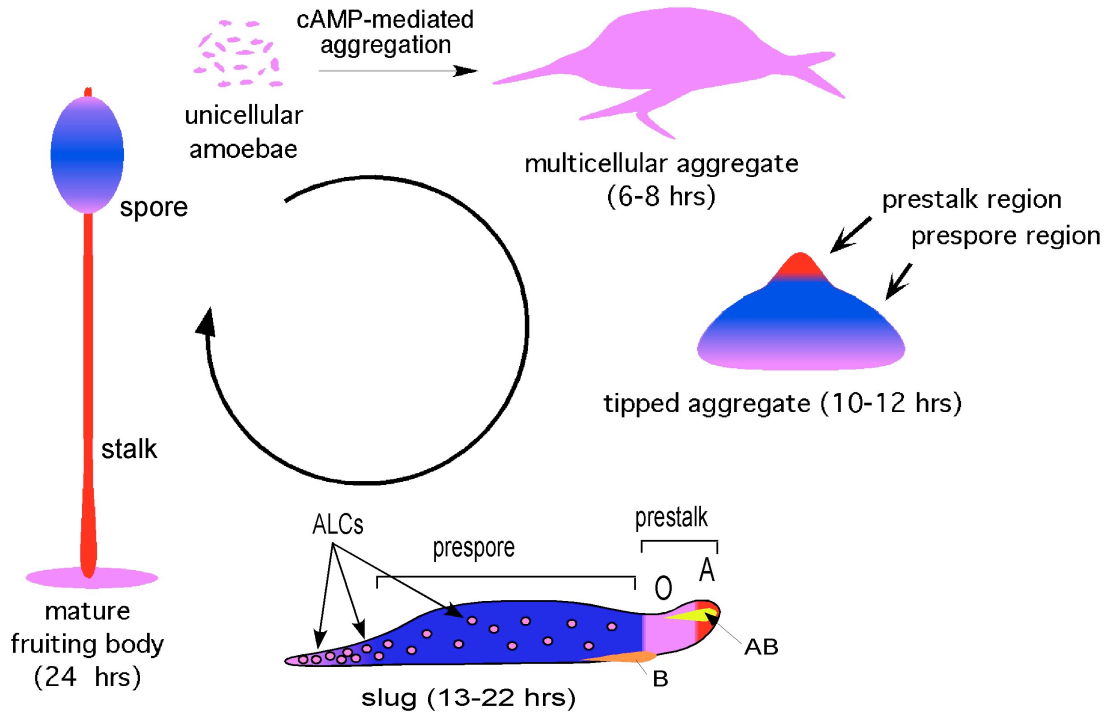


Fig. 3: The developmental life cycle of *Dictyostelium*.

are small populations that localize to the core interior and the intersection between prespore and prestalk regions, respectively. ALCs are prestalk cells but they are scattered through prespore region and finally form the lower cup and basal disc. In addition to the differences in distribution, the prestalk subgroups can also be distinguished by the expression of the cell type-specific genes (Williams, 2006). The *ecmA* gene is expressed in pstA and ALC cells but repressed in pstO cells. The *ecmB* gene is expressed in pstAB, pstB and ALC but repressed in pstA at early development. After 20 hours of starvation, the slugs culminate the developmental process by forming a stalk tube (differentiated prestalk cells) and a mass of spores (differentiated prespore cells). Differentiation is

terminated by the death of stalk cells and encapsulation of spores. The entire developmental cycle takes approximately 24 hrs to complete.

ERK function and regulation in *Dictyostelium*

Dictyostelium has only two MAPKs, ERK1 and ERK2, that both are expressed during growth and development. ERK1 and ERK2 shared 30-40% of sequence identity with each other and with MAPKs of other organisms (Goldberg et al., 2006; Maeda et al., 1996; Sobko et al., 2002). Unlike mammalian cells, *Dictyostelium* does not have receptor tyrosine kinases but has over 50 G protein-coupled receptors that might regulate ERK pathways. This organism encodes 12 different G α subunits, but only one G β and G γ subunit resulting in the possible formation of 12 different heterotrimeric G proteins that couple with the GPCRs (Fey et al., 2009). cAMP receptors (cAR1 and cAR3) are well-characterized GPCRs that play important role during aggregation. When binding to cAMP, the receptor cAR1 stimulates the phosphorylation of ERK2 at the conserved TEY motif through uncharacterized mediators. The duration of this phosphorylation depends on the level of extracellular cAMP. Although G α 2 and G $\beta\gamma$ subunits associate with the cAR1, *ga2*- and *g β* - cells only partially reduced ERK2 activation suggesting that ERK2 might be activated by other additional pathways that are independent of G proteins when activated downstream of the cAR1 receptor (Brzostowski and Kimmel, 2006).

erk2- cells can chemotax to cAMP but can not aggregate. A previous study has shown that ERK2 negatively regulates RegA, an intracellular cAMP-specific phosphodiesterase (Maeda et al., 2004). Therefore, cAMP does not accumulate in *erk2*- cells and consequently, *erk2*- cells do not aggregate (Segall et al., 1995). However, *erk2*- cells can co-aggregate with wild-type cells to form chimeras due to the presence of cAMP pulses from the wild-type cells. In these chimeras, *erk2*- cells are excluded from the prespore region of the developing slugs suggesting a role for ERK2 in spore development. This role of ERK2 might be connected to ERK2 regulation of intracellular cAMP and

PKA activity that regulates spore differentiation. In addition to extracellular cAMP, ERK2 is also activated in response to folate. In nature, folate allows cells to forage for new bacterial food sources. Folate stimulates the phosphorylation of ERK2 through an undefined receptor. However, this receptor requires Ga4 subunit to transduce its signal because the *ga4-* cells cannot cause folate-stimulated ERK2 phosphorylation (Hadwiger et al., 1994). *erk2-* cells have slightly decreased chemotaxis to folate suggesting that ERK2 has a minor role in chemotaxis to both cAMP and folate.

There is very little information on the function and regulation of ERK1. The activation of ERK1 is transient and disappears 30 seconds after stimulation with cAMP, the time at which ERK2 activation begins to peak (Sobko et al., 2002). The phosphorylation at the conserved TEY motif of ERK1 is not detected by the commercially-available antibodies that detect the phosphorylations of ERK2 and many MAPKs from other organisms. The inability of these antibodies to bind to phosphorylated ERK1 perhaps results from differences in the flanking residues of ERK1 TEY motif or perhaps ERK1 undergoes an atypical phosphorylation (Hadwiger and Nguyen, 2011). MEK1 (MAP2K) is required for cAMP-stimulated activation of ERK1 but not ERK2. Furthermore, the loss of MEK1 results in some developmental phenotypes which are similar to those of *erk1-* cell suggesting that MEK1 and ERK1 might function in the same pathway (Ma et al., 1997).

Until recently, the ERK1 and ERK2 signaling pathways in *Dictyostelium* were not well defined. The goal of this research was to use the genetic and biochemical approaches available with *Dictyostelium* to characterize the function and the specificity in signaling pathways of ERK1 and ERK2. The results from this study will hopefully provide insights into general mechanisms of MAPK signaling and help guide the investigation of MAPK-associated diseases such as cancer, cardiovascular diseases and obesity.

Outline of the dissertation

The research was prompted by the recognition of D-motifs that mediate MAPK docking in Gα4, Gα5 and other Gα subunits. The identification of D-motifs led to studies on the interactions of ERKs with Gα subunits. Using immunoprecipitation and pull-down assays, we demonstrated that the Gα4 subunit interacts with ERK2 through a D-motif. Although a physical interaction between Gα5 subunit and ERK1 could not be determined due to the lethal effect of Gα5 subunit, the genetic analysis of *erk1*- and Gα5 mutants suggested that ERK1 and the Gα5 subunit function in a common signaling pathway that is different from that of the Gα4 subunit-ERK2 signaling pathway. These studies are presented in chapter 2 and chapter 3. In the subsequent study, we addressed the question of whether ERK1 and ERK2 had redundant or distinct functions during growth and development. While *erk2*- cells have been already characterized, we created *erk1*- and *erk1-erk2*- cells to inspect function and regulation of ERK1. Our results showed that ERK1 and ERK2 have distinct roles in the G protein-mediated pathways. This study is presented in chapter 4. We then continued searching for ERK-associated proteins with the goal of better understanding ERK signaling pathways. Using mass spectrometry, combined with the genetic analysis, we successfully defined two distinct ERK pathways. The Gα5 subunit-ERK1 pathway regulates nuclear localization of transcription factor STATc resulting in the repression of the prestalk gene *ecmA*, while the Gα2/ Gα4 subunits-ERK2 pathway regulates function of another transcription factor STATa and expression of the *ecmB* gene. The regulation of STATa also includes the down regulation of the phosphodiesterase RegA. These results are presented in chapters 5 and 6.

Chapters 2, 3 and 4 are the manuscripts that have been published in Developmental Biology (Nguyen and Hadwiger, 2009), Microbiology (Raisley et al., 2010) and Cellular Signaling (Nguyen et al., 2010), respectively. All of these journals have provided permission for these data to be presented in this dissertation. The author of this dissertation has provided the following contributions to the published data. In chapter 2, the author performed interaction assays of the wild-type and altered Gα4 subunits with ERK2 as well as development and chemotaxis assays. In chapter 3, the author created

erk1- cell and performed genetic assays to show the functional relationship between ERK1 and the Gα5 subunit. In chapter 4, the author conducted complementation, development and chemotaxis assays on the ERKs and G protein mutants. The author also performed ERK activation assays and characterized the intracellular distribution of ERKs in this chapter.

Chapters 5 and 6 include the manuscripts submitted to Molecular Biology of the Cell (Hoai-Nghia Nguyen and Jeffrey Hadwiger) and Cellular Signaling (Hoai-Nghia Nguyen and Jeffrey Hadwiger). In these chapters, the author performed pull-down assays to isolate ERK-associated proteins, knocked out *regA* gene in several mutants and determined the intracellular distributions of the transcription factors (STATc and STATa) as well as the expressions of *ecmA* and *ecmB* genes in mutants.

CHAPTER II

THE $G\alpha 4$ PROTEIN SUBUNIT INTERACTS WITH THE MAPK ERK2 USING A D-MOTIF THAT REGULATES DEVELOPMENTAL MORPHOGENESIS IN *Dictyostelium*

Abstract

G protein $G\alpha$ subunits contribute to the specificity of different signal transduction pathways in *Dictyostelium discoideum* but $G\alpha$ subunit-effector interactions have not been previously identified. The requirement of the *Dictyostelium* $G\alpha 4$ subunit for MAP kinase (MAPK) activation and the identification of a putative MAPK docking site (D-motif) in this subunit suggested a possible interaction between the $G\alpha 4$ subunit and MAPKs. *In vivo* association of the $G\alpha 4$ subunit and ERK2 was demonstrated by pull-down and co-immunoprecipitation assays. Alteration of the D-motif reduced $G\alpha 4$ subunit-ERK2 interactions but only slightly altered MAPK activation in response to folate. Expression of the $G\alpha 4$ subunit with the altered D-motif in *ga4⁻* cells allowed for slug formation but not the morphogenesis associated with culmination. Expression of this mutant $G\alpha 4$ subunit was sufficient to rescue chemotactic movement to folate. Alteration of the D-motif also reduced the aggregation defect associated with constitutively active $G\alpha 4$ subunits. These results suggest $G\alpha 4$ subunit-MAPK interactions are necessary for developmental morphogenesis but not for chemotaxis to folate.

Introduction

G protein-coupled receptors activate many different cellular responses in eukaryotes using a wide variety of signal transduction pathways (Milligan and Kostenis, 2006; Neves et al., 2002; Simon et al., 1991). Most known pathways include the activation of heterotrimeric G proteins ($G\alpha\beta\gamma$) that disassociate into a $G\alpha$ subunit and a $G\beta\gamma$ dimer capable of regulating

the function of downstream effectors (Hamm, 1998). The $G\alpha$ subunit provides specificity for receptor coupling and when activated the $G\alpha$ releases the $G\beta\gamma$ dimer (Conklin et al., 1993). In some signaling pathways, the interaction of the $G\alpha$ subunit with a specific effector (e.g., adenylyl cyclase, phospholipase C, etc.) has been established but in most pathways the interactions the $G\alpha$ subunit with downstream effectors remains to be determined (Neer and Clapham, 1988; Neves et al., 2002). Identifying $G\alpha$ subunit interactions with other signaling proteins will likely provide important insights with respect to pathway specificity and cellular responses mediated by G proteins.

The genome of the soil amoebae *Dictyostelium discoideum* encodes 12 different $G\alpha$ subunits (*gpa* genes) and some of these subunits have been shown to function in pathways that regulate chemotactic responses and a variety of processes associated with the developmental life cycle of this organism (Brandon et al., 1997; Brzostowski et al., 2002; Hadwiger and Firtel, 1992; Hadwiger et al., 1996; Kumagai et al., 1991). Genetic analysis has indicated the $G\alpha_4$ subunit is required for chemotaxis to folate and the promotion of spore cell development during multicellular development whereas the closely related $G\alpha_5$ and $G\alpha_2$ subunits are required for different or even opposing functions (Hadwiger and Firtel, 1992; Hadwiger et al., 1994; Hadwiger et al., 1996; Kumagai et al., 1991; Natarajan et al., 2000). The $G\alpha_2$ subunit is required for cAMP chemotaxis and cell aggregation and the $G\alpha_5$ subunit inhibits folate chemotaxis and promotes prestalk cell development (Hadwiger et al., 1996; Kumagai et al., 1991). The $G\alpha_4$ subunit and these other $G\alpha$ subunits are presumed to couple to the same $G\beta\gamma$ dimer because only single genes have been identified for the $G\beta$ and $G\gamma$ subunits in the *Dictyostelium* genome and so only the receptor and the $G\alpha$ subunit are likely to be the determinants of pathway specificity (Lilly et al., 1993; Zhang et al., 2001). A previous study of chimeric $G\alpha$ subunits suggests the $G\alpha_4$ subunit and other $G\alpha$ subunits can contribute to the specificity of downstream responses, perhaps through interactions with signaling components other than the $G\beta\gamma$ dimer (Hadwiger,

2007). However, $G\alpha$ subunit interactions with effectors in *Dictyostelium* have not been previously described.

Downstream in many G protein-mediated signaling pathways is the activation of MAP kinases (MAPKs) that can phosphorylate both cytoplasmic and nuclear targets to regulate cell growth and differentiation (Caunt et al., 2006; Chen and Thorner, 2007; Goldsmith and Dhanasekaran, 2007). *Dictyostelium* has only two MAPKs, ERK1 and ERK2 that belong to a subclass of MAPKs known as extracellular signal-regulated kinases (Goldberg et al., 2006). ERK1 regulates cell aggregate size and the timing of gene expression during the development and ERK2 is necessary for cAMP-mediated cellular aggregation and prespore development (Gaskins et al., 1996; Gaskins et al., 1994; Segall et al., 1995; Sobko et al., 2002). In cAMP-stimulated cells, ERK2 is required for cAMP accumulation through the inhibition of the phosphodiesterase RegA (Maeda et al., 2004; Segall et al., 1995). MAPKs, including the *Dictyostelium* ERK1 and ERK2, contain a common docking (CD) site that allows them to associate with their activators, MAPK kinases (MAPKKs) and substrates (Tanoue et al., 2000). The CD sites contribute to interactions with MAPK docking sites (D motifs) on other proteins to tether and facilitate interactions (Grewal et al., 2006; Remenyi et al., 2005). In some G protein signaling pathways, MAPK activation occurs via the release of $G\beta\gamma$ dimers that transduce signals through Ras proteins and kinase cascades that include MAPKKs and MAPKK kinases (MAPKKKs) (Belcheva and Coscia, 2002; Chen and Thorner, 2007). This type of signal transduction pathway has been extensively studied in the yeast (*Saccharomyces cerevisiae*) mating response pathway and recently the $G\alpha$ subunit, Gpa1, of this pathway has been shown to contain a D-motif that allows for direct interaction between the Gpa1 subunit and the MAPK Fus3 (Metodiev et al., 2002). These interactions are thought to regulate a small change in the cytoplasmic/nuclear distribution of Fus3 and promote adaptation to mating pheromone signaling (Blackwell et al., 2003).

In this study we characterized the role of the Gα4 subunit in MAPK function in *Dictyostelium*. Gα4 subunit-ERK2 interactions were analyzed using a yeast two-hybrid assay and a pull-down assay in *Dictyostelium*. The primary structure of the Gα4 subunit was analyzed for D motifs in regions that might be exposed to interactions with other proteins. Gα4 mutants with an altered D-motif were tested for the ability to interact and activate MAPKs and the ability to rescue developmental morphology and chemotaxis of *ga4-* mutants. The results of this study support a role for Gα4 subunit-MAPK interactions in developmental processes such as cell differentiation.

Materials and methods

Strains and media

All *Dictyostelium* strains used in the study were derived from the wild-type strain KAx3. The *ga4-*, Gα4HC, and JH8 strains were previously described (Hadwiger and Firtel, 1992). The *erk2-* strain was created in JH8 (*pyr5-6-*) cells using a gene disruption construct previously described and generously provided by J. Segall (Albert Einstein College of Medicine, Bronx, NY) and the *Dictyostelium* Stock Center. The *erk2-* gene disruption was verified by genomic DNA blot analysis and the *erk2-* mutant displayed the phenotypes previously described for *erk2-* cells in other strain backgrounds (Segall et al., 1995). Cells were grown in axenic HL5 medium or on bacterial lawns of *Klebsiella aerogenes* (Watts and Ashworth, 1970). DNA vectors were electroporated into cells as previously described (Hadwiger, 2007). Transformed cells were selected and maintained in medium containing 3-10 µg/ml of the drug G418 or 3 µg/ml of the drug blasticidin S and drug selection was removed several hours prior to analysis. Folate solutions were prepared by neutralizing folic acid with NaHCO₃. The modeling of the Gα4 subunit structure was conducted using the Swiss-Model program and displayed using the PyMOL Molecular Graphics System 2008 (DeLano Scientific) (Arnold et al., 2006).

Recombinant DNA constructs

The wild-type *Ga4* gene (also designated as *gpaD*) in the vector pBluescriptIISK+ (Stratagene) was previously described (Hadwiger, 2007). Alterations to the *Ga4* subunit D-motif, *Ga4*R107E,R108E subunit (designated *Ga4d*-), were created from the wild-type *Ga4* cDNA in the vector pBluescriptIISK+ using the Gene Tailor PCR mutagenesis system (Invitrogen) and overlapping oligonucleotides: (sense strand: 5'-CAAAGAGCAGCAAATGTACTcgaggaAACTATTGGTAATGAACC) and (antisense strand: 5'-GTACATTTGCTGCTCTTTGTTTATTTTC) (lower case nucleotides differ from those of the template DNA). In addition to substituting two arginine codons with two glutamate codons, one silent mutation was introduced to create *XhoI* site thus allowing the mutant gene to be detected by restriction enzyme digestion analysis. A gene encoding a constitutively-active *Ga4*Q200L subunit (designated *Ga4**) was also created from the wild-type *Ga4* gene by PCR mutagenesis using overlapping oligonucleotides (sense strand: 5'-GATTAGATTAAAGATTGTAGAcGTCGGTGGTCtAAGATCTCAAAGAAGAAAATGG) and (antisense strand: 5'-CAGAATTTACATTTGATAAGATTAGATTAAAGATTGTAG). A silent mutation, creating an *AatII* site for restriction enzyme analysis verification, was also part of the PCR mutagenesis. This mutation conferring constitutive activity was also created in the gene encoding the *Ga4d*- subunit resulting in a gene encoding a *Ga4d*-,* subunit. All mutations created in this study were verified by sequence analysis and each mutant *Ga4* gene was then inserted into the *Dictyostelium* expression vector pDXA-GFP2 (*Ddp2*-based plasmid), replacing the *HindIII/XbaI* fragment that contained the *GFP2* reading frame downstream of the *act15* promoter (Levi et al., 2000). The *Ddp2*-based vectors integrate into the genome unless the cell also contains the pREP vector. Each *pact15/Ga4* gene construct was also transferred as *SalI/XbaI* fragments into the *Ddp1*-based pTX-GFP vector (extrachromosomal vector) replacing the *pact15/GFP2* gene (Levi et al., 2000).

To create Myc-tagged wild-type and mutant *Gα4* subunits the various *Gα4* genes were modified with a 5'-*Bam*HI and a 3'-*Xba*I restriction site using PCR and the oligonucleotides: (sense strand 5'- gccggcggatccATGAGATTCAAGTGT TTTGGATCAG) and (antisense strand 5'-cggcgctctagaTTAGAAAGTGT TCTAATGCTTGAGATAAAATTGTTTGTCTAAC). Each amplified *Gα4* gene was inserted at *Bam*HI and *Xba*I sites into a pBluescriptIISK+ vector containing the *Hind*III-Myc-*Bam*HI linker created from the oligonucleotides: (sense strand 5'- AGCTTATGGAACAAAAATTATTATCAGAAGAAGATTTAG) and (antisense strand 5'- GATCCTAAATCTTCTTCTGATAATAATTTTGTTCATA). This linker adds the amino acid sequence MEQKLLSEEDLGS to the amino terminus of each *Gα4* gene (underlined residues represent the Myc epitope). Each gene was then transferred into the *Dictyostelium* expression vector pDXA-GFP2 as described above. A His6-tagged ERK2 was constructed by PCR amplification of an *ERK2* (also designated as *erkB*) cDNA kindly provided by J. Segall using the oligonucleotides: (sense strand 5'- cgcaagcttggatccctcgagacacaATGTCATCTGAAGATATAGATAAACATG) and (antisense strand 5' – GCGGTCGACTCTAGATTATGTTGATAAAGTTGGAGCAGTTGTACT). The amplified *ERK2* gene was inserted into the TOPO vector (Invitrogen) and then transferred into *Xba*I and *Xho*I sites of the *Dictyostelium* expression vector pDXA-HC containing His6-tag (Manstein et al., 1995).

MAPK activation assay

Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (5×10^7 cells/ml). The cell suspension was shaken for 1 hr and then stimulated with 50 μM folate. Cells were harvested by mixing with SDS-PAGE loading buffer and boiled. Samples (8×10^7 cells/lane) were subjected to SDS-PAGE and immunoblot analysis using a rabbit α -phospho-

p44/p42 MAPK antibody and secondary goat HRP-conjugated α -rabbit IgG antibody for chemiluminescence detection (Cell Signaling Technology).

Development and chemotaxis assays

Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10^8 cells/ml), before spotting on nonnutrient plates (phosphate buffer, 1.5% agar) for development or chemotaxis as described (Hadwiger et al., 1996). Cell development was analyzed using a dissecting microscope or fluorescence microscopy. Chemotaxis assay were performed by spotting droplets of cell suspension (10^7 to 10^8 cells/ml) on nonnutrient plates followed by the spotting of 1 μl of folate solutions (10^{-2} to 10^{-4} M) approximately 2-3 mm away from the cell droplet. Cell movement was monitored with a dissecting microscope. All strains were treated identically for each experiment.

Pull-down assays

Cells grown to 3×10^6 cells/ml were collected and washed in phosphate as described above. Pellets were suspended at 5×10^8 cell/ml in lysis buffer (300 mM NaCl, 50 mM sodium phosphate) containing a protease inhibitor cocktail (Sigma). Cells were lysed by freezing at -80°C for 30 min, thawed on ice, vortexed, and centrifuged at 14,000 rpm for 5 sec to eliminate insoluble materials. Cell extracts were incubated with Talon Co^{2+} resin (Clontech) for 20 min in ice with gentle shaking. Extract-treated resins were washed three times on spin columns with wash buffer (300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazol and 10% glycerol) and finally eluted using an elution buffer (300 mM NaCl, 50 mM sodium phosphate, 150 mM imidazol). The elutants were subjected to SDS-PAGE and western blotting. Blots were incubated with the primary mouse α -Myc antibody and then a secondary goat HRP-conjugated α -mouse IgG before bioluminescence detection.

Immunoprecipitation assays

Cells were prepared and lysed as described above for the pull-down assays. Cell extracts were treated with Protein A/G-agarose (25% v/v) (Santa Cruz Biotechnology) for 30 min on ice for preclearing treatment according to the manufacturer's instructions. The collected supernatants were incubated with mouse anti-Myc antibody for 1 hr and then with Protein A/G-Agarose for 1 hr on ice. The agarose was precipitated and washed 2 times with 1 ml of lysis buffer. The resulting immune complexes were subjected to SDS PAGE and western blotting. Blots were incubated with the primary rabbit α -His antibody and then a secondary goat HRP-conjugated α -rabbit IgG before bioluminescence detection.

Results

G α 4 function is required for MAPK activation

While several G α subunit genes have been identified in *Dictyostelium* genome, none of the encoded subunits have been shown to specifically interact with downstream signaling proteins that would define their role in pathway specific responses. Several reports have indicated a requirement for the G α 4 subunit for the activation of ERK2 in the response to folate whereas the G α 2 subunit is not required for ERK2 activation in response to cAMP suggesting the G α 4 subunit might directly regulate the activity of this MAPK (Maeda et al., 1996; Maeda and Firtel, 1997). Verification of the requirement of the G α 4 subunit in MAPK activation was examined through the detection of the phosphorylated MAPK in wild-type and *ga4*- cells (Fig.4). Only a low level of MAPK activation was observed (band with approximate molecular weight of 42,000) after folate stimulation of *ga4*- cells compared to the level observed in wild-type cells. A slightly higher level of phosphorylated MAPK was detected in *erk2*- cells and this detected band might be residual ERK2 produced from the leakiness of the *erk2*- allele as suggested by others (Kosaka and Pears, 1997). This detected band might also correspond to phosphorylated ERK1 because it

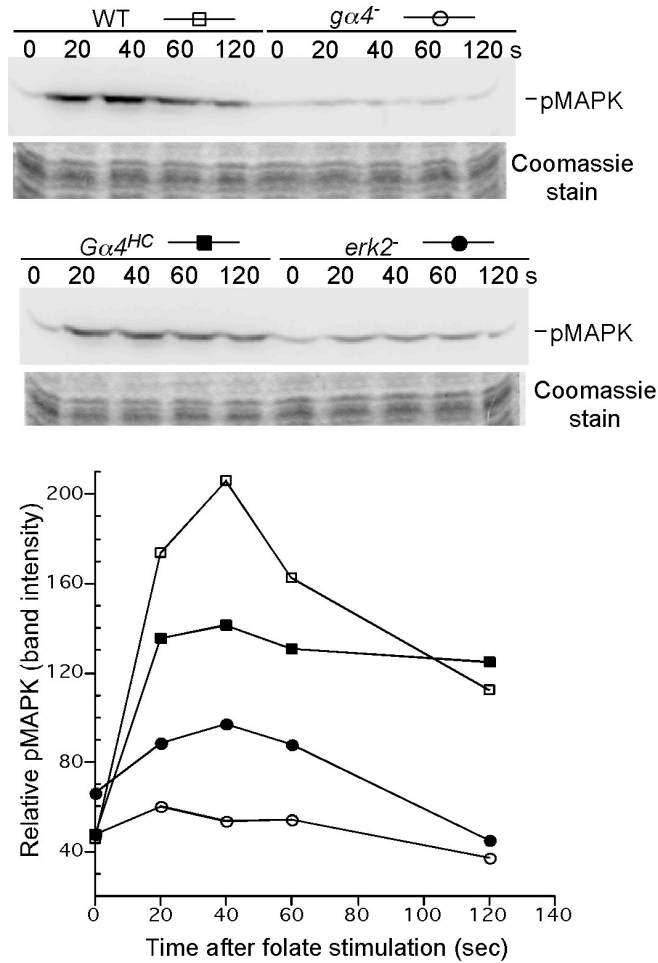


Fig. 4: Folate stimulated MAPK activation in wild-type and mutant cells. Wild-type cells (WT), *ga4*- cells (*ga4*-), and *Ga4* subunit over-expression (high copy gene number) cells (*Ga4HC*), and *erk2*- cells (*erk2*-) were grown, washed, and suspended in phosphate buffer as described in the Material and methods. Cell suspensions (5×10^7 cells/ml) were stimulated with $50 \mu\text{M}$ folate and harvested at the times indicated. Cell extracts were subjected to immunoblot analysis using an α -phospho-p44/p42 MAPK antibody. The band (pMAPK) corresponds to phosphorylated ERK2 and possibly phosphorylated ERK1 kinase. Coomassie stained gels are provided as a loading control (lower panels). The immunoblot analysis was identical for both gels and pMAPK band intensity is indicated in line graph below (symbols indicated above blots). Data represents one of at least three experiments with similar results.

also contains the highly conserved TEY segment that is phosphorylated by MAPKKs and recognized by the α -phospho-p42/p44 antibody. Therefore, we chose to designate the detected band as pMAPKs (phosphorylated ERK1/ERK2) because the possible presence of phosphorylated ERK1. In addition, MAPK activation was also analyzed in G α 4HC cells that over-express the G α 4 gene due in a high copy number of G α 4 genomic sequence. The over-expression of the G α 4 subunit did not increase the level of phosphorylated MAPKs suggesting G α 4 subunit expression is not a limiting factor.

D-motif in the G α 4 subunit

The requirement for G α 4 subunit in the folate stimulation of ERK2 might be due to a physical interaction between the G α 4 subunit and ERK2 as a G α subunit-MAPK interaction has been previously reported in the pheromone response pathway in yeast (Metodieff et al., 2002). To assess whether or not the G α 4 subunit might interact with ERK2, a search for potential D-motifs [K/R1-2 - spacer(1-6) - I/L-X-I/L] in the G α 4 subunit revealed a putative D-motif spanning residues 107-118 (Fig. 5A). The G α 4 subunit did not contain a D-motif near the amino terminus where the yeast Gpa1 D-motif exists and also where several other *Dictyostelium* G α subunits have putative D-motifs. Only one other *Dictyostelium* G α subunit, G α 5, had a potential D-motif in the same region as the G α 4 subunit and this motif had an unusually long spacer region compared to other known D-motifs. To assess whether the G α 4 D-motif at positions 107-118 was on the surface of the G α 4 subunit, a modeling program was used to predict a 3-dimensional model of the G α 4 subunit based on the crystallographic studies of another G α subunit (Fig. 5B). The D-motif is predicted to be part of loop on a surface of the G α 4 subunit located away from the predicted binding surfaces for receptors or the G $\beta\gamma$ dimer suggesting this D-motif is accessible to other proteins such as MAP kinases. Another potential D-motif was found at positions 25-37 in the G α 4 subunit but this region is not likely to function as a MAPK docking site because the region overlaps with the highly conserved G1 region that contributes to the pocket for guanine

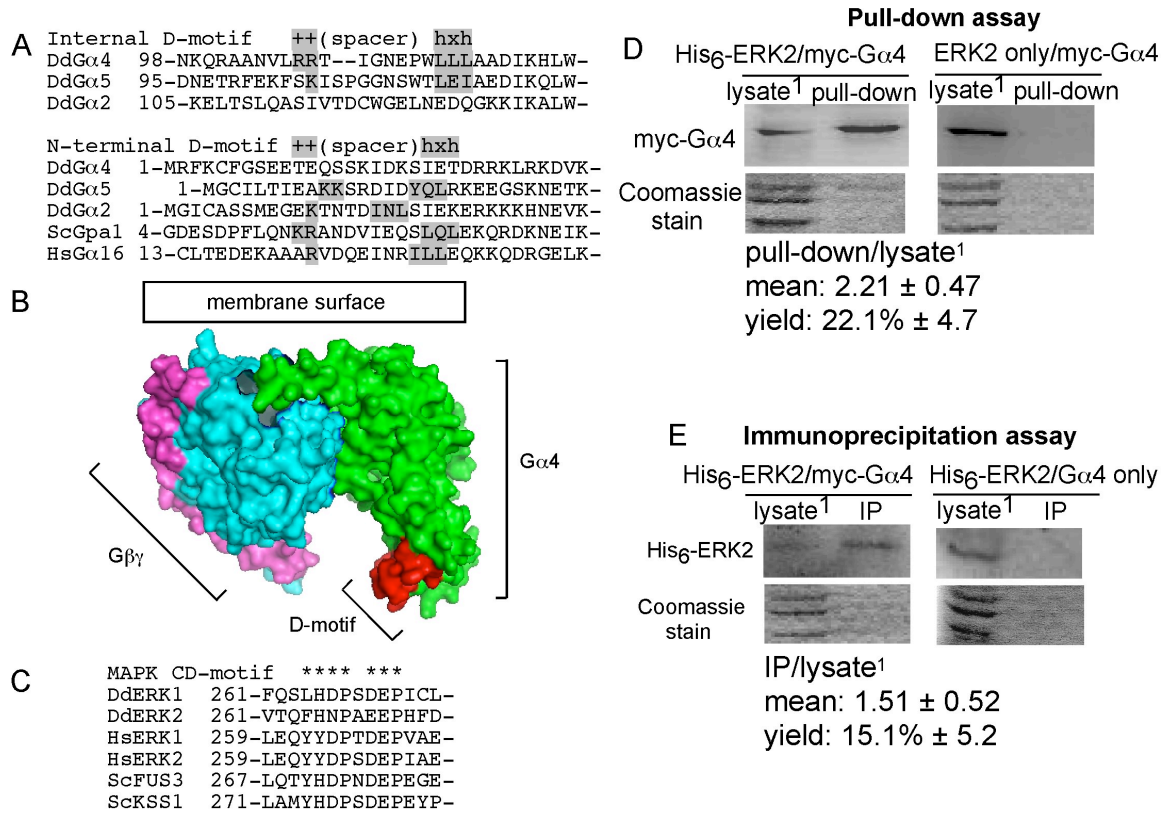


Fig. 5: (A) Alignments of D-motifs in Gα subunits. Primary sequence of the *Dictyostelium* Gα4 (DdGα4), Gα5 (DdGα5), Gα2 (DdGα2), yeast Gpa1 (ScGpa1), and human Ga16 (HsGa16) Gα subunits starting at the designated residue number. The row above the alignments indicates D-motif features with positively-charged (+), hydrophobic (h), and variable (x) residues. D-motif residues in the Gα subunits are shaded. **(B)** Modeling of Gα4 subunit structure and D-motif. A structure model of the Gα4 subunit was created using the SWISS-MODEL workspace and PyMOL program and the structure was modeled using the determined structure of the mammalian heterotrimeric G protein consisting of Gαi1, Gβ1, and Gγ2 subunits with GDP bound. The Gα4 subunit (green), Gβ subunit (blue), and Gγ subunit (magenta) are shown with respect to the membrane surface. The D-motif of the Gα4 subunit is indicated (red) on the lower portion of the Gα4 subunit. **(C)** Alignment of CD-motifs in MAPKs. Primary sequence of the

Dictyostelium MAPKs (DdERK1 and DdERK2), human MAPKs (HsERK1 and HsERK2), and yeast MAPKs (ScFUS3 and ScKSS1) starting at the designated residue number. Highly conserved regions are indicated by asteriks above alignment. (D) Detection of Ga4 subunit association with ERK2 kinase in *Dictyostelium*. *erk2⁻* cells expressing either the His₆-ERK2 or ERK2 kinase were transformed with a vector expressing the Myc-Ga4 subunit. Cell lysates were mixed with a Co²⁺ resin to pull-down the His₆-ERK2 kinase and associated proteins prepared as described in the Materials and Methods section. Untreated lysates and pull-down assay elutants were subjected to SDS-PAGE and immunoblot analysis using an α -Myc-epitope antibody (upper panels). Coomassie stained gel is provided as a loading control (lower panels). ¹ The lysate lane in each panel represents 1/10th of the cell extract used in the pull-down assay (pull-down). The mean pull-down/lysate¹ ratio of band intensity and standard deviation was determined from 3 experiments and then used to estimate the approximate pull-down yield of Myc-Ga4 subunit from the cell extract. (E) Detection of ERK2 association with the Ga4 subunit in *Dictyostelium*. *erk2⁻* cells expressing the His₆-ERK2 were transformed with a vector expressing the Myc-Ga4 subunit or the untagged Ga4 subunit. Immunoprecipitations were performed as described in the Materials and Methods section. Untreated lysates and immunoprecipitates (IPs) were subjected to SDS-PAGE and immunoblot analysis using an α -His-epitope antibody (upper panels). Coomassie stained gel is provided as a loading control (lower panels). ¹ The lysate lane in each panel represents 1/10th of the cell extract used in the immunoprecipitation. The immunoprecipitate/lysate¹ ratio of band intensity and standard deviation was determined from 4 experiments and then used to estimate the approximate co-immunoprecipitation yield of His₆-ERK2.

nucleotide binding (Conklin and Bourne, 1993). The D-motifs found in *Dictyostelium* Ga subunits can potentially interact with either ERK1 and ERK2 because both MAPKs contain conserved regions, such as the CD-motif, that have been implicated in D-motif interactions (Fig.

5C) (Tanoue and Nishida, 2003). However, other regions of MAPKs are also known to contribute to D-motif binding.

Analysis of Gα4-ERK2 interaction in vivo

Possible Gα4 subunit-ERK2 interactions in *Dictyostelium* were investigated using a pull-down assay that examined the presence of Myc-tagged Gα4 subunit in the extraction of a His₆-tagged ERK2 protein. A vector expressing a Myc-tagged Gα4 subunit (Myc-Gα4) was transformed into an *erk2*- strain already containing an integrated expression vector for a His₆-tagged ERK2 (His₆-ERK2). In these cells, the His₆-ERK2 was sufficient to allow for developmental aggregation indicating the His₆-tag does not destroy ERK2 function (data not shown). The His₆-ERK2 kinase was pulled down from cell lysates using Co²⁺ resin and washed and the elutant from the resin was subjected to immunoblot analysis for the presence of the Myc-Gα4 subunit. The level of the Myc-Gα4 subunit detected in elutants was approximately 2-fold greater than that found in 1/10 of the total cell lysate (~22% yield) indicating that the Gα4 subunit and ERK2 are capable of interacting *in vivo* (Fig. 5D). To determine if the Myc-Gα4 subunit in the pull-down elutants was dependent on the presence of His₆-ERK2, the Myc-Gα4 subunit was also expressed in *erk2*- cells that expressed ERK2 without the His₆-tag. Using the same Co²⁺ resin pull-down conditions, no Myc-Gα4 subunit was detected in the elutant indicating the Myc-Gα4 association with the pull-down assay is dependent on the His₆-ERK2.

The Gα4 subunit-ERK2 interaction was also demonstrated by the detection of the His₆-ERK2 in immunoprecipitates of myc-Gα4 subunit. In *erk2*- cells transformed with the His₆-ERK2 and myc-Gα4 subunit expression vectors, the Myc-Gα4 subunits were immunoprecipitated using α-Myc antibodies and the resulting immune complexes contained the His₆-ERK2 (Fig. 2D). The His₆-ERK2 was not detected in cells expressing the untagged Gα4 subunit indicating that the presence of the His₆-ERK2 in the immunoprecipitations was dependent on the Myc-Gα4 subunit.

The ability to detect ERK2 in Gα4 subunit-immunoprecipitates provides further support for the association of these proteins *in vivo*.

Alteration of the D-motif reduces Gα4 subunit association with ERK2

A study of the D-motif in the yeast Gpa1 subunit has indicated that the replacement of the positively charged residues with negatively charged residues reduces the Gpa1 subunit interactions with the MAP kinase Fus3 and reduces the ability of the Gpa1 subunit to promote cellular adaptation to mating pheromone (Metodieff et al., 2002). Therefore a similar alteration was created in the Gα4 subunit D-motif through the replacement of the two arginine residues with glutamate residues (Gα4R107E, R108E referred to as Gα4d-) and the Myc-tagged version of this Gα4d- subunit was assessed for associations with ERK2 using the previously described His₆-ERK2 pull-down assay. The level of Gα4d- subunit in the elutants was nearly equal that found in 1/10th of the total cell lysate (~9% yield) indicating a large reduction (~ 2.5-fold) in the association with His₆-ERK2 compared to the wild-type Gα4 subunit (Fig. 6). This result suggests the D-motif alteration impairs but does not eliminate the interaction between the Gα4 subunit and ERK2.

In yeast the Gpa1 subunit-Fus3 interaction is enhanced by the activated state of the Gpa1 subunit and so a constitutively active Gα4Q200L subunit (referred to as Gα4*) was also tested in the His₆-ERK2 pull-down assay. The level of Myc-Gα4* subunit in the elutants were similar to that found in 1/10th of the total cell lysate (~9% yield) indicating a reduced association with the His₆-ERK2 kinase compared to that of the Myc-Gα4 subunit (Fig. 6). This 3-fold difference in yield suggests the activation of the Gα4 subunit might reduce interactions with ERK2. To determine if the association of the constitutively active Gα4* is dependent on the D-motif, a mutant Gα4R107E, R108E, Q200L subunit (referred to as Gα4d-*) containing both the docking

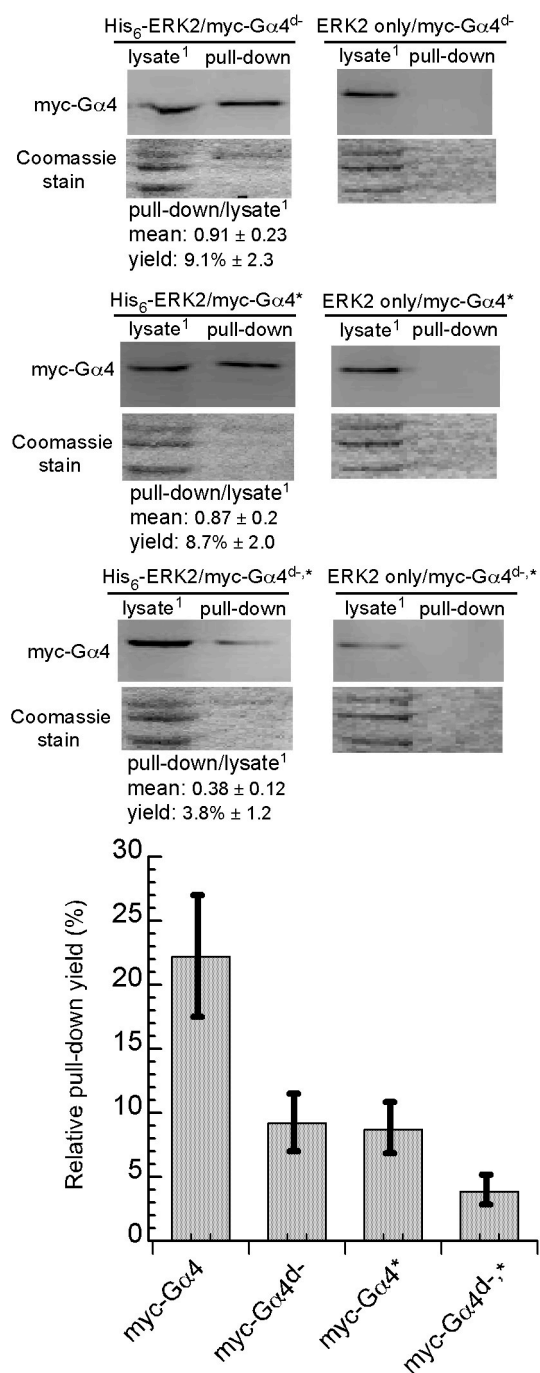


Fig. 6: Association of mutant Ga4 subunits with the ERK2 kinase in *Dictyostelium*. *erk2-* cells expressing either the His₆-ERK2 or ERK2 were transformed with a vector expressing either the Myc-Gα4 subunit, Myc-Gα4^{d-} subunit, Myc-Gα4^{*} subunit, or Myc-Gα4^{d-,*} subunit. The Co²⁺ resin pull-down assays were conducted and analyzed as described in Fig. 5. All blots, including

those in Fig. 5, were probed together in the same antibody solutions. Differences in Myc-tagged *Gα* subunit expression in untreated lysates likely reflect variations in the copy number of the *Gα* subunit expression vector. The same *erk2*- strains expressing either the His₆-ERK2 or ERK2 were used to generate all of the Myc-*Gα* subunit expressing strains to limit differences in His₆-ERK2 or ERK2 expression levels. 1 The lysate lane in each panel represents 1/10th of the cell extract used in the pull-down assay (pull-down). Coomassie stained gel is provided as a loading control (lower panels). The mean pull-down/lysate1 ratio of band intensity was determined from 3 experiments and then used to estimate the approximate pull-down yield of Myc-*Gα4* subunit from the cell extract. Average yields (including data in Fig. 5) displayed in bar graph where error bars represent standard deviations.

site alteration and constitutively active mutation was tested in the His₆-ERK2 pull-down assay. The levels of the Myc-tagged *Gα4d*-,* subunit associated with the elutants were nearly 3-fold less than that found in 1/10th of the total cell lysate (~4% yield) indicating a dependence on the D-motif. In all assays, the association of the mutant Myc-*Gα4* subunits with the elutants was dependent on the His₆-tagged ERK2.

Alteration of the D-motif in the Gα4 subunit affects MAPK activation

The *Gα4* subunit-ERK2 interaction might be important for the activation of ERK2 because of the requirement for *Gα4* subunit function in this process. The stimulation of *ga4*- cells expressing the *Gα4d*- subunit with folate resulted in MAPK activation but the level of pMAPKs was slightly reduced compared to that observed for *ga4*- cells expressing the wild-type *Gα4* subunit (Fig. 7). This slight reduction in MAPK activation suggests the *Gα4* D-motif only provides a minor contribution to the activated state of MAPKs, possibly through the interaction with ERK2. The expression of the *Gα4** subunit or the *Gα4d*-,* subunit in *ga4*- cells also allowed for MAPK activation but the activation was significantly reduced in both strains compared to cell

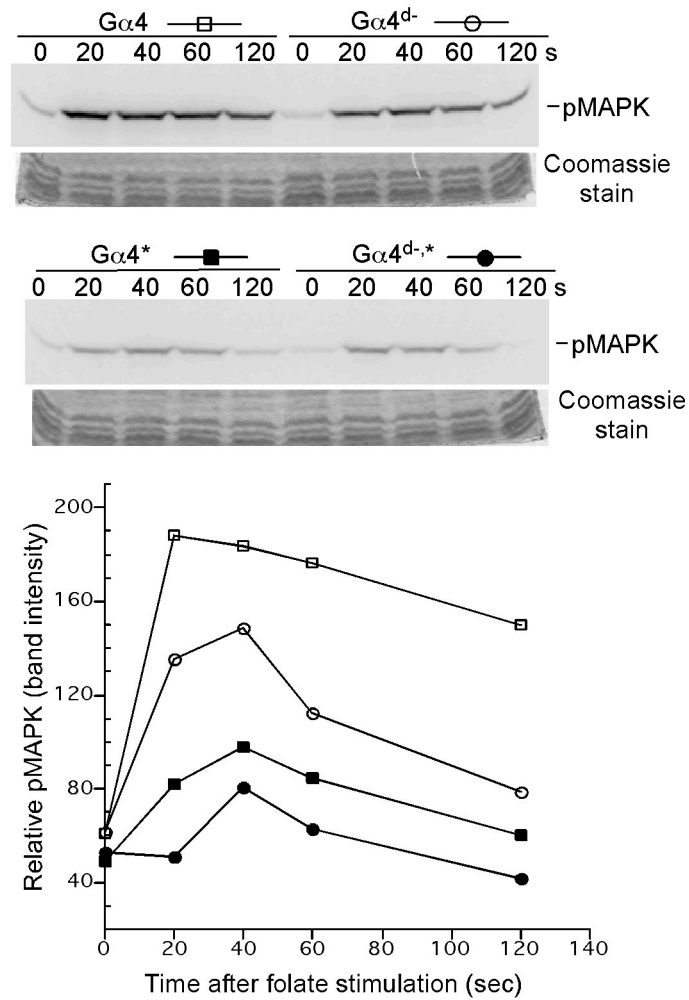


Fig. 7: Folate stimulated MAPK activation in *Ga4* D-motif mutants. *ga4-* cells expressing the *Ga4* subunit vector (*Ga4*), *Ga4d-* vector (*Ga4d-*), *Ga4** vector (*Ga4**), *Ga4d-,** (*Ga4d-,**) were prepared and assayed for folic acid stimulation of MAPK activation as described in Fig. 1. Cell extracts were subjected to immunoblot analysis and probed with α -phospho-p44/p42 MAPK antibody to detect the level of phosphorylated ERK2 (phospho-ERK2). The band (pMAPK) corresponds to phosphorylated ERK2 and possibly phosphorylated ERK1 kinase. The immunoblot analysis was identical for both gels and pMAPK band intensity is indicated graph below (symbols indicated above blots). Coomassie stained gel is provided as a loading control (lower panels). Data represents one of at least three experiments with similar results.

expressing the wild-type Gα4 subunit. The duration of MAPK activation was also noticeably shorter in cells expressing the Gα4* or Gα4d-,* subunit as the level of activated MAPK dropped considerably before the 2 min time point in these cells implying a possible reduction of MAPKK activity or increase in phosphatase activity.

Alteration of the D-motif in the Gα4 subunit affects development

To assess the function of the Gα4 D-motif during development, *ga4-* cells expressing the Gα4d- subunit or other Gα4 subunits were examined during development on nonnutrient agar. Starved *ga4-* cells aggregate and form anterior tips composed of prestalk cells but these tips extend from the mound leaving behind most prespore cells (Fig. 8A). As one tip moves away from the mound, subsequent tips can develop and repeat the process. Prespore cells are typically scattered throughout the mound but some move with the anterior prestalk cells (Hadwiger and Firtel, 1992; Hadwiger et al., 1994; Srinivasan et al., 1999). The expression of the wild-type Gα4 subunit in *ga4-* cells allows for wild-type development with slug migration and mature fruiting body formation (i.e., spore mass supported by a stalk) indicating a complete rescue of development. The *ga4-* cells expressing the Gα4d- subunit aggregated into mounds but this process often involved ring-shaped intermediates as the loose aggregates organized into more compact mounds. Similar aggregate reorganization was also observed in wild-type cells but this process was much more apparent in the aggregates expressing the Gα4d- subunit. The Gα4d- subunit expressing mounds developed into finger-like structures or slugs but these structures did not undergo culmination to form fruiting bodies. Rather, these structures developed a bulge near the anterior end of the slug where spores eventually formed. However the amount of spores observed by visual inspection in these structures was typically much lower than the spore

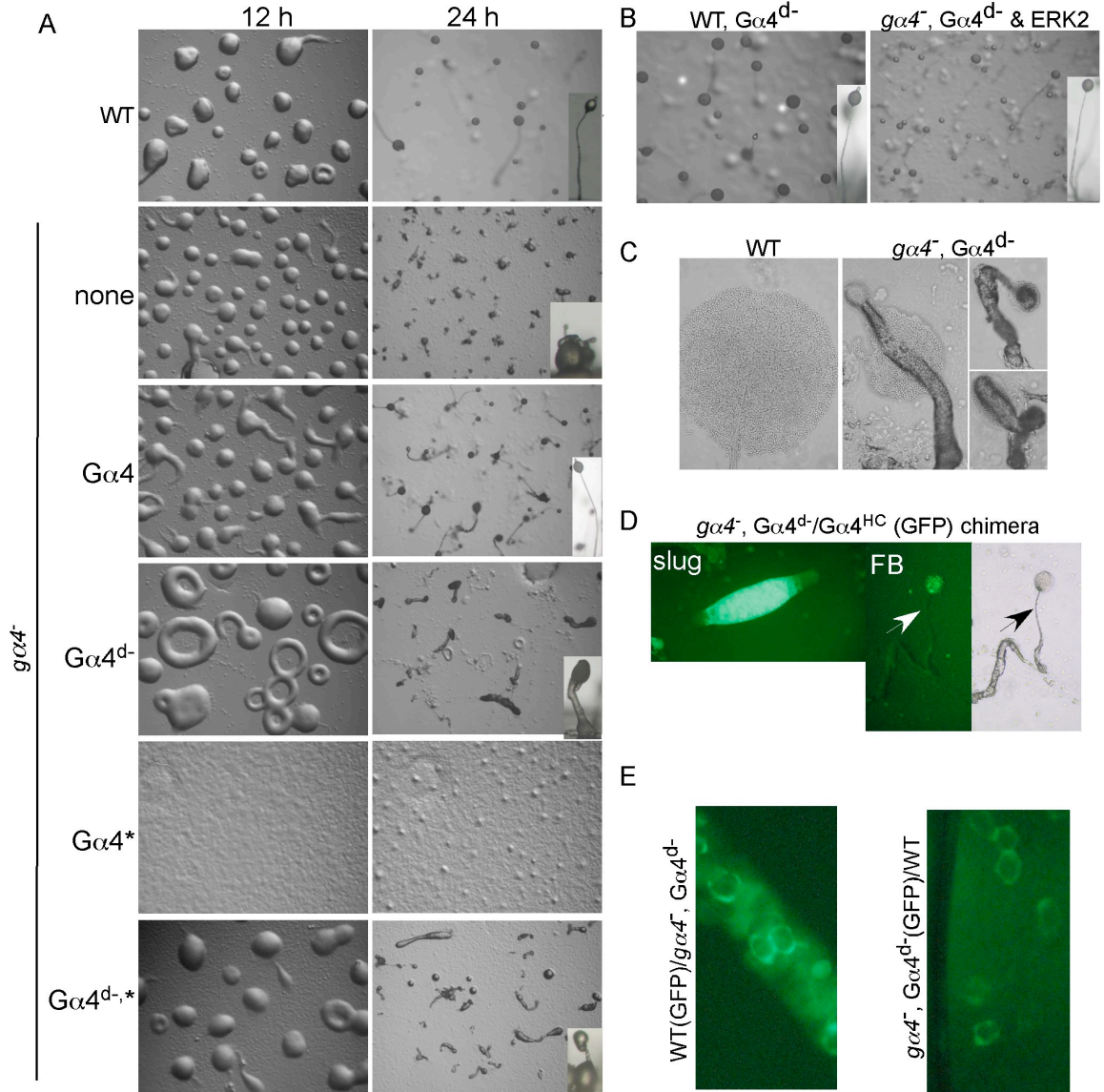


Fig. 8: Developmental phenotypes of *Ga4* D-motif mutants. Cells were grown and plated for development as described in the Materials and Methods section. (A) Development of wild-type cells (WT), *Ga4*⁻ cells without an expression vector (none) and *Ga4*⁻ cells with the *Ga4* subunit (*Ga4*), the *Ga4*^{d-} subunit (*Ga4*^{d-}), the *Ga4*^{*} subunit (*Ga4*^{*}), and *Ga4*^{d-,*} subunit (*Ga4*^{d-,*}) expression vectors. Photographs (10X magnification) were taken from overhead at 12 hrs and 24 hrs after cell plating. Insets on the second column are images of individual structures at 24 hrs photographed (20X magnification) from the side. Fruiting body structures of *Ga4*⁻ cells with the *Ga4* vector resembled those of wild-type fruiting bodies and *Ga4*⁻ cells with the *Ga4*^{*} vector did

not development beyond small aggregates. (B) Wild-type cells expressing the Ga4^{d-} vector (WT, Ga4^{d-}), Ga4⁻ cells expressing the Ga4^{d-} subunit with increased selective pressure (Ga4⁻, Ga4^{d-}, high G418), and Ga4⁻ cells expressing the Ga4^{d-} and ERK2 expression vectors (Ga4⁻, Ga4^{d-}, ERK2) at 24 hrs of development. Insets were created as described above. (C) Dispersed spores from the top of a wild-type (WT) fruiting body and the internal spore region of Ga4⁻ aggregates expressing the Ga4^{d-} subunit. (D) Chimeras of Ga4⁻ cells expressing the Ga4^{d-} subunit and Ga4^{HC} cells expressing GFP mixed in a 1:1 ratio. GFP fluorescence located in the prespore region of migratory a slug (left panel - 20X magnification). GFP fluorescence (center panel - 10X magnification) located in spore mass but not in stalk (designated by arrow) of fruiting body lying on agar surface (bright field image in right panel). (E) GFP-expressing vacuolated stalk cells in chimeras composed of Ga4⁻ cells expressing the Ga4^{d-} subunit and wild-type (WT) cells (1:1) ratio. Fluorescent wild-type stalk cells in upper stalk region (left panel) and fluorescent Ga4⁻ stalk cells expressing the Ga4^{d-} subunit in the lower stalk region.

production in a fruiting body of wild-type cells (Fig. 8C). The anterior tips of the Ga4d- expressing structures remained intact but often drooped over as the bulge of spores formed. Wild-type cells expressing the Ga4d- subunit displayed normal development with fruiting body formation indicating that Ga4d- subunit does not affect the development of cells expressing a wild-type Ga4 subunit (Fig. 8B). Interestingly, ga4- aggregates without any Ga4 subunit expression vector can achieve structures similar to the Ga4d- aggregates when development occurs after cells have grown on bacterial lawns or in shaking bacterial cultures (data not shown). The basis of this conditional developmental morphology of ga4- cells is not known but the development of ga4- cells expressing the Ga4d- subunit does not appear to be affected by whether cells are grown in axenic medium or on bacteria (data not shown).

Expression of the constitutively active Ga4* subunit in ga4- cells resulted in impaired aggregation and so development did not proceed beyond loosely-formed small aggregates (Fig.

5A). This phenotype was similar to the aggregation defect described for Gα4* subunit expression from the Gα4 promoter and this aggregation deficiency was previously attributed to the inhibition of cAMP chemotaxis (Srinivasan et al., 1999). Similar aggregation defects have also been observed for other constitutively active Gα subunits, including Gα2* and Gα5* subunits. The expression of the Gα4d-* subunit in *ga4-* cells did not inhibit aggregation as did the Gα4* subunit. Rather, the Gα4d-* subunit expressing cells formed aggregates that produced extended tip structures similar to that of *ga4-* cells. The ability of these cells to aggregate suggests that the D-motif alteration removes the inhibition of aggregation associated with the constitutively active Gα4* subunit.

Development of cells expressing the Gα4d- subunit can be rescued by increased ERK2 or Gα4d- subunit expression

Protein-protein interactions that are compromised due to changes in protein structure can often be enhanced through increasing the concentration of one or both proteins (Hadwiger et al., 1989a; Hadwiger et al., 1989b). Consistent with this idea, increased selection (> 10 µg/ml G418) for the Gα4d- subunit expression vector in *ga4-* cells allowed for fruiting body development (data not shown). An increase in ERK2 expression might also stabilize Gα4d- subunit-MAPK interactions and so to test this idea *ga4-* cells expressing the Gα4d- subunit without G418 selection were co-transformed with an ERK2 expression vector and a blasticidin S resistance vector. Transformants were selected using only blasticidin S to avoid increasing Gα4d- subunit expression. Multiple clones were found that could complete the fruiting body process suggesting that ERK2 over-expression can rescue developmental morphogenesis (Fig. 5B). The rescue of developmental morphogenesis with increased Gα4d- subunit or ERK2 expression implies that the developmental defects associated with the Gα4d- subunit are likely due to impaired Gα4 subunit-MAPK interactions.

Defects in Gα4d- stalk development are not cell autonomous

The inability of *ga4-* cells expressing the Gα4d- subunit to undergo fruiting body development suggests possible defects in both spore and stalk cell development. However, a previous analysis of *ga4-* cells has indicated that Gα4 function is necessary primarily for spore development and that the lack of stalk development is due to the lack of extracellular signaling (Hadwiger and Firtel, 1992; Hadwiger and Srinivasan, 1999). To determine if *ga4-* cells expressing the Gα4d- subunit can form stalks, these cells were mixed with Gα4HC cells expressing GFP to form chimeric aggregates. Previous reports have shown that Gα4HC cells are capable of forming spores but not stalk cells in chimeras with wild-type or *ga4-* cells (Hadwiger and Srinivasan, 1999). The chimeric aggregates of Gα4d- subunit expressing cells and Gα4HC cells were capable of forming fruiting bodies but none of the Gα4HC cells were found in the stalk regions indicating that the stalks are composed of Gα4d- subunit expressing cells (Fig 8D). This result suggests the absence of stalk formation in aggregates of *ga4-* cells expressing the Gα4d- subunit is due to the lack of an intercellular signal that can be provided by cells with a wild-type Gα4 subunit.

Stalk formation was also observed in chimeras of *ga4-* cells expressing the Gα4d- subunit mixed with wild-type cells. Either the Gα4d- expressing cells or the wild-type cells were labeled using a GFP expression vector and the stalks of the resulting chimeric fruiting bodies were examined for fluorescent cells. Both wild-type and Gα4d- expressing cells were found in the stalks and capable of developing into vacuolated stalk cells in which the GFP was excluded from the center of the cell (Fig. 8E). The stalks of these chimeras typically contained more wild-type cells than Gα4d- expressing cells even though the chimeric aggregates contained equal amounts of both strains implying that Gα4d- expressing cells might have a reduced preference for stalk cell development.

Chemotaxis to folate

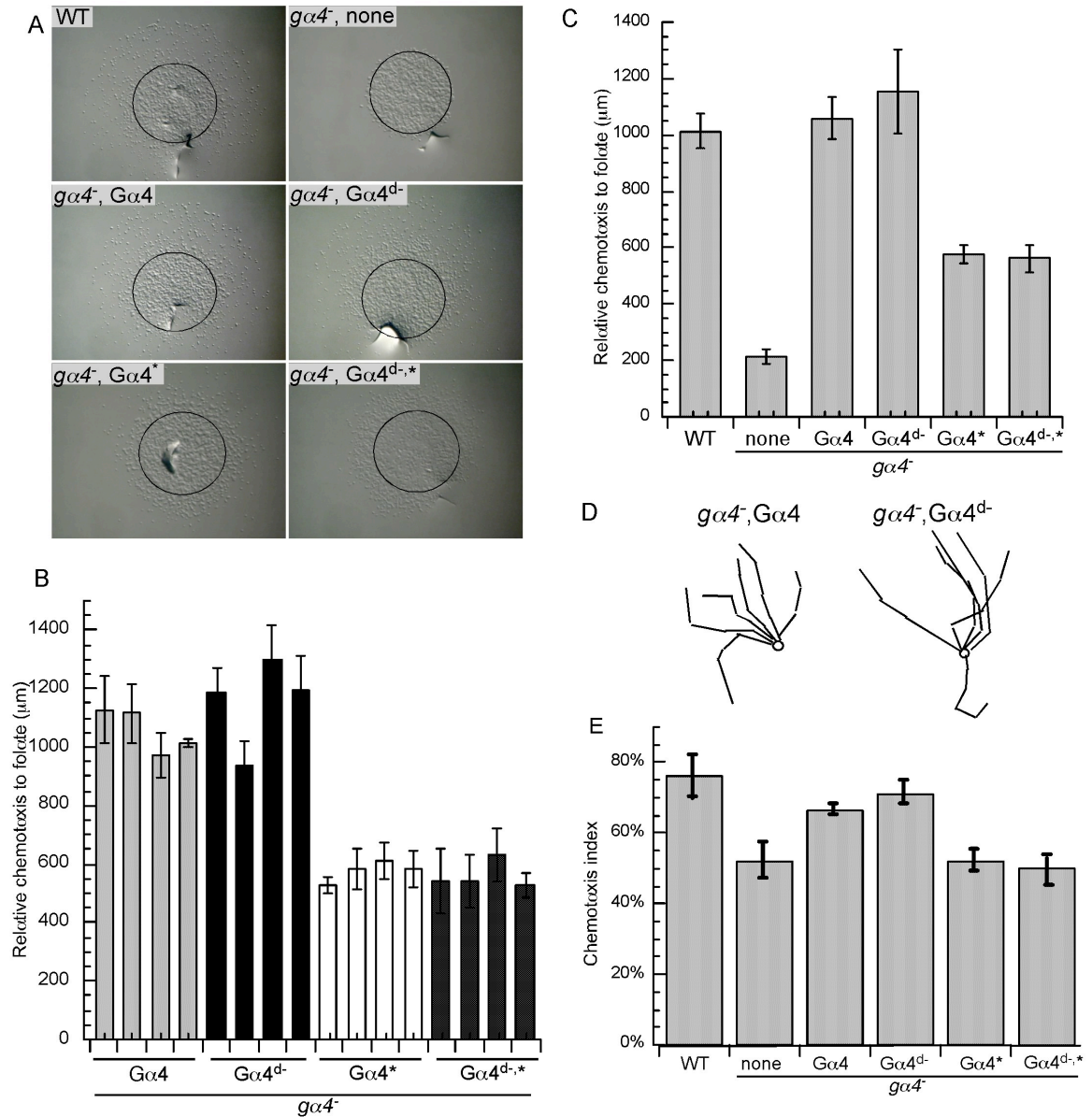


Fig. 9: Chemotaxis of Ga4 D-motif mutants. Wild-type cells (WT), Ga4⁻ cells without an expression vector (none) and Ga4⁻ cells with the Ga4 (Ga4), Ga4^{d-} (Ga4^{d-}), Ga4* (Ga4*), and Ga4^{d-,*} (Ga4^{d-,*}) subunit expression vectors were prepared and plated for chemotaxis as described in the Materials and methods section. (A) Micrographs of chemotaxis assays. In all panels the source of folate is above (upper side) the cell droplet. Deformations in agar were created by a needle and used as a reference points to align images captured at different times

during the assay. Cell distribution was photographed 3 hrs later using a dissecting microscope (20X magnification) and indirect light. The drawn circle indicates the original cell droplet perimeter. Each micrograph is representative of several assays with similar results. (B) Chemotaxis distance traveled by individual clones after 3 hrs. The maximum distance from the original cell droplet perimeter to the leading edge of migrating cells in the direction of the folate source was recorded. Each value is the mean of six independent cell droplets and the error represents the standard deviation. (C) The average chemotaxis distance traveled by individual clones described in part (B) compared to wild-type and $G\alpha 4^-$ cells. Each value is the mean of four clones and the error represents the standard deviation. (D) Migration maps of $g\alpha 4^-$ cells with or without $G\alpha$ subunit expression vectors. Low density cell suspension of 5×10^6 cells/ml were plated for chemotaxis assays and then 2.5 hours after the initial plating time-lapse photography was used to follow the movement of cells over a 30 minute period. Images corresponding to 5 min intervals were used to trace the movement of individual cells. Only cells with minimal or no cell-cell contact were chosen for creating maps. Multiple paths representing different cells of the each strain are shown in each panel and the initial point of each path is designated with an open circle. All initial points were positioned at a central location in each panel with the orientation of the folate source at the top of each panel. Data is representative of one of two experiments with similar results. (E) Chemotaxis index of cells after 3 hrs. Chemotaxis index was determined as the number cells outside the original drop perimeter on the side facing the source of folate divided by the total number of cells outside the original droplet perimeter. Each index was determined as the mean of 6 separate droplets and the error bars represent the standard deviation.

Previous studies have shown the $G\alpha 4$ subunit is essential for chemotactic responses to folate and related pterin compounds allowing cells to search out nearby bacterial food sources (Hadwiger et al., 1994). MAPK function has also been implicated in folate chemotaxis as *erk2*-cells have been reported to have reduced chemotactic responses to folate (Wang et al., 1998). To

determine if the G α 4 D-motif is important for chemotactic responses, *ga4*- cells expressing the G α 4 or G α 4d- subunit were analyzed for the ability to chemotax to folate in an above agar assay. The expression of the G α 4d- subunit in *ga4*- cells restored folate chemotaxis similar to that observed for cells expressing the G α 4 subunit (Fig. 9A-C). The analysis of individual cell movement using time-lapse photography also suggested G α 4d- expressing cells move similar to those cells expressing the G α 4 subunit (Fig. 9D). The chemotaxis index of cells expressing the G α 4d- was also as high as that observed for cells expressing the G α 4 subunit but less than that for wild-type cells (Fig. 9E).

The chemotaxis of cells expressing the G α 4* subunit to folate was significantly less than that observed for cells expressing the wild-type G α 4 subunit (Fig. 9A-C). However, expression of the G α 4* subunit increased the overall movement of cells compared to *ga4*- cells and the direction of cell movement appeared to be random as indicated by the chemotaxis index (Fig. 9E). This inability to direct cell movement up the folate gradient suggests the G α 4* subunit might affect the reception of the chemotactic signal or interfere with the ability of the cell to establish directed movement up the chemotactic gradient. However, the nearly equal movement of cells regardless of the proximity to the source of folate implies that cell movement does not depend on the folate stimulus. A similar chemotaxis phenotype was observed for *ga4*- cells expressing the G α 4d-,* subunit suggesting the D-motif alteration does not change the cell movement phenotype associated with the G α 4* subunit (Fig. 9A&B).

Discussion

Stimulated G protein-coupled receptors are known to activate MAPKs through transduction pathways that involve G $\beta\gamma$ dimers and Ras proteins and this mechanism also exists in *Dictyostelium* as the G β subunit and rasD protein can be necessary for MAPK activation (Belcheva and Coscia, 2002; Chen and Thorner, 2007; Knetsch et al., 1996; Maeda and Firtel,

1997). In response to folate, the $G\alpha 4$ subunit presumably triggers MAPK activation through the release of the $G\beta\gamma$ dimer as suggested by the requirement of $G\beta$ for this activation. Our results indicate the $G\alpha 4$ subunit can also have a minor effect on MAPK activation through direct interactions with a MAPK using a D-motif. The contribution of the $G\alpha 4$ subunit D-motif to MAPK activation is difficult to assess because the $G\alpha 4d$ - alteration only reduced but did not completely eliminate $G\alpha 4$ subunit-ERK2 interactions. The recruitment of a MAPK to the $G\alpha 4$ subunit D-motif could localize the MAPK to a region with enhanced MAPKK activity or reduced phosphatase activity. While the relationship of $G\alpha 4$ subunit-MAPK binding to MAPK activation is not completely resolved, it is clear that alterations in the $G\alpha 4$ subunit D-motif can have important developmental consequences with respect to cell differentiation and morphogenesis.

The location of the D-motif in the $G\alpha 4$ subunit is unusual compared to most other *Dictyostelium*, yeast, and human $G\alpha$ subunits analyzed for D-motifs but this feature might be associated with the ability of the $G\alpha 4$ subunit to facilitate MAPK activation whereas other $G\alpha$ subunits, such as the *Dictyostelium* $G\alpha 2$ subunit or the yeast $G\alpha 1$, are not required for MAPK activation. The amino terminal location of D-motifs found in other $G\alpha$ subunits might prevent access to MAPK docking unless the $G\alpha$ subunit is activated, as previously demonstrated for the yeast $G\alpha 1$ subunit-Fus3 interaction, and this restriction might limit the possible functions associated with the interaction (Metodiev et al., 2002). The availability of the D-motif, even when the $G\alpha 4$ subunit is associated with the receptor and $G\beta\gamma$ dimer, could potentially localize or position a MAPK prior to the stimulation of the pathway. In support of this idea, cells expressing the constitutively active $G\alpha 4^*$ subunit had reduced ERK2 interactions and MAPK activation compared to cells expressing the wild-type $G\alpha 4$ subunit. The $G\alpha 4$ subunit, like many $G\alpha$ subunits, has a cysteine residue near the amino terminus that is likely to be palmitoylated as a mechanism to keep the $G\alpha 4$ subunit anchored in the membrane and so an interactive MAPK would likely be tethered near the membrane (Wedegaertner, 1998). The $G\alpha 4$ D-motif is predicted

to be distal to the membrane surface whereas the amino terminal D-motifs of other G α subunits are located proximal to the membrane surface. Such differences in D-motif location could impact MAPK orientation with respect to other regulatory proteins (e.g., MAPKKs and phosphatases) or MAPK substrates at the membrane surface. Alternatively, all of the G α subunits with D-motifs might serve a common function such as restricting the movement of MAPK into the nucleus.

The *ga4*- cells expressing the G α 4d- subunit, like *ga4*- cells, have reduced spore production and deficiencies in signals for stalk formation suggesting that G α 4 subunit-MAPK interactions are important for these developmental processes. ERK2 is a likely candidate for these interactions because previous chimera development and gene expression studies indicate ERK2 is necessary for prespore development (Gaskins et al., 1996). As for the production of extracellular signals for stalk formation, a recent report has inferred G α 4 function in the production of extracellular signals such as GABA and SDF-2 necessary for spore development and so perhaps these or other signals also coordinate stalk formation during the final stages of spore development (Anjard et al., 2009). The developmental defects observed for G α 4d- expressing aggregates are not likely due to insufficient of G α 4d- subunit expression because the developmental defects occur with cells that are completely rescued in folate chemotaxis. Rather, defects in development might reflect specific G α 4 functions that depend on MAPK interactions.

One major developmental distinction between *ga4*- cells expressing the G α 4d- subunit compared to *ga4*- cells is that a greater proportion of the mound moves with the anterior tip, allowing the formation of a slug rather than just a tip extension. The mechanism for the movement of cells in the central and posterior regions of aggregates might share some similarities to the chemotactic movement of cells to folate since neither movement requires G α 4 subunit-MAPK interactions. The lack of a G α 4-MAPK interaction for cell movement was not expected because an earlier report indicated *erk2*- cells have reduced folate chemotaxis (Wang et al., 1998). We have confirmed this defect in recently created *erk2*- strains but found only a slight

reduction in folate chemotaxis using the same assay described in the Materials and methods section (Hadwiger, unpublished data). We have also examined *erk1*- cells and found these cells also chemotax to folate suggesting neither of the MAPKs is essential for this response. Therefore, the ability of *ga4*- cells expressing the $G\alpha 4$ - subunit to chemotax to folate or to follow the anterior tip in multicellular development is consistent with MAPKs playing only a minor role in chemotactic responses to folate.

The expression of the constitutively active $G\alpha 4^*$ subunit results in an aggregation defect that disappears if the D-motif is altered suggesting the defect in aggregation requires $G\alpha 4$ subunit-MAPK interaction. Previous studies have shown that the $G\alpha 4^*$ subunit inhibits cAMP accumulation and ERK2 increases cAMP accumulation in cells stimulated by extracellular cAMP (Maeda et al., 2004; Segall et al., 1995; Srinivasan et al., 1999). These observations suggest the $G\alpha 4^*$ subunit impacts cell aggregation and cAMP accumulation possibly through interactions with ERK2. Our results indicate the $G\alpha 4^*$ subunit has a relatively weak association with ERK2 but this association is further reduced by alterations in the D-motif suggesting that the $G\alpha 4^*$ subunit could possibly inhibit ERK2 to limit the accumulation of cAMP during cell aggregation. Constitutively active $G\alpha 2^*$ and $G\alpha 5^*$ subunits also interfere with cell aggregation and both of these subunits contain putative D-motifs at their amino terminus (Okaichi et al., 1992; Srinivasan et al., 1999). Alteration of the amino terminal D-motif reduces the aggregation defect associated with the constitutively active $G\alpha 5^*$ subunit (Raisley, et al., unpublished data).

Both folate and cAMP stimulate MAPK activation in *Dictyostelium* but many distinctions are emerging as these pathways become more characterized. Only folate-stimulated MAPK activation requires both the $G\alpha 4$ and $G\beta$ subunit function whereas neither the $G\alpha 2$ or $G\beta$ subunit are required in the response to cAMP stimulation (Brzostowski and Kimmel, 2006; Maeda et al., 1996; Maeda and Firtel, 1997). These results are surprising because the $G\alpha 2$ and $G\beta$ subunits have been shown essential for cAMP chemotactic responses and none of the other 11 $G\alpha$ subunits

have been directly associated with cAMP receptor. Even though the mechanism for cAMP-stimulated MAPK activation does not require the Gα2 subunit, the putative D-motif near the amino terminus of this subunit could possibly tether MAPKs and regulate downstream responses to cAMP. Defining the effect of potential Gα2 subunit-MAPK interactions on MAPK regulation and function will likely provide additional insights into the distinctions between folate and cAMP-stimulated MAPK regulation.

CHAPTER III

Gα5 SUBUNIT-MEDIATED SIGNALING REQUIRES A D-MOTIF AND THE MAPK ERK1 IN *DICTYOSTELIUM*

Abstract

The *Dictyostelium* Gα5 subunit has been shown to reduce cell viability, inhibit folate chemotaxis and to accelerate tip morphogenesis and gene expression during multicellular development. Alteration of the D-motif [mitogen-activated protein kinase (MAPK) docking site] at the amino terminus of the Gα5 subunit or the loss of the MAPK ERK1 diminished the lethality associated with the over-expression or constitutive activation of the Gα5 subunit. The amino terminal D-motif of the Gα5 subunit was also found necessary for the reduced cell size, small aggregate formation and precocious developmental gene expression associated with Gα5 subunit over-expression. This D-motif also contributed to the aggregation delay in cells expressing a constitutively-active Gα5 subunit but the D-motif was not necessary for the inhibition of folate chemotaxis. These results suggest that the amino terminal D-motif is required for some but not all phenotypes associated with elevated Gα5 subunit functions during growth and development and that ERK1 can function in Gα5 subunit-mediated signal transduction.

Introduction

Signal transduction through G protein-coupled receptors activates many downstream regulatory proteins and leads to a wide variety of cellular response. Receptors and sometimes

G proteins are pathway specific but many other signaling proteins function in multiple G protein-mediated pathways suggesting that signal-specific responses involve receptor and/or G protein interactions with specific downstream signaling components. The soil amoebae *Dictyostelium discoideum* uses several different G protein-mediated pathways during growth and development to regulate cell movement and differentiation and therefore this organism offers a useful system to study the interactions between pathway-specific and common signaling components during development (Firtel, 1996; Firtel et al., 1989)

The *Dictyostelium* G α 5 subunit functions in a signaling pathway that inhibits folate chemotaxis, promotes small aggregate size and accelerates tip formation and gene expression during development (Hadwiger et al., 1996; Natarajan et al., 2000). The activator and receptor for this pathway remain to be determined but a recent study of chimeric G α subunits suggests the functional specificity of the G α 5 subunit is not limited to the specificity of receptor coupling but requires interactions with other signaling components (Hadwiger, 2007). The G α 5 subunit primary sequence is closely related to the G α 4 subunit, a subunit required for folate chemotaxis, except for a region near the amino terminus of the G α 5 subunit where a putative mitogen-activated protein kinase (MAPK) docking site, known as a D-motif, exists suggesting that interactions with MAPKs might be important for G α 5 specific responses (Hadwiger, 2007; Remenyi et al., 2005). An interaction between the amino terminal D-motif of a G α subunit and a MAPK has been previously reported in the budding yeast *Saccharomyces cerevisiae*, where the interaction of the Gpa1 G α subunit and the Fus3 MAPK facilitates the G α subunit mediated-adaptation to pheromone stimulation (Blackwell et al., 2003; Metodiev et al., 2002). In *Dictyostelium*, a D-motif in the central region of the G α 4 subunit has been recently shown to be important for interactions with the ERK2 MAPK and for G α 4 function late in the culmination stage of development (Nguyen and Hadwiger, 2009).

The *Dictyostelium* genome contains genes for only two MAPKs, ERK1 and ERK2 (extracellular-signal regulated kinases), and both kinases can be activated in G protein-mediated signaling pathways. ERK1 can be activated in response to cAMP and loss of ERK1 results in small aggregate size during development (Sobko *et al.*, 2002). ERK2 is rapidly phosphorylated and activated in response to either cAMP or folate and the loss of ERK2 prevents aggregation and reduces prespore gene expression during development (Brzostowski & Kimmel, 2006; Knetsch *et al.*, 1996; Maeda *et al.*, 1996; Maeda & Firtel, 1997). Both ERK1 and ERK2 contain sequence similarity with MAPKs from other organisms in a common docking (CD) domain suggesting they also interact with proteins containing D-motifs (Gaskins *et al.*, 1994; Segall *et al.*, 1995).

In this study, the role of the D-motif at the amino terminus of the G α 5 subunit was explored by analyzing the phenotypes of G α 5 subunits with alterations in this D-motif. Growth and developmental phenotypes associated with G α 5 subunit over-expression or constitutive activation were found to depend on the amino terminal D-motif. Sensitivity to elevated G α 5 subunit expression and function during vegetative growth was found to be absent in *erk1*⁻ but not *erk2*⁻ cells indicating a specific requirement of ERK1 in G α 5 subunit-mediated signaling. The G α 5 subunit D-motif alteration did not eliminate the inhibition of folate chemotaxis suggesting this D-motif plays an important role in some but not all G α 5 functions during the growth and development.

Materials and methods

Strains and media

All of the *Dictyostelium* strains were isogenic to the wild-type strain KAx3 except where noted in the mutants. The G α 5⁻ strain (JH257) has been previously described (Hadwiger *et al.*, 1996). The *erk2*⁻ strain (JH697) was created from the JH8 strain using the pAK240Cla gene disruption construct previously described (Segall *et al.*, 1995). The pAK240Cla gene construct

was kindly provided by J. Segall (Albert Einstein College of Medicine, Bronx, NY) and the *Dictyostelium* Stock Center. Disruption of the *ERK2* (*erkB*) locus was verified by genomic DNA blot analysis (data not shown). The *erkI*⁻ strain (JH1058) was created in KAx3 cells by disrupting the *ERK1* (*erkA*) gene with an insertion of the blasticidin S resistance gene as described (Nguyen et al., unpublished data). Cells were grown in axenic HL5 medium or on bacterial lawns. DNA vectors were electroporated into cells as previously described (Hadwiger, 2007). Transformed cells were grown in medium containing G418 and drug selection was removed several hours prior to analysis. The modeling of the Gα5 subunit structure was conducted using the Swiss-Model program and displayed using the PyMOL Molecular Graphics System 2008 (DeLano Scientific).

Recombinant DNA constructs

The wild-type Gα5 gene, replacing the GFP gene, was expressed from the *act15* promoter (pJH510) in the pTX-GFP vector (Ddp1-based plasmid) as previously described (Hadwiger, 2007). Alterations to the Gα5 subunit D-motif, Gα5^{K10E,K11E} subunit (designated Gα5^{d-}), were created in a *HindIII/PstI* fragment of the *Gα5* gene in the vector pT7T318U (Pharmacia) by PCR mutagenesis of the entire vector using the following overlapping oligonucleotides: (sense strand: 5'-gcgccgATCGATATCAATTAAGAAAAGAAGAAGGAAG) and (antisense strand: 5'-gcgccgaTCGATGTCTCTTGATTcCTcTGCTTCAATTGTTAATATACAACCC) (lower case nucleotides differ from those of the template sequence). These mutations were confirmed by sequence analysis and this mutant segment of *Gα5* gene was used to replace the same segment of the wild-type *Gα5* gene in the expression vectors. The constitutively-active Gα5^{Q198L} (designated Gα5^{*}) subunit expressed from the *act15* promoter (pBR515) was created by replacing a *PstI/BclI* fragment of the wild-type *Gα5* constructs with a fragment from a *Gα5*^{Q198L} gene previously created in a genomic fragment (Hadwiger et al., 1996). This mutation was also combined with the mutations in the Gα5 D-motif to create the combined mutant Gα5^{d-*} subunit expression vector (pBR558). To create this gene the *HindIII/PstI* fragment of the *Gα5*^{d-} gene was used to

replace the beginning of the *Gα5** open reading frame. All *Gα5* expression constructs were also created in the pDXA-GFP2 vector (Ddp2-based plasmid) that integrates into the genome unless co-electroporated with the pREP vector because plasmid copy number can sometimes influence the phenotypes of cells (Levi et al., 2000). Similar results were obtained with both vector systems but only the results from the pTX vector are shown.

Cell growth and drug selection

Comparison of cell viability for cells expressing wild-type and mutant *Gα5* subunits was assessed by either counting the number of transformants after electroporation or growing established strains in different concentrations of drug selection. Briefly, cells were electroporated with equal amounts of wild-type and mutant *Gα5* expression vectors, plated onto petri dishes with HL5 medium, and after 24 hrs the cells were subjected to G418 drug selection ranging from 1-10 mg/ml. Drug resistant colonies were counted 10 -14 days after drug selection.

Comparisons of cell size were accomplished by growing equal concentrations of different strains, harvesting cells by centrifugation, washing cells in phosphate buffer and weighing cell pellets. Protein content of cells treated with Triton X-100 (0.01% final concentration) was conducted using a Bradford protein assay (Bradford, 1976).

Development and chemotaxis assays

Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10^8 cells/ml or 2×10^7 cells/ml), before spotting on nonnutrient plates (phosphate buffer, 1.5% agar) for development. Alternatively, cells were spotted on to Whatman-50 filters soaked in phosphate buffer for developmental RNA isolation. Total RNA was isolated, separated on formaldehyde-denaturing agarose gels, and blotted to membranes as described (Hadwiger *et al.*, 1996). Radioactive probes were created using the random primer method as described

(Feinberg and Vogelstein, 1983). Hybridized blots were detected using autoradiography. Ribosomal RNA bands on blots were detected by ultraviolet-light shadowing before hybridization to verify consistency in sample loading and blotting.

Chemotaxis assays were performed by spotting droplets (~ 0.5 ml) of cell suspensions (10^8 cells/ml) on nonnutrient plates allowing absorption of liquid into the agar followed by the spotting of 1 ml droplets of 1 mM folate solutions approximately 2-3 mm away from the cell droplet as previously described (Hadwiger, 2007). Images of the cell droplet perimeter and the location of the leading edge of cells migrating toward the folate source after 3 hrs were recorded using a dissecting microscope and a digital camera. Scarring of the agar surface with a needle allowed for the early and late images to be accurately aligned.

Results

Docking site motifs in Ga subunits

A region near the amino terminus of the $G\alpha 5$ subunit was identified as a potential site of functional specificity due to sequence diversity with respect to the $G\alpha 2$ and $G\alpha 4$ subunits and further inspection of this region revealed a D-motif (Hadwiger, 2007). This D-motif is located near a highly conserved guanine nucleotide interaction domain and at a position analogous to the previously characterized D-motif of the yeast mating response Ga subunit, Gpa1 (Holbrook and Kim, 1989; Metodiev et al., 2002) (Fig. 1A). The sequence of this motif is similar to the consensus sequence [K/R]₁₋₃-X₁₋₆-[L/I]-X-[L/I] found in MAP2Ks and other proteins that dock with MAPKs (Grewal et al., 2006). The $G\alpha 5$ D-motif contains a tyrosine residue (Y20) at a position typically occupied by a leucine or isoleucine residue but the aromatic ring of the tyrosine side group likely contributes to the hydrophobic nature of this region. A survey of the other eleven *Dictyostelium* Ga subunit sequences revealed putative D-motifs on the $G\alpha 2$, $G\alpha 3$, and $G\alpha 11$ subunits in this same region suggesting that MAPKs might interact with multiple $G\alpha$

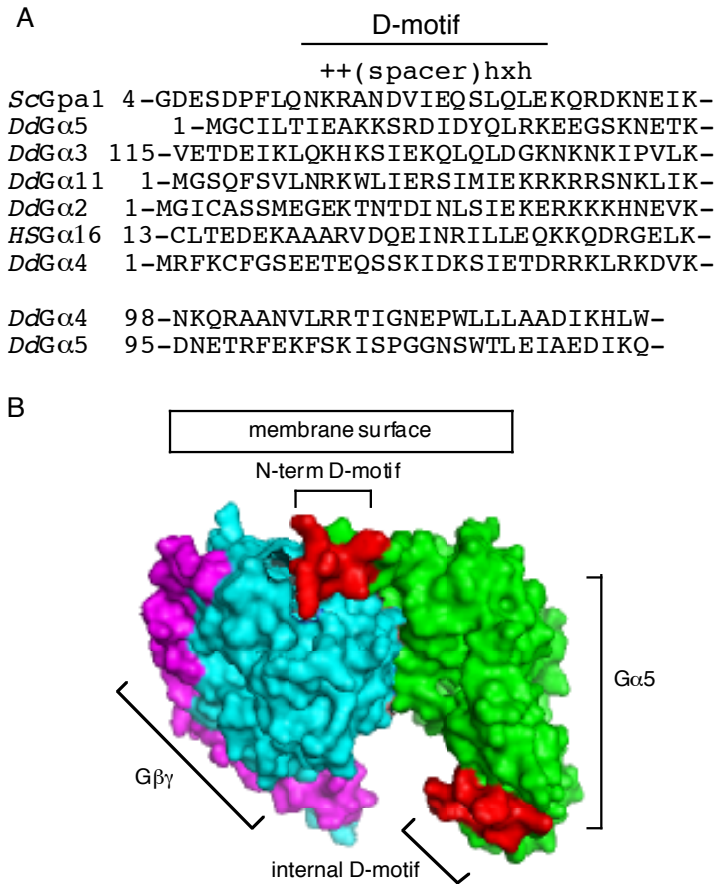


Fig. 10: Location of D-motifs in the Ga5 subunit. (A) Alignment of D-motifs in Ga subunits. Primary sequence of the yeast (Sc) Gpa1, Dictyostelium (Dd) Ga5, Ga3, Ga11, Ga2, Ga4, and human (Hs) Ga16 subunits starting at the designated residue number. Consensus sequence for D-motifs is designated in the top row with basic (+), hydrophobic (h), and nonconserved (x) residues. D-motif residues in Ga subunits are shaded. Sequence information for the Dictyostelium Ga subunits was obtained using dictyBase (<http://www.dictybase.org/>). (B) Modeling of Ga5 subunit structure and D-motifs. A structure model of the Ga5 subunit was created using the SWISS-MODEL workspace and PyMOL program and the structure was modeled using the determined structure of the mammalian heterotrimeric G protein consisting of Gai1, Gβ1, and Gγ2 subunits with GDP bound. The Ga5 subunit (green), Gβ subunit (blue), and Gγ subunit (magenta) are shown with respect to the membrane surface. The amino terminal (N-term) and internal D-motifs of the Ga5 subunit are indicated (red) on the Ga5 subunit.

subunits in this organism. The human $G\alpha 16$ (murine $G\alpha 15$) subunit also has a D-motif near the amino terminus (Wilkie et al., 1992). The *Dictyostelium* $G\alpha 4$ subunit does not contain a D-motif near the amino terminus but does have a D-motif at a more internal site (residues 117-134) where there is a second possible D-motif in the $G\alpha 5$ subunit. Both the amino terminal and internal D-motifs of the $G\alpha 5$ subunit are predicted to be located in exposed regions of the subunit based on structural modeling of the $G\alpha 5$ subunit (Fig. 10B).

Alteration of the amino terminal D-motif reduces the lethal effects of $G\alpha 5$ subunit over-expression

To determine if the amino terminal D-motif in the $G\alpha 5$ subunit plays a role in G protein-mediated signaling, the first two lysine residues of this motif were changed to glutamate because an analogous change in the yeast $G\alpha 1$ subunit was previously reported to reduce both $G\alpha 1$ -Fus3 interactions and $G\alpha 1$ -mediated down regulation of the pheromone response (Metodiev *et al.*, 2002). The $G\alpha 5$ D-motif mutant subunit, $G\alpha 5^{K10E,K11E}$ ($G\alpha 5^d$) and the wild-type $G\alpha 5$ subunit were electroporated into $G\alpha 5^-$ and wild-type cells to examine phenotypes associated with D-motif alterations. Electroporation of the $G\alpha 5^d$ subunit vector into cells typically resulted in at least a 10-fold higher number of viable transformants compared to the wild-type $G\alpha 5$ subunit unless the drug selection for the vectors was reduced to less than 2 mg/ml G418. Increasing the G418 concentration resulted in death of clonal transformants expressing the $G\alpha 5$ but not the $G\alpha 5^d$ subunit suggesting the $G\alpha 5^d$ subunit is less detrimental to vegetative growth than the wild-type $G\alpha 5$ subunit.

Stimulation of the $G\alpha 5$ mediated pathway with an exogenous signal has not been possible because the signal for this pathway remains unknown. However, the mutant $G\alpha 5^{Q198L}$ ($G\alpha 5^*$) subunit contains an alteration known to impair the GTPase activity of $G\alpha$ subunits resulting in a constitutively-active $G\alpha$ subunit (Hadwiger *et al.*, 1996). Expression of the $G\alpha 5^*$ subunit

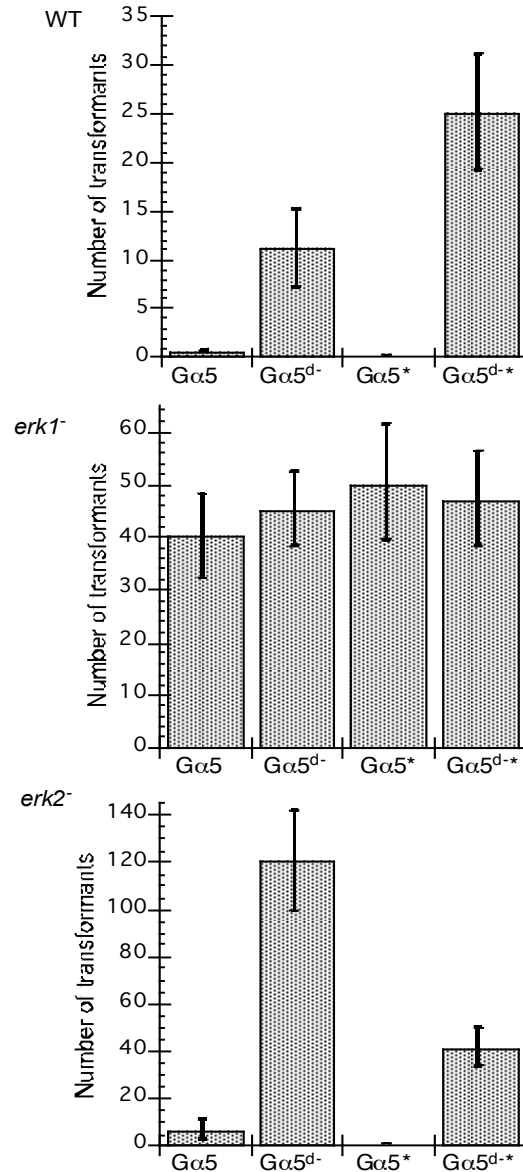


Fig. 11: Survival of wild-type, *erk1*⁻ and *erk2*⁻ cells transformed with Ga5 subunit expression vectors. The strains were electroporated in side-by-side experiments with equal concentrations of Ga5, Ga5^{d-}, Ga5* and Ga5^{d-*} expression vectors and transformants were selected at 3 mg/ml G418. The numbers of viable transformants in different sections of the transformation plates were counted after 10-14 days of drug selection. Data is the average count from 4 different plate sections and error bars represent the standard deviation of these counts. Results are representative of 3 independent electroporations. Transformation results of *ga5*⁻ cell were similar

to that of wild-type cells and strains electroporated without DNA did not produce viable transformants (data not shown).

typically resulted in 10-fold fewer viable transformants compared to the expression of the wild-type Gα5 subunit consistent with earlier studies that suggest Gα5 subunit activation is detrimental to vegetative growth. Combining the D-motif mutations with the constitutively-active mutation, resulted in a Gα5^{d-*} subunit expression vector that typically generated 100-fold more viable transformants than the Gα5* vector suggesting the D-motif is important for the lethal effect of the Gα5* subunit on vegetative cells. The increased viability of cells expressing the Gα5^{d-*} subunit also correlated with the ability of clonal transformants to survive at higher G418 concentrations.

erk1⁻ cells are not sensitive to Gα5 expression vectors

The increased yield in viable transformants with the Gα5^{d-} subunit expression vector in *ga5⁻* and wild-type cells could possibly result from decreased interactions with MAPKs. To test this possibility, the various Gα5 subunit mutants were transformed into *erk1⁻* and *erk2⁻* cells and the numbers of viable transformants were determined after selection with 3 mg/ml G418 (Fig.11). *erk2⁻* cells transformed with either the Gα5 or Gα5* subunit expression vectors gave very few viable transformants compared to those cells transformed with Gα5^{d-} or Gα5^{d-*} subunits. In contrast, *erk1⁻* cells produced a similar number of transformants with all of the Gα5 subunit expression vectors suggesting these cells are insensitive to the detrimental effects of the Gα5 and Gα5* subunits. All *erk1⁻* transformants and the *erk2⁻* cells expressing the Gα5^{d-} or Gα5^{d-*} subunits were also resistant to higher drug selection (> 10 mg/ml G418). These results indicate the requirement of ERK1 but not ERK2 for the lethality associated with over-expression of the Gα5 or Gα5* subunits implying ERK1 functions downstream in the Gα5 subunit-mediated signaling pathway during vegetative growth.

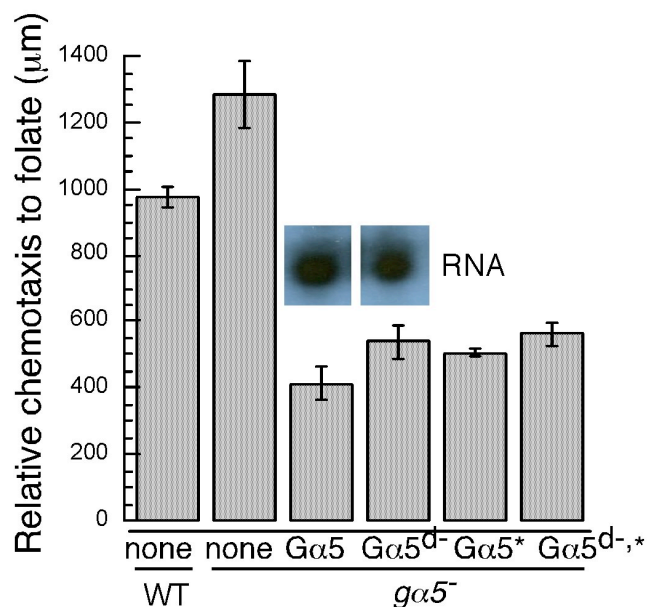


Fig. 12: Chemotaxis of wild-type and Ga5 mutant cells to folate. Chemotaxis assays were conducted as described in the Methods section and measurements were determined for the distance traveled by the leading edge of cells. Measurements of the maximum distance of cell migration from the edge of original cell droplet to the leading edge of cells migrating toward the source of folate after 3 hrs. Chemotaxis of wild-type cells with no expression vector (WT), ga5⁻ cells with no expression vector, wild-type Ga5 subunit expression vector, Ga5^{d-} subunit expression vector, Ga5^{*} subunit expression vector, or Ga5^{d-,*} subunit expression vector. The chemotaxis data are the mean of six individual assays of representative clones and the error bars represent standard deviation of these measurements. RNA gel blot of Ga5 and Ga5^{*} expression in clones used for chemotaxis and other assays (inset). Each lane was loaded with 8 μg total RNA and blot was hybridized with a Ga5 probe (lanes are from same blot).

Ga5 subunit inhibition of folate chemotaxis does not require the amino-terminal D-motif

Over-expression of the Ga5 subunit has been shown to inhibit chemotaxis to folate as indicated by the increased chemotactic movement of ga5⁻ cells and the reduced chemotactic movement of cells overexpressing the Ga5 subunit (Natarajan *et al.*, 2000). Clonal transformants

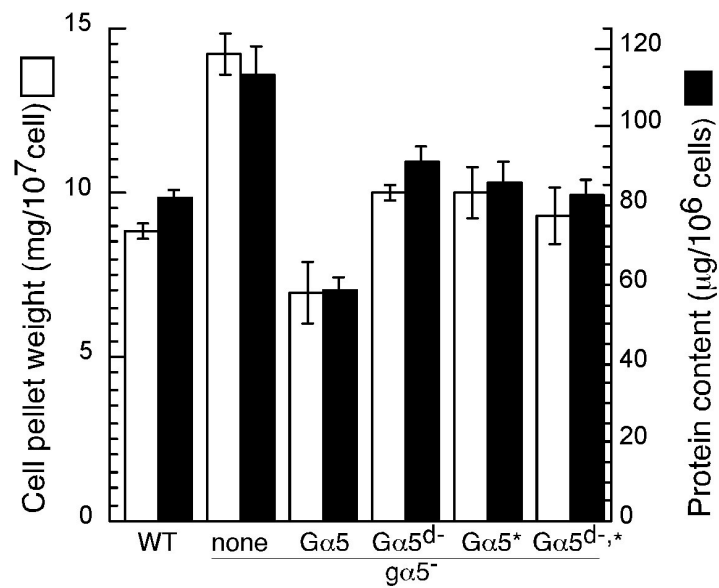
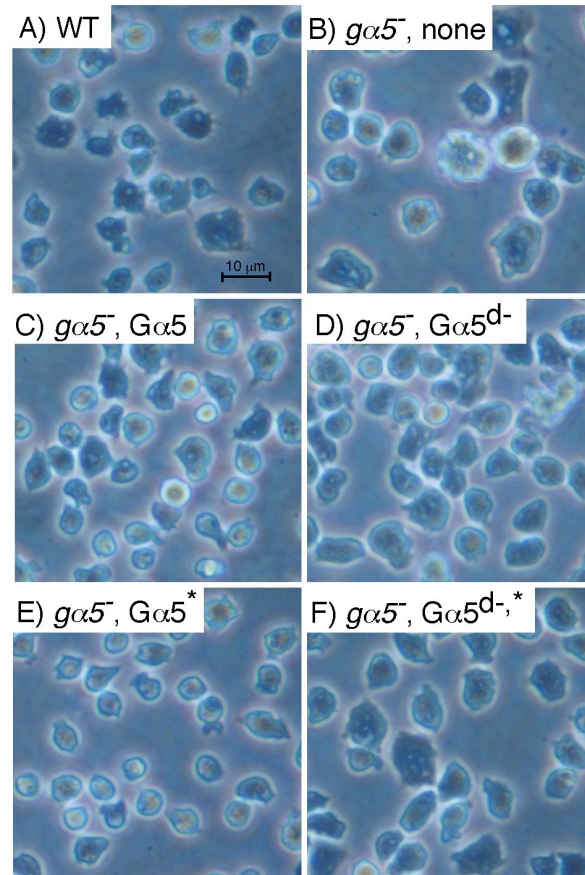


Fig.13: Wild-type and $G\alpha 5$ mutant cell sizes. Cells were grown in HL5 medium on plastic petri dishes and photographed using an inverted phase-contrast microscope at 200X magnification

(upper panels). (A) Wild-type cells with no expression vector. (B) *ga5*⁻ cells with no expression vector. (C) *ga5*⁻ cells with wild-type *Ga5* subunit expression vector. (D) *ga5*⁻ cells with *Ga5d*⁻ subunit expression vector. (E) *ga5*⁻ cells with *Ga5*^{*} subunit expression vector. (F) *ga5*⁻ cells with *Ga5d*⁻,^{*} subunit expression vector. Comparisons of cell pellet weights (open columns) and protein content (closed columns) were conducted as described in Methods to assess differences in cell size (graph). Cultures of vegetative wild-type (WT) and *ga5*⁻ cells with no vector (none) or a vector expressing one of the various *Ga5* subunits (*Ga5*, *Ga5d*⁻, *Ga5*^{*}, or *Ga5d*⁻,^{*}) were counted to determine cell density and then assayed for pellet weight (wet) and protein content. Data is the average three different measurements and the error bars represent the standard deviation of these measurements.

over-expressing the *Ga5* subunit were inhibited in their chemotactic movement to folate similar to that previously reported and cells over-expressing the *Ga5*^{d-} subunit were also capable of inhibiting chemotaxis (Fig. 12). The level of chemotaxis inhibition correlates with *Ga5* subunit expression level and so clonal transformants with similar levels of *Ga5* and *Ga5*^{d-} subunit expression were compared in the chemotaxis assay and all subsequent analyses. Increasing the *Ga5*^{d-} subunit expression through higher levels of drug selection did not significantly change the growth or developmental phenotypes. Cells expressing the *Ga5*^{*} or *Ga5*^{d-,*} subunit were also inhibited with respect to folate chemotaxis indicating that the altered amino terminal D-motif does not eliminate chemotaxis inhibition.

Reduction of large cell phenotype by Ga5 subunit D-motif mutants

Cells lacking the *Ga5* gene typically appear larger than wild-type cells when attached to culture plates suggesting the mutant cells are either larger or more capable of extensive spreading (Fig. 13). To quantify cell size differences, wild-type and *ga5*⁻ cells carrying various *Ga5* subunit expression vectors were counted and then analyzed for cell weight and protein content. The large

cell phenotype of $ga5^-$ cells is reversed when the Gα5 subunit is over-expressed in these cells. Over-expression of the Gα5^{d-} subunit decreased cell size to near that of wild-type cells but not to the extremes observed for Gα5 subunit expression indicating that the alteration to the amino terminal D-motif reduces but does not eliminate the ability of the Gα5 subunit to decrease cell size. The expression of the Gα5* or Gα5^{d-*} subunits in $ga5^-$ cells also reduced cell size to nearly that of wild-type cells.

Gα5 subunit amino-terminal D-motif is important for early tip formation

Expression of the wild-type Gα5 subunit in $ga5^-$ cells resulted in noticeably smaller aggregates with precocious tip formation (9 h into development) compared to the aggregates formed by wild-type or $ga5^-$ cells (Fig. 14). This same level of accelerated development is not observed for $ga5^-$ cells expressing the Gα5^{d-} subunit but the Gα5^{d-} subunit expression does correct for the delayed development observed for $ga5^-$ cells. Expression of the Gα5* subunit in $ga5^-$ cells resulted in a greater delay of aggregate formation than observed for $ga5^-$ cells with no expression vectors. This phenotype is likely attributed to an inhibition of cAMP chemotaxis previously observed for cells expressing constitutively-active Gα5*, Gα4* or Gα2* subunits (Srinivasan *et al.*, 1999). The expression of the Gα5^{d-*} subunit did not delay the timing of aggregate formation of $ga5^-$ cells suggesting the altered D-motif reduces the aggregation delay associated with the Gα5* subunit. Therefore, the developmental morphologies associated with elevated and constitutive Gα5 subunit activity were diminished when the amino terminal D-motif was altered suggesting MAPK interactions might be important for the timing of these developmental processes.

Gα5 subunit amino terminal D-motif affects the timing of gene expression during development

The importance of the Gα5 subunit D-motif in developmental morphology and the established role of MAPKs in gene regulation suggest the D-motif might also contribute to the

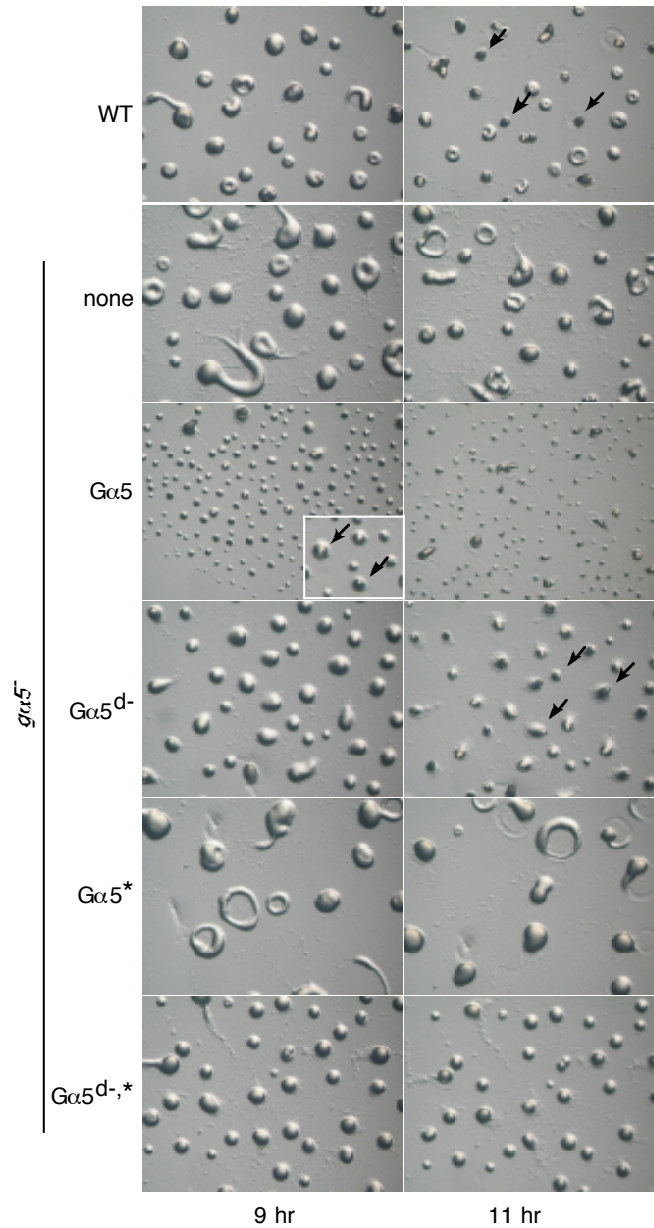
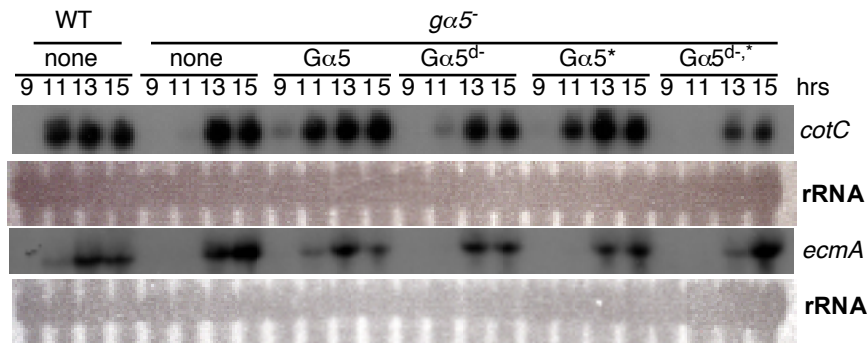


Fig. 14: Developmental morphology of wild-type and $ga5^-$ cells with or without $Ga5$ subunit expression vectors. Cells were grown in axenic medium and washed with phosphate buffer and then cell suspensions (2×10^7 cells/ml) were plated for development as described in Methods. Developing cell aggregates of wild-type cells (WT) and $ga5^-$ cells with no expression vector (none) or a vector expressing the $Ga5$, $Ga5^{d-}$, $Ga5^$, or $Ga5^{d-,*}$ subunit were photographed 9 and 11 h after initial plating using a digital camera and dissecting microscope (20x magnification).*

The inset in the 9 h image of Ga5 expressing cells is a portion of this image 2X magnified.

Arrows indicate representative mounds with tip development.

regulation of developmental gene expression. To examine developmental gene expression, RNA was isolated from *ga5⁻* cells that express wild-type or mutant Ga5 subunits during tip formation (9-15 hrs after the onset of starvation) (Fig. 15). Expression of the prespore-specific gene *cotC* was delayed in *ga5⁻* cells and slightly accelerated in cells expressing the wild-type Ga5 subunit as compared to wild-type cells, consistent with the results from previous studies (Hadwiger *et al.*, 1996). Cells containing the Ga5^{d-} or Ga5^{d-,*} subunit expressed *cotC* with kinetics similar to *ga5⁻* cells suggesting the Ga5 amino terminal D-motif is important for the early onset of prespore specific gene expression. The expression of the *cotC* gene in cells expressing the Ga5* subunit was similar to that of wild-type cells even though these cells have a delay in aggregation. The effect of wild-type and mutant Ga5 subunits on the expression of the prestalk-specific gene, *ecmA*, was much more subtle than that observed for the prespore gene. Only slight differences were observed in the timing and level of *ecmA* expression.



*Fig. 15: Developmental gene expression in wild-type and *ga5⁻* cells with or without Ga5 subunit expression vectors. Cells were grown in axenic medium and washed with phosphate buffer and then cell suspensions (1×10^8 cells/ml) were plated for development as described in Methods. Total RNA was isolated from cells at times indicated (hours after starvation) and subjected to RNA blot analysis using *cotC* (prespore-specific) or *ecmA* (prestalk-specific) probes as described*

in Methods. Each lane was loaded with 8 mg of total RNA. UV shadowing of rRNA band shown as loading control (under each blot). All lanes from each panel were run on the same gel and then blotted and hybridized together.

Discussion

The identification of a D-motif in the amino terminus of the Gα5 subunit that impacts vegetative and developmental functions suggests that Gα5 subunit-MAPK interactions contribute to signaling pathways in *Dictyostelium*. The molecular modeling of the Gα5 subunit predicts the amino terminal D-motifs is positioned near the Gβγ dimer and receptor binding sites suggesting this motif might not be available for MAPK interactions when the Gα subunit is inactive and bound by the Gβγ dimer and receptor. Support for this concept is provided by the increase in Gpa1-Fus3 interactions when yeast cells are stimulated by mating pheromone (Metodieff *et al.*, 2002). However, molecular modeling of the Gα5 subunit structure predicts the internal D-motifs of Gα5 might be available in the inactive state and this is supported by the interactions of the internal D-motif of the Gα4 subunit with ERK2 in the absence of pathway activation (Nguyen and Hadwiger, 2009). Attempts to analyze the Gα5 subunit and ERK1 interactions in *Dictyostelium* have been hampered by the inability to express sufficient levels of epitope-tagged wild-type and constitutively-active Gα5 subunits in *Dictyostelium*, presumably due to the lethal effects of these subunits (Nguyen and Hadwiger, data not shown).

The requirement of the amino terminal D-motif for the lethality associated with Gα5 subunit suggests that MAPK interactions are important for this phenotype and this concept is further supported by the specific requirement of ERK1 for this phenotype. In addition, ERK1 expression vectors can also affect cell viability (Nguyen *et al.* unpublished data). The mechanism(s) responsible for the loss of viability due to Gα5 or ERK1 function remains to be

determined but this mechanism could possibly involve a loss in nutrient uptake similar to that accompanying the transition from vegetative growth to multicellular development.

The amino terminal D-motif of the G α 5 subunit is important for the formation of small aggregates with precocious tip development and cell type specific gene expression. The G α 5 subunit-mediated tip development might also require ERK1 function because *erk1⁻* aggregates are often delayed or blocked in tip development and over-expression of the G α 5 subunit in *erk1⁻* cells does not accelerate tip development (Nguyen et al., unpublished data). The spatial expression patterns of the G α 5 subunit and ERK1 appear to be very similar with both proteins expressed from anterior-like cells (ALCs) found near the posterior of slugs (Gaskins *et al.*, 1994; Hadwiger *et al.*, 1996). However, the small aggregate size phenotype cannot be readily attributed to the activation of ERK1 function because the loss of ERK1 results in a similar phenotype. How the G α 5 subunit D-motif affects the timing of prespore-specific gene expression is also not clear but MAPKs often contribute to the regulation of gene expression through the phosphorylation of cytoplasmic or nuclear substrates (Caunt et al., 2006; Raman et al., 2007). Previous studies implicate *Dictyostelium* ERK2 function in prespore gene expression and the indirect activation of cAMP-dependent protein kinase (PKA), a regulator of prespore gene expression (Hopper et al., 1995). Therefore, the contribution of G α 5 subunit signaling through ERK2 or other regulatory components cannot be excluded.

The presence of D-motifs in the amino terminus of other G α subunits in *Dictyostelium*, yeast and mammalian implies that MAPK interactions with G α subunits might be wide spread among eukaryotes. The requirement of ERK1 for G α 5 subunit-mediated phenotypes and the previously determined association of ERK2 and G α 4 subunit suggests that interactions between G proteins and MAPKs might be specific but we cannot exclude the possibility of other signaling combinations (Nguyen and Hadwiger, 2009). Variations in D-motif sequence and location might

contribute to the specificity of $G\alpha$ subunit-MAPK interactions and these interactions are likely to play an important role in signaling specificity downstream of G protein coupled receptors.

CHAPTER IV

MAP KINASES HAVE DIFFERENT FUNCTIONS IN DICTYOSTELIUM G PROTEIN-MEDIATED SIGNALING

Abstract

Extracellular signal regulated kinases (ERKs) are a class of MAP kinases that function in many signaling pathways in eukaryotic cells and in some cases, a single stimulus can activate more than one ERK suggesting functional redundancy or divergence from a common pathway. *Dictyostelium discoideum* encodes only two MAP kinases, ERK1 and ERK2, that both function during the developmental life cycle. To determine if ERK1 and ERK2 have overlapping functions, chemotactic and developmental phenotypes of *erk1*- and *erk2*- mutants were assessed with respect to G protein-mediated signal transduction pathways. ERK1 was specifically required for Gα5-mediated tip morphogenesis and inhibition of folate chemotaxis but not for cAMP-stimulated chemotaxis or cGMP accumulation. ERK2 was the primary MAPK phosphorylated in response to folate or cAMP stimulation. Cell growth was not altered in *erk1*-, *erk2*- or *erk1-erk2*-mutants but each mutant displayed a different pattern of cell sorting in chimeric aggregates. The distribution of GFP-ERK1 or GFP-ERK2 fusion proteins in the cytoplasm and nucleus was not grossly altered in cells stimulated with cAMP or folate. These results suggest ERK1 and ERK2 have different roles in G protein-mediated signaling during growth and development.

Introduction

Extracellular signal response kinases (ERKs) are the prototypical MAP kinases

(MAPKs) that function in many different signal transduction pathways including those regulated by G protein-coupled receptors and tyrosine kinase receptors (Belcheva and Coscia, 2002; Chen and Thorner, 2007). Multiple ERKs are expressed in a wide range of eukaryotes suggesting that individual ERKs might have different functions in a single species but conserved functions among different species. Mammals, yeast and *Dictyostelium* genomes encode multiple ERKs with a highly conserved TEY sequence that can be phosphorylated (both T and Y residues) upon activation by MAP2Ks (Gaskins et al., 1994; Goldberg et al., 2006). The simultaneous activation of multiple ERKs in response to a single stimulus opens the possibility that ERK paralogs might have overlapping or redundant functions. Genetic analysis in mice indicate the closely related ERK1 and ERK2 proteins have different roles in development (Lloyd, 2006). Loss of ERK2 results in an embryonic lethal phenotype whereas the loss of ERK1 has only subtle phenotypes such as defects in T cell maturation. Also, the down regulation of ERK2 but not ERK1 inhibits the rapid proliferation of tumor cells (Vantaggiato et al., 2006). The ERK orthologs in yeast, Fus3 and Kss1, are both activated in response to mating pheromone but genetic analysis indicates that only Fus3 is required for efficient mating (Chen and Thorner, 2007). Therefore, the simultaneous activation of multiple ERKs might not represent redundancy in signaling but rather divergence of a signaling pathway to regulate multiple responses.

The *Dictyostelium* genome encodes only two MAPKs, ERK1 and ERK2, that share 37% sequence identity and both are expressed during vegetative growth and multicellular development (Gaskins et al., 1996). During the aggregation phase of development, external cAMP activates ERK2 allowing it to phosphorylate and inhibit the cAMP-specific phosphodiesterase, RegA, so that the cAMP signal can be relayed to other cells. *erk2*- cells can chemotax to cAMP, although with low efficiency at high concentrations, but clonal populations of these cells do not form aggregates when starved (Segall et al., 1995). ERK2 is also important for the prespore cell specific gene expression and differentiation (Gaskins et al., 1996). ERK2 is also activated in

response to folate and *erk2*- cells exhibit a slight reduction in folate chemotaxis (Maeda and Firtel, 1997; Wang et al., 1998). Less is known about the function of ERK1 but previous studies have reported *erk1*- cells to be defective in cAMP chemotaxis and to form small aggregates during development (Sobko et al., 2002). ERK1 can be activated in response to cAMP and this activation is mediated by the MAP2K, MEK1 (Sobko et al., 2002). While the *erk1*- and *erk2*- mutants have differences with respect to developmental phenotypes the specificity of ERK1 and ERK2 function in different G protein mediated signaling pathways has not been defined.

Many different G protein-mediated signaling pathways exist in *Dictyostelium* and several of them play important roles in growth and development. Responses to cAMP are mediated through cAMP receptors and the G protein containing the G α 2 subunit (Kumagai et al., 1989; Saxe et al., 1991). cAMP stimulation is responsible for the aggregation phase of development and aids in the establishment of prespore and prestalk cell zones in the aggregate. Response to folate or related pterin compounds is mediated through a pathway using the G α 4 subunit and this pathway allows cells to chemotax to bacterial food sources (Hadwiger et al., 1994). This G α 4 subunit-mediate pathway is also important for the localization and development of prespore cells in multicellular aggregates and the morphogenesis associated with fruiting body formation (Hadwiger and Firtel, 1992). Responses to folate are inhibited by another G protein pathway using the G α 5 subunit and the signal activating this pathway is unknown (Natarajan et al., 2000). The G α 5 subunit helps to regulate cell size, growth, and the rate of morphogenesis after aggregate formation (Hadwiger et al., 1996). All three of these G α subunits presumably couple to a common Gbg dimer and function in pathways that affect ERK function (Wu et al., 1995; Zhang et al., 2001). All three Ga subunits also contain known or putative MAPK docking sites (D-motifs) (Nguyen and Hadwiger, 2009). The G α 4 subunit is required for folate stimulated ERK2 activation and recently the G α 4 subunit has been shown to associate with ERK2. The lethality associated

with G α 5 subunit over-expression requires a MAPK docking motif and ERK1 function and the G α 2 subunit is not required for ERK2 activation in response to cAMP (Raisley et al., 2010).

In this report we describe an analysis of ERK1 and ERK2 function with respect to different G protein-mediated signaling pathways. Strains defective in ERK1 or ERK2 or both ERKs were analyzed for complementation or suppression with ERK expression vectors. The ERK mutants were also assessed for chemotaxis, development, and sensitivity to G α subunit over-expression. The phosphorylation of ERKs in response to folate stimulation was examined in ERK mutants and the distribution of ERK1 and ERK2 in the cell was monitored using GFP fusions. These analyses indicate very different functional roles for ERK1 and ERK2 with respect to signaling pathways mediated by G proteins.

Materials and methods

Strains and media

All *Dictyostelium* strains used in the study were derived from the wild-type strain KAx3. The *ga4*-, *ga5*-, *erk2*- strains were previously described. The *ERK1(erkA)* locus was disrupted in KAx3 and *erk2*- strains using an *ERK1* gene construct with a blasticidin S resistance (Bsr) gene inserted into the coding region and *erk1*- mutants were identified using primers specific to the ERK1 and Bsr sequences for PCR analysis and sequencing. Strains were labeled with GFP using the expression vector pTX-GFP2. Cells were grown in axenic HL5 medium or on bacterial lawns of *Klebsiella aerogenes* (Watts and Ashworth, 1970). DNA vectors were electroporated into cells as previously described (Hadwiger, 2007). Transformed cells were selected and maintained in medium containing 3-10 μ g/ml of the drug G418 or 3 μ g/ml of the drug blasticidin S and drug selection was removed several hours prior to analysis. Folate solutions were prepared by neutralizing folic acid with NaHCO₃.

Recombinant DNA constructs

An *ERK1(erkA)* gene with flanking restriction enzymes sites was created from an *ERK1* cDNA, kindly provided by the *Dictyostelium* cDNA project in Japan and National BioResource Project. The open reading frame was amplified using PCR with oligonucleotides (sense strand: 5'-CGCGGATCCCTCGAGAATTAATGCCACCACCACCAACAAGTG) and (antisense strand: 5'-GCGGTCGACTCTAGATTAATTTTAAATTGATTGTTGATTTACTTGTTG) to produce an *ERK1* gene with *Bam*HI and *Xho*I sites 5' to the start codon and *Xba*I and *Sal*I sites 3' to the stop codon. The *Hind*III/*Sal*I digested PCR product was inserted into the same sites of the vector pBluescriptIIISK+ (Stratagene) and verified by sequence analysis. To create an ERK1 expression vector, the *ERK1* gene construct was transferred as *Sal*I/*Xba*I fragment into the Ddp2-based vector pDXA-3H vector (Manstein et al., 1995). This *act15* promoter driven *ERK1* gene construct was then inserted into the Ddp1-based *Dictyostelium* expression vector pTX-GFP2, replacing the *Sal*I/*Xba*I fragment that contained a GFP gene (Levi et al., 2000). Similar results were obtained using either the Ddp1- and Ddp2-based vectors but only data from Ddp1 vectors is shown. The *ERK1* gene was also transferred as *Xho*I/*Xba*I fragment into the vector pTX-GFP to create the GFP-ERK1 expression vector. To create an *ERK1* gene disruption, the 3' *Hind*III fragment was deleted from the *ERK1* gene and a *Bam*HI fragment containing the *Bsr* gene was inserted into the unique *Bcl*I site in the *ERK1* coding region. This construct was excised from the vector as a *Bam*HI-*Hind*III fragment and electroporated into cells. PCR verification of the *ERK1* gene disruption used oligonucleotides specific to the *Bsr* gene (antisense strand: 5'-CTGCAATACCAATCGCAATGGCTTCTGCAC) and a region of the *ERK1* gene outside the sequence used in the disruption construct (antisense strand: 5'-GTATGGTGCCTGTGGATCTTCAGGATG). The PCR product was sequenced to confirm disruption of the *ERK1* locus. Multiple *erk1*- and *erk1-erk2*- clones with similar phenotypes were identified but the data represents the analysis of a single representative clone of each mutant. ERK2 and GFP-ERK2 expression vectors were constructed as described for the ERK1 expression vectors except that a *Hind*III/*Xba*I *ERK2(erkB)* gene fragment was inserted into the same sites of

the pDXA-GFP2 vector and then the *act15* promoter driven *ERK2* gene was transferred into the pTX-GFP2 vector. The Ddp1-based Gα4 and Gα5 subunit expression vectors were previously described (Hadwiger, 2007).

Development and chemotaxis assays

Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10^8 cells/ml), before spotting on nonnutrient plates (phosphate buffer, 1.5% agar) for development or chemotaxis as described (Hadwiger et al., 1996). Cell development was analyzed using a dissecting microscope or fluorescence microscopy. Above-agar assays were used to measure chemotaxis as previously described (Hadwiger et al., 1996). Briefly, the folate chemotaxis assay was performed by spotting droplets of cell suspension (10^7 to 10^8 cells/ml) on nonnutrient plates followed by the spotting of 1 µl of folate solutions (10^{-2} to 10^{-4} M) approximately 2-3 mm away from the cell droplet. For the cAMP chemotaxis assay, cells were shaken for 4 hours with the addition of 100 nM cAMP every 15 min and then spotted on nonnutrient plates followed by the spotting of 1 µl of cAMP (10^{-4} M). Cell movement was monitored with a dissecting microscope. All strains were treated identically for each experiment.

Cyclic GMP assays

cGMP concentration in *Dictyostelium* was determined using a radioimmunoassay as previously described (Kumagai et al., 1991). Cells were grown to mid-log phase, washed twice in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH) and deposited on nonnutrient plates for starvation. After 6 hours of starvation, cells were collected and bubbled with air for 10 min prior to treatment with 1 mM cAMP. At times indicated, the cellular responses were terminated by addition of perchloric acid and then the samples were neutralized with ammonium

sulfate. The concentration of cGMP in each sample was determined using a radioimmunoassay kit (Amersham).

Sensitivity to expression vectors assays

Strains were electroporated with the same amount of each vector and each electroporation was performed in duplicate. The number of transformants was determined on each plate section after 7-10 days of drug selection. Electroporations of strains with no DNA were used as controls. After transformants were identified at a low drug selection (3 μ l/ml G418), culture plates were treated with increasing selection of drug selection and the number of surviving clones was determined.

MAPK activation assay

MAPK activation was determined as previously described (Nguyen and Hadwiger, 2009). Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (5×10^7 cells/ml). For folate responses, cell suspensions were shaken for 1 hr and then stimulated with 50 mM folate. For cAMP responses, cell suspensions were shaken for 6 hrs and pulsed with 100 nM cAMP every 15 min after the first 3 h. After this preconditioning to cAMP, cell suspensions were stimulated with 100 nM cAMP. Stimulated cells were treated with SDS-PAGE loading buffer at times indicated and boiled. Cell lysates were subjected to SDS-PAGE (8×10^7 cells/lane) and immunoblot analysis was conducted using a rabbit α -phospho-p44/p42 MAPK antibody and secondary goat HRP-conjugated α -rabbit IgG antibody for chemiluminescence detection (Cell Signaling Technology). Densitometry of immunoblot bands was determined using Image J software (NIH).

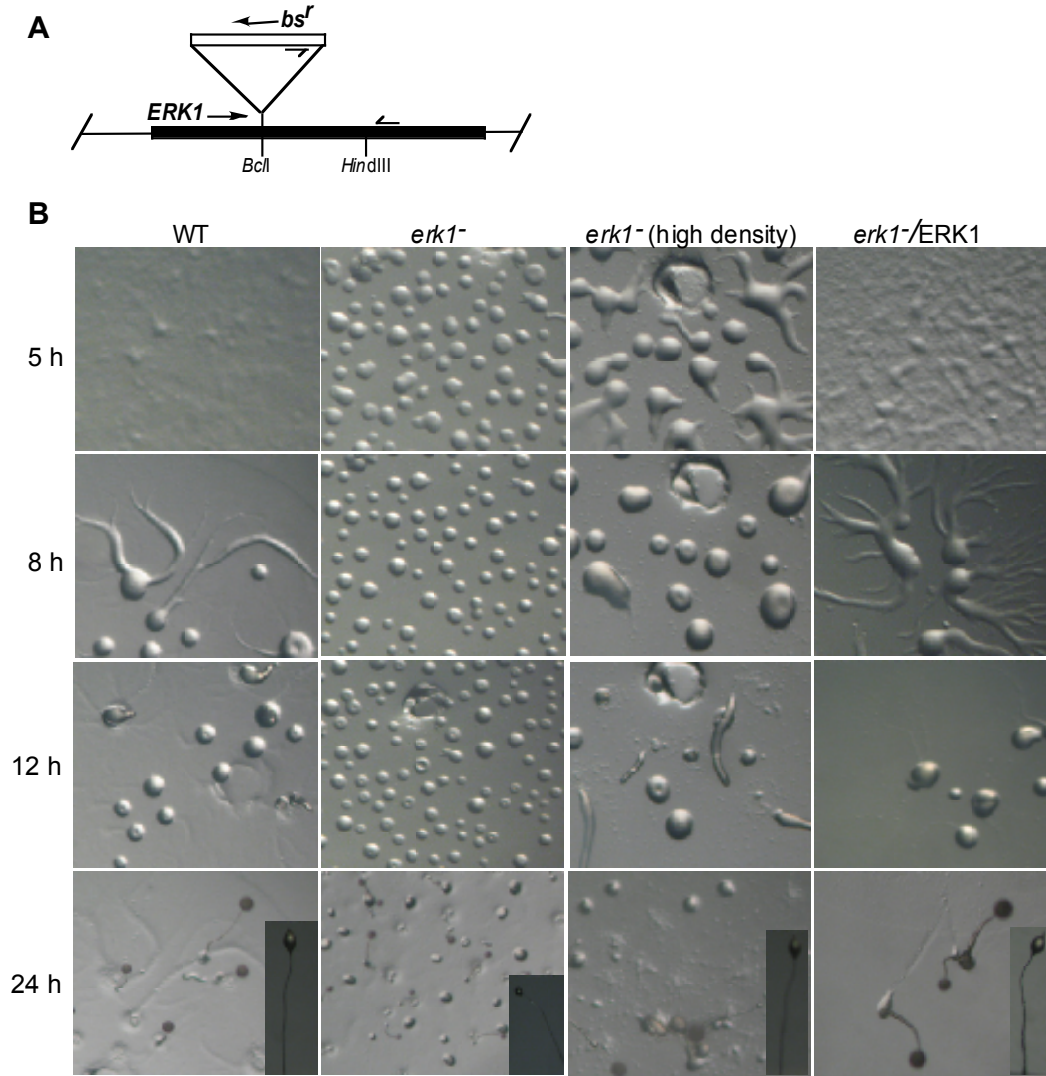
Results

erk1- cells have altered aggregate morphology and are inefficient in tip morphogenesis

To compare ERK1 and ERK2 functions in growth and development, *ERK1* and *ERK2* gene disruptions were created in strains with the same genetic background to reduce strain-specific phenotypes. An *erk2-* strain previously created using a REMI allele (PYR5-6 selection) insertion into the JH8 strain (KAx3 *pyr5-6-* strain) was used in all analyses of ERK2 function (Nguyen and Hadwiger, 2009). The *ERK1* gene was disrupted in the KAx3 strain using a blasticidin resistance gene insertion into a unique *BclI* site within the central part of the coding region and starvation of *erk1-* clones resulted in small aggregates as previously described by others (Fig. 16) (Sobko et al., 2002). However, a more detailed inspection of the *erk1-* cell aggregation revealed relatively short aggregation streams compared to that of wild-type cells. In addition, the timing of mound formation was accelerated with mounds forming as early as 5 hrs and most of these aggregates were either delayed or completely blocked in tip formation (movement of prestalk cells to the top of the aggregate). Aggregates that produced tips were capable of completing development but the size of the mature fruiting bodies was small due to the limited number of cells in the aggregate. At higher cell densities the aggregates of *erk1-* cells were nearly that of wild-type cells. The introduction of an ERK1 expression vector rescued of the wild-type aggregate size and development indicating the phenotypes are ERK1 specific.

erk1- cells have normal cAMP responses

The aggregation phenotype of *erk1-* cells suggests that cell-cell signaling might be compromised possibly due to defects in responses to the external cAMP signal. Therefore, starved *erk1-* cells were examined for cAMP chemotaxis using an above-agar assay and *erk1-* cells were found to travel nearly the same distance as wild-type cells in most experiments suggesting that *erk1-* and wild-type cells have similar capabilities for cAMP chemotaxis (Fig. 17A). However, many *erk1-* cells moved in directions other than toward the source of cAMP



*Fig. 16: ERK1 gene disruption and developmental phenotypes. A) Disruption of the ERK1 locus at the BclI site with the blasticidin S resistance gene (Bsr). PCR verification of the gene disruption used primers indicated by angles. B) Developmental phenotypes of wild-type cells (WT), *erk1*⁻ cells, *erk1*⁻ cells at five times cell density (high density), and *erk1*⁻ cells containing an ERK1 expression vector (*erk1*⁻, ERK1). Cells were grown and plated for development at 1×10^7 cells/ml as described in section 2.3 and photographs (10x magnification) were taken from overhead at 5, 8, 12 and 24 h after cell plating. Insets are images of individual structures at 24 h photographed (20x magnification) from the side. At the lower cell density the fruiting bodies of *erk1*⁻ cells were smaller than those of wild-type cells.*

suggesting an altered chemotactic response compared to wild-type cells. Tracking the movement of individual cells in an above agar assay revealed that *erk1*- cells typically travel faster than wild-type cells but that *erk1*- cells were also less directed toward the cAMP source compared to wild-type cells (Fig. 17B). An earlier report had indicated that another *erk1*- strain was defective in directional movement and also speed but this previous study had examined the movement of individual cells toward a micropipet filled with cAMP (Sobko et al., 2002). The above agar chemotaxis assay assesses the movement of cells in a large population and therefore a subpopulation of cells with a defect in speed might not be detected. However, the above-agar assay indicates that many *erk1*- cells are capable of rapid cell movement in response to cAMP. *erk1*- cells were also labeled with a GFP expression vector and mixed with wild-type cells for an above-agar cAMP chemotaxis assay and the ratio of *erk1*- cells to wild-type cells in the leading edge of chemotaxing cells remained constant indicating both wild-type and *erk1*- cells have similar chemotaxis rates (Fig. 17C). In addition, *erk1*- cell responsiveness to external cAMP was also assayed with respect to cGMP accumulation because the loss of MEK1 reduces cGMP production. *erk1*- cells were found to produce similar levels of cGMP compared to that of wild-type cells indicating that ERK1 function is not necessary for cGMP accumulation (Fig. 17D). This result also suggests that MEK1 regulates cGMP levels through a pathway that does not require ERK1 function.

ERK1 and ERK2 function are required for different developmental processes but not cell growth

In mammals, ERK function has been associated with cell growth and development because down regulation of ERK2 function reduces the rapid proliferation of tumor cells and loss of ERK2 results in an embryonic lethal phenotype (Bessard et al., 2008; Meloche and Pouyssegur, 2007). *Dictyostelium* has only two MAPKs and so an *erk1-erk2*- double mutant strain was created to examine phenotypes in growth and development and to identify possible redundant or oppositional relationships that might exist between the two ERKs. The resulting

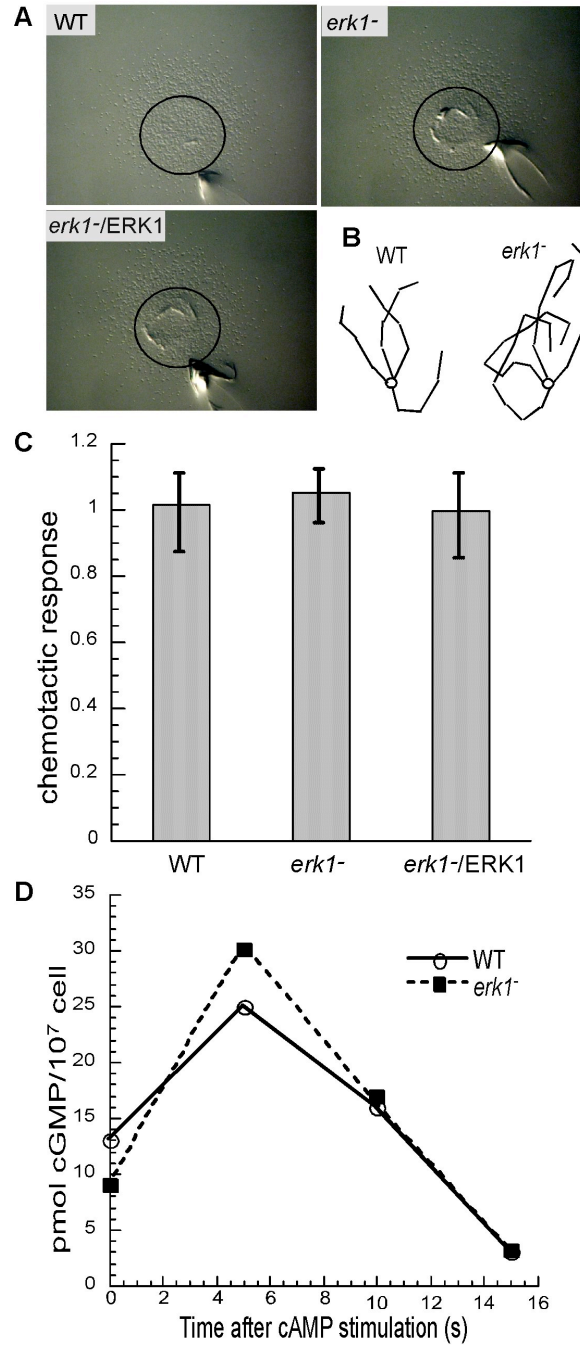


Fig. 17: Chemotaxis response *erk1*- cells to cAMP. Cells were prepared and plated for cAMP chemotaxis as described in section 2.3. A) Micrographs of wild-type (WT), *erk1*-, and *erk1*- cells with an *ERK1* expression vector (*ERK1*) in cAMP chemotaxis assays after 4 hrs (20X magnification). In all panels the source of cAMP is above (upper side of image) the cell droplet and deformations in agar were created by a needle and used as a reference points to align

images captured at different times during the assay. The drawn circle indicates the original cell droplet perimeter. Each micrograph is representative of several assays with similar results. (B) Migration maps of individual wild-type and *erk1*- cells in cAMP chemotaxis assays. Low-density cell suspensions of 5×10^6 cells/ml were plated for chemotaxis assays and then 3.5 hours after the initial plating time-lapse photography was used to follow the movement of cells over a 30 min period. Images corresponding to 5 min intervals were used to trace the movement of individual cells. Only cells with minimal or no cell-cell contact were chosen for creating maps. Multiple paths representing different cells of the each strain are shown and the initial point of each path is designated with an open circle. All initial points were positioned at a central location with the orientation of the cAMP source at the top of the maps. Data is representative of one of two experiments with similar results. (C) Wild-type (WT), *erk1*- and *erk1*- (*ERK1*) cells expression vector were labeled with a GFP expression vector, mixed with unlabeled wild-type cells in a 1:4 ratio, and subjected to a cAMP chemotaxis assay. The percentage of fluorescent cells at the leading edge of the chemotaxis assay was divided by the percentage of fluorescent cells plated for each mixed population. The results are the mean of 3 independent assays and the error bars represent the standard deviation. (D) cAMP-stimulated cGMP accumulation in wild-type (open circles) and *erk1*- (filled squares) cells. The assays were conducted as described in section 2.4. The concentration of cGMP was normalized with respect to the protein concentration in each cell extract and the results are the average of two assays.

erk1-erk2- mutant was viable suggesting that ERK function is not required for vegetative growth. The *erk1*-, *erk2*-, and *erk1-erk2*- mutants were tested for growth in axenic and bacterial cultures. Growth rates of all the ERK mutants and wild-type cells were similar in both the axenic and bacterial cultures suggesting that ERK function is not required for vegetative growth of cells (Fig. 18A & B). The starvation of the *erk1-erk2*- cells resulted in an aggregation defect identical to *erk2*- cells indicating the loss of ERK1 does not suppress the *erk2*- phenotype (Fig. 18C). The

expression of ERK2 but not ERK1 in the *erk1-erk2*- cells rescued developmental aggregation but the aggregates displayed the small aggregate phenotype associated *erk1*- cells. Under high drug selection (5 mg/ml G418), ERK1 expression was not sufficient to rescue the aggregation defect of *erk2*- cells but ERK2 expression in *erk1*- cells did allow for larger aggregates to form at low cell densities. The ERK1 expression vector in wild-type cells delayed development starting at the aggregation phase but only when the vector was selected at higher drug concentrations (10 mg/ml). The ERK2 expression vector blocked the aggregation of wild-type cells when drug selection was relatively high (10 mg/ml). The impaired development of wild-type cells expressing ERK1 or ERK2 vectors suggests the over-expression of ERK1 or ERK2 interferes signaling pathways involved with development.

Cell sorting altered by ERK function

The developmental phenotypes of *erk1*-, *erk2*-, and *erk1-erk2*- cells suggest ERK1 and ERK2 might be required for different processes in cell differentiation or cell sorting. To examine the role of the ERKs in these processes, ERK mutants were labeled using GFP expression vectors and then monitored during development in the presence of wild-type cells. GFP-labeled *erk1*- cells were found primarily in the anterior half of chimeric slugs and these cells later contributed to both the stalk and spore mass of the mature fruiting body (Fig. 19). The *erk1*- cells were very sparse in the posterior of slugs, a region typically composed of anterior-like cells (ALCs) and late developing prespore cells. This pattern of cell sorting suggests that *erk1*- cells have enhanced capability to migrate to the anterior of developing aggregates and that the wild-type cells are providing extracellular signaling necessary to overcome the delay or block in anterior tip formation observed with *erk1*- clonal aggregates. GFP-labeled *erk2*- cells aggregated with wild-type cells but many of the *erk2*- cells in these aggregates were left behind as the mounds developed into slugs. The *erk2*- cells retained in the slug were found primarily in the prestalk O

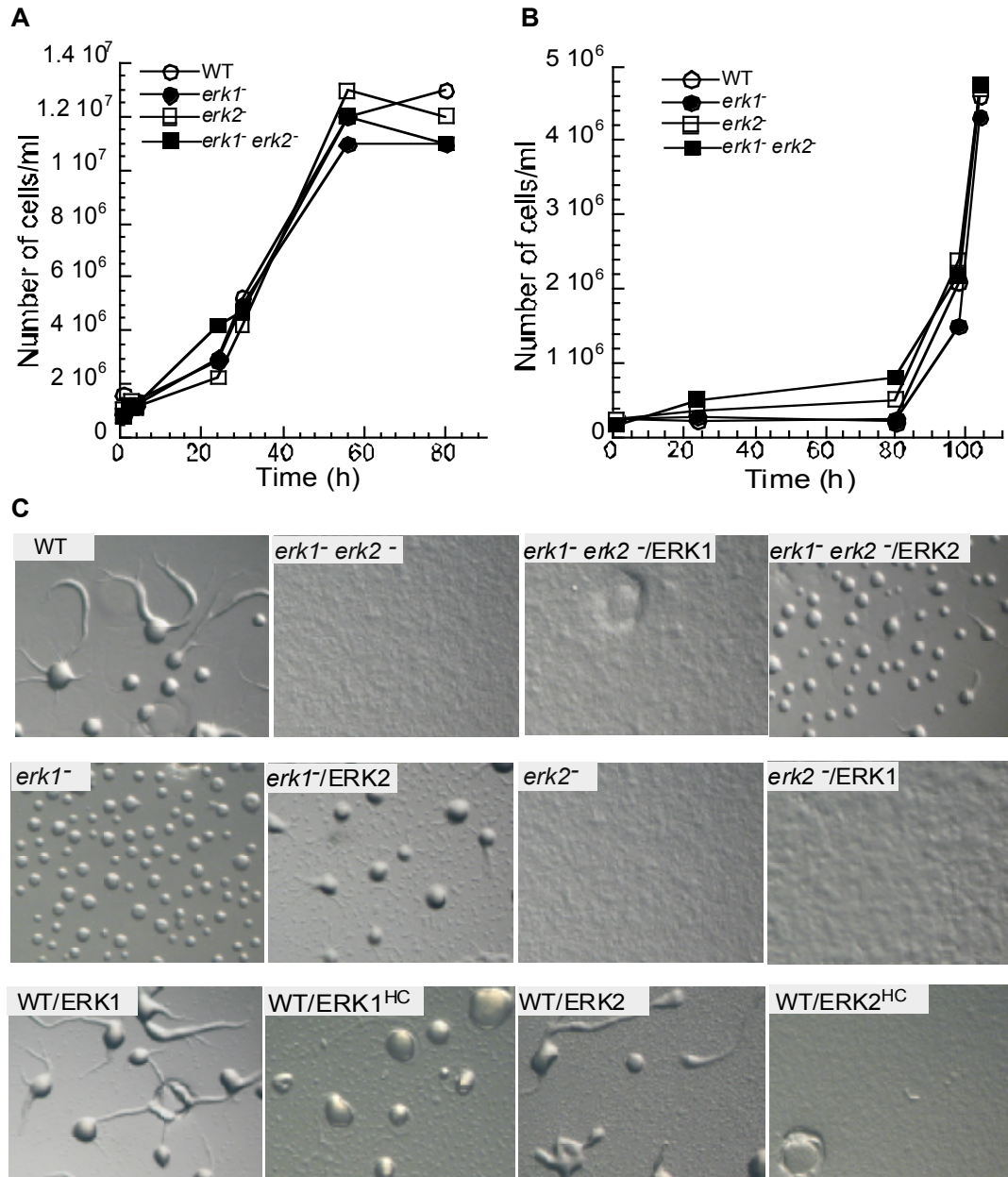


Fig. 18: Growth and development of ERK mutants. Growth curves of wild-type (open circles), *erk1*⁻ (filled circles), *erk2*⁻ (open squares), *erk1 erk2*⁻ (filled squares). Cells were inoculated at 2×10^5 cells into flasks of HL5 medium (axenic) or *Klebsiella aerogenes* (bacterial) culture and then shaken at 200 rpm. Cells were counted using a hemacytometer at the times shown. A) Cell concentrations during growth in axenic medium. B) Cell concentrations during growth in a bacterial suspension. The counting of *Dictyostelium* was hampered in the bacterial suspensions

*at high cell density due to the formation of cell aggregates. Data shown for a typical experiment repeated three times. C) Developmental phenotypes of ERK mutants with or without ERK expression vectors. Cells were grown and plated for development as described in section 2.3 and photographs (10x magnification) were taken from overhead after 12 h. (Upper panel) Wild-type (WT) and *erk1⁻erk2⁻* cells (with or without the ERK1 or ERK2 expression vector). (Middle panel) *erk1⁻* cells (with or without the ERK2 expression vector) and *erk2⁻* cells (with or without the ERK1 expression vector). (Lower panel) Wild-type (WT) cells with ERK1 or ERK2 expression vectors selected at 3 mg/ml or 10 mg/ml (HC) G418.*

region near the anterior but not in the extreme anterior, designated as the prestalk A region. The low level of *erk2⁻* cells in the central prespore region of the slugs was consistent with earlier reports of *erk2⁻* sorting and deficiencies in spore development (Gaskins et al., 1996; Segall et al., 1995). The *erk2⁻* cells in the prestalk O region contributed to the upper and lower cups around the spore mass of the fruiting body but these cells were relatively scarce in the spore mass or stalk. Similar to *erk2⁻* cells, many of the GFP-labeled *erk1-erk2⁻* cells found in chimeric aggregates were left behind during slug formation. However, the *erk1-erk2⁻* cells retained in slugs were found primarily in the prestalk A region rather than the prestalk O region and these cells contributed to both the stalk and the cups surrounding the spore mass.

ERK mutants are differentially sensitive to Ga subunit over-expression

Other studies have indicated that G protein mediated pathways activate ERK signaling in *Dictyostelium* and in some cases the Ga subunits of these pathways contain D-motifs for MAPK interactions (Knetsch et al., 1996; Maeda and Firtel, 1997). A D-motif in the Ga5 subunit has been determined to be important for the lethality associated with over-expression or constitutive activation of the Ga5 subunit suggesting that MAPK interactions are necessary for this phenotype

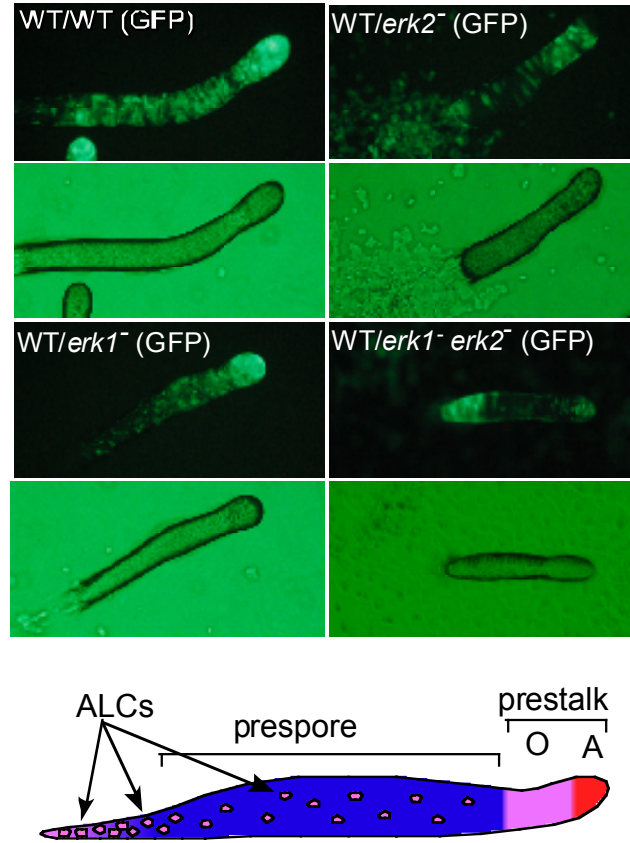


Fig. 19: Spatial distribution of ERK mutants in chimeras with wild-type cells. Wild-type (WT), $erk1^{-}erk2^{-}$, $erk1^{-}$, and $erk2^{-}$ cells were labeled with a GFP expression vector and mixed with wild-type cells (1:4 ratio) before plating for development as described in section 2.3. GFP fluorescence in migratory slugs was photographed at 16 h after cell plating (20x magnification). Lower panels are bright field photographs of the same slugs. Diagram of cell type specific regions in migratory slugs is displayed below panels.

(Raisley et al., 2009). To compare the sensitivity to Gα subunit over-expression, ERK mutants were transformed with a Gα5 or Gα4 subunit expression vector and the number of viable transformants was determined. Many viable transformants were found when $erk1^{-}$ and $erk1^{-}erk2^{-}$ cells were transformed with the Gα5 subunit vector but very few wild-type and $erk2^{-}$ cell transformants survived suggesting the Gα5 subunit lethality is specifically mediated through ERK1 and not ERK2 (Fig. 20). In contrast, transformation of a Gα4 subunit expression vector

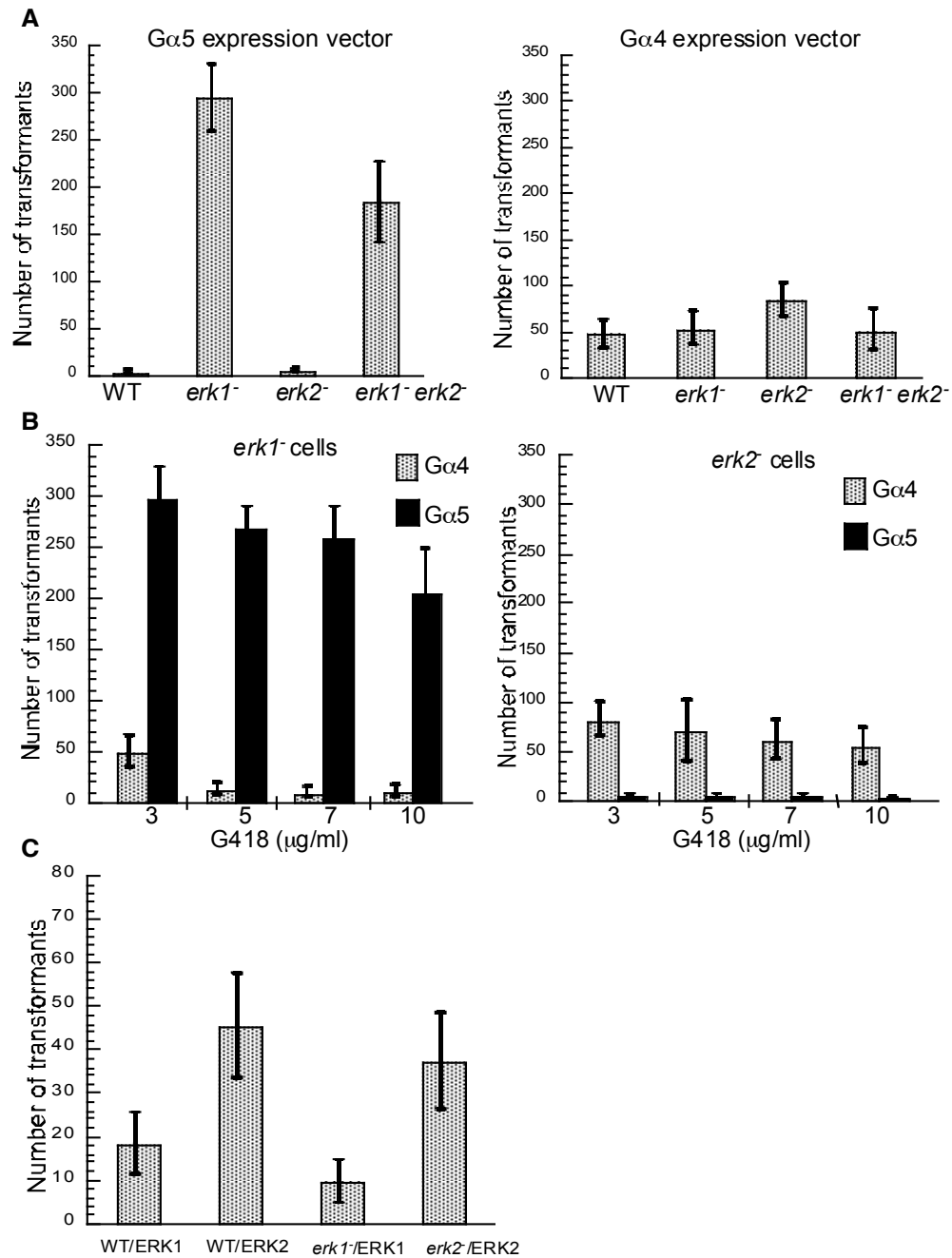


Fig. 20: Viable transformants expressing Ga subunit or ERK expression vectors. Cells were electroporated in side-by-side experiments with equal DNA concentrations of the expression vectors. The number of viable transformants was counted in different sections of the transformation plates after 10-12 days of drug selection as described in section 2.5. A) Number of

wild-type (WT), *erk1*⁻, *erk2*⁻ or *erk1 erk2*⁻ cells transformed with a *Gα5* or *Gα4* subunit expression vector after selection with 3 mg/ml of G418. B) Number of viable *erk1*⁻ or *erk2*⁻ transformants with either a *Gα4* or *Gα5* subunit expression vector after selection with different concentrations of G418. C) Number of viable wild-type (WT), *erk1*⁻ and *erk2*⁻ transformants with ERK1 or ERK2 expression vectors after selection with 5 mg/ml of G418. Data is the average count from 4 different plate sections and error bars represent the standard deviation of these counts. Results are representative of multiple independent electroporations. Wild-type and mutant cells were also electroporated without DNA resulting in no viable transformants (data not shown).

resulted in a similar number of transformants regardless of the ERK mutations. Increasing the drug selection had little impact on the survival of *erk1*⁻ cells transformed with the *Gα5* subunit vector but substantially reduced the number of *erk1*⁻ cells transformed with the *Gα4* subunit vector. In contrast, most *erk2*⁻ cells transformed with the *Gα4* subunit vector retained viability at the higher drug selection. Some of the few viable *erk2*⁻ transformants with the *Gα5* subunit expression vector were not affected by increased drug selection but this might be attributed to mutations or rearrangements in the *Gα5* subunit expression allowing the G418 resistance gene to amplify without increasing *Gα5* subunit function. Earlier studies have shown a strong selection against *Gα5* function, often resulting in *Gα5* subunit vector rearrangements (Hadwiger et al., 1996). Transformation of an ERK1 or ERK2 expression vector into wild-type or ERK mutants consistently resulted in fewer transformants with the ERK1 expression vector suggesting ERK1, like the *Gα5* subunit, might be detrimental to cells when over-expressed.

ERK1 is required for Gα5 subunit folate chemotaxis inhibition and tip morphogenesis

The possibility that *Gα5*-mediated signals regulate ERK1 function suggests ERK1 might be necessary for *Gα5* specific phenotypes such as the inhibition of folate chemotaxis. Like *Gα5*-

cells, *erk1*⁻ cells have a slightly enhanced chemotactic response to folate (Fig. 21A). Over-expression of the Gα5 subunit inhibits folate chemotaxis but not in *erk1*⁻ cells suggesting ERK1

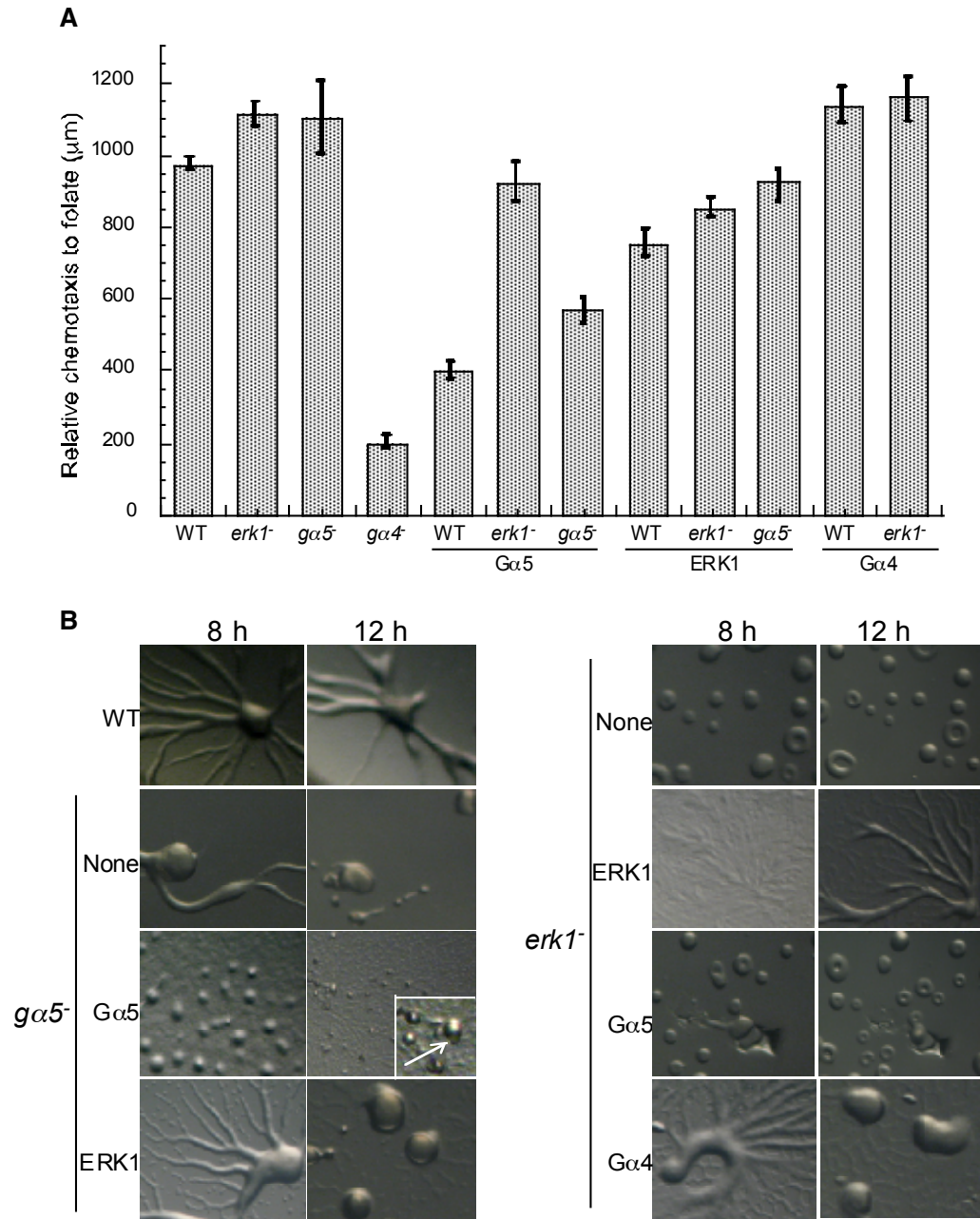


Fig. 21: Folate chemotaxis and development of ERK and Gα subunit mutants. (A) Folate chemotaxis distances traveled by wild-type, *erk1*⁻, *gα5*⁻ and *gα4*⁻ cells with or without Gα subunit and ERK1 expression vectors. Cells were prepared and plated for folate chemotaxis as described

in section 2.3. At 3 h after cell plating, the maximum chemotaxis distance from the original cell droplet perimeter to leading edge of migrating cells in the direction of the folate source was recorded. Each value is mean of six independent cell droplets and errors represent the standard deviation. (B) Developmental phenotypes of $ga5^-$ and $erk1^-$ cells with and without $G\alpha$ subunit and ERK1 expression vectors at 8 and 12 h after cell plating. Expression vectors were selected at 5 mg/ml G418 except for $G\alpha5$ expression vector in $ga5^-$ cells (2 mg/ml G418) because of the lethal effects of $G\alpha5$ subunit expression. Inset of $ga5^-/G\alpha5$ subunit 12 hr photograph is a 3X magnification of one section displaying a tipped aggregate (indicated by arrow).

is required for this inhibition. Likewise, ERK1 over-expression inhibits folate chemotaxis but not in $ga5^-$ cells further supporting that the $G\alpha5$ subunit and ERK1 function in the same pathway. The over-expression of the $G\alpha4$ subunit in wild-type and $erk1^-$ cells enhances folate chemotaxis suggesting the $G\alpha4$ subunit-mediated responses to folate do not require ERK1 function.

Both ERK1 and the $G\alpha5$ subunit affect tip morphogenesis suggesting these proteins might function in a common signaling pathway to regulate this process. To examine this possibility, $ga5^-$ and $erk1^-$ cells carrying expression vectors for the $G\alpha5$ subunit and ERK1 were monitored for tip formation. The $G\alpha5$ subunit expression vector promotes small aggregate formation and accelerated tip formation in $ga5^-$ cells even under low drug selections of 2 mg/ml G418 (Fig. 21B). This $G\alpha5$ subunit-mediated precocious tip formation is not observed in $erk1^-$ cells expressing the $G\alpha5$ subunit vector even under high drug selection (10 mg/ml) indicating ERK1 is required for this phenotype. However, over-expression of the $G\alpha4$ subunit delayed aggregation in $erk1^-$ cells, as previously described for wild-type cells, indicating this $G\alpha4$ subunit-mediated phenotype does not require ERK1 function (Hadwiger and Firtel, 1992). Over-expression of ERK1 resulted in a delay in aggregation in wild-type and $erk1^-$ cells but not in $ga5^-$ cells suggesting this ERK1-mediated phenotype is dependent on $G\alpha5$ subunit function.

Phosphorylation of ERKs in response to folate and cAMP

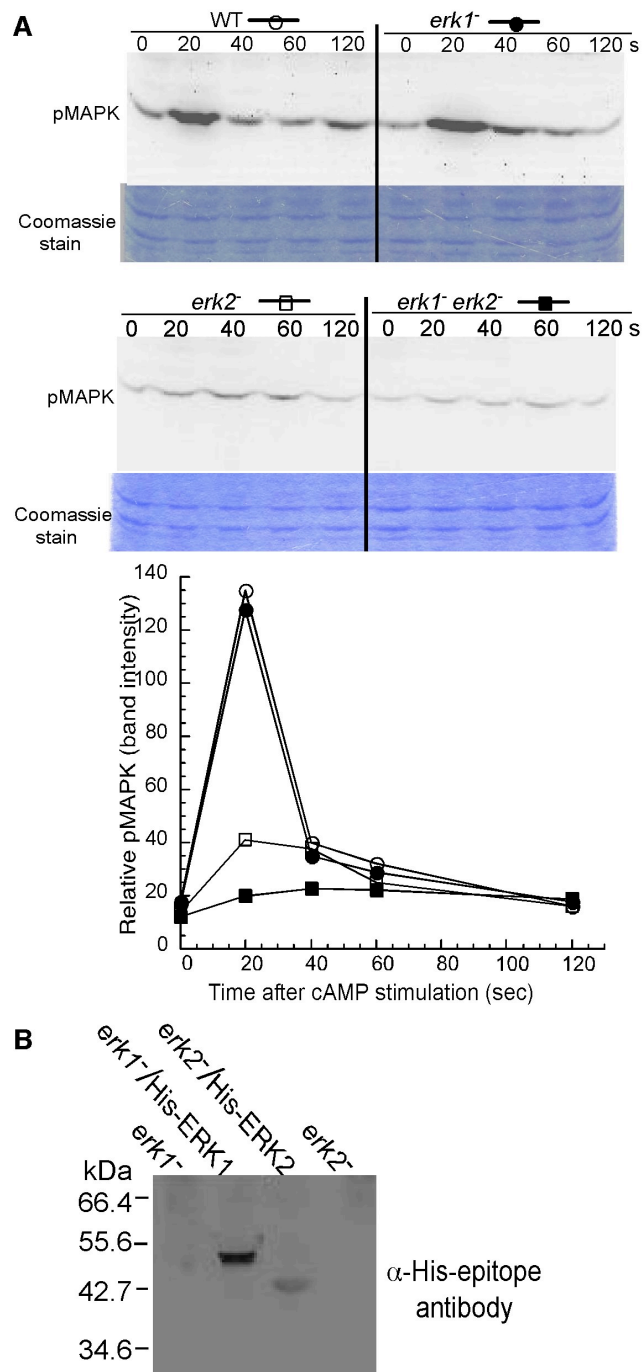


Fig. 22: cAMP-stimulated MAPK activation in wild-type and mutant cells. A) Wild-type, erk1-, erk2-, and erk1-erk2- cells were grown, washed, and suspended in phosphate buffer as described in section 2.3. Cells were starved and pulsed with cAMP in shaking culture to induce cAMP

responsiveness. Cell suspensions (5×10^7 cells/ml) were stimulated with 100 nM cAMP and harvested at the times indicated. Cell extracts were subjected to immunoblot analysis using an α -phospho-p44/p42 MAPK antibody. Coomassie stained gel is provided as a loading control (lower panels). The phospho-MAPK (pMAPK) band intensity is indicated in line graph below (symbols indicated above blots). Data represents one of at least two experiments with similar results. B) Western blot of cell extracts from *erk1*- cells, *erk1*- cells expressing His₆-ERK1, *erk2*- cells expressing His₆-ERK2, and *erk2*- cells using an α -His tag antibody.

Previous reports have indicated that cAMP stimulation of *Dictyostelium* results in the activation of ERK1 and ERK2 and some of these studies have used antibodies generated against the conserved activation site (human phospho-p44/42 MAP kinase -TEY region) to detect phosphorylated MAPKs (Maeda and Firtel, 1997). Using these antibodies a band approximately 42 kDa is detected from wild-type cells and this band is greatly reduced in *erk2*- or *erk1-erk2*- cells but not in *erk1*- cells, suggesting this band corresponds to phosphorylated ERK2 and not ERK1 (Fig. 22A). The detection of a weak band in *erk2*- and *erk1-erk2*- cells possibly represents leakiness of the *erk2*- allele or another phosphorylated protein. ERK1 is predicted to be approximately 48 kDa but these antibodies do not detect a band of this size after cAMP stimulation even though ERK1 is slightly more conserved than ERK2 to the human ERKs in this region. A protein blot of cells expressing His₆-tagged ERK1 or ERK2 confirms that ERK1 migrates slightly slower than ERK2, as expected for the difference in protein size (Fig 22B). Therefore the inability to detect ERK1 after cAMP stimulation is likely due to either a very weak phosphorylation of ERK1 or another mechanism for ERK1 activation.

Stimulation of *Dictyostelium* with folate results in rapid phosphorylation of ERK2 (Nguyen and Hadwiger, 2009). Consistent with earlier studies this phosphorylation requires G α 4 subunit function and ERK2 expression as indicated by the large reduction in the detected protein from *ga4*- and *erk2*- cells (Fig.24). The phosphorylation of ERK2 in *erk1*- cells was similar to

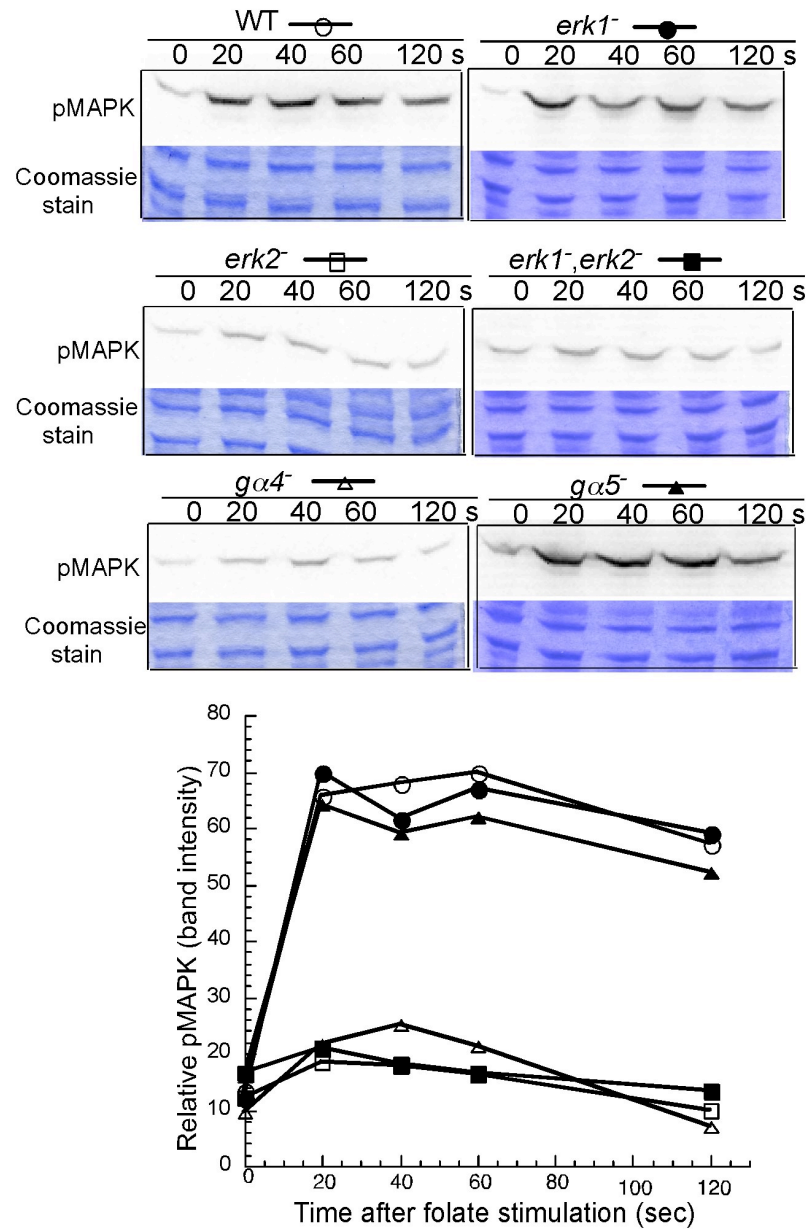


Fig. 23: Folate-stimulated MAPK activation in wild-type and mutant cells. Wild-type, *erk1*-, *erk2*, *erk1-erk2*-, *ga4*- and *ga5*- cells were grown, washed, and suspended in phosphate buffer as described in section 2.3. Cell suspensions (5×10^7 cells/ml) were shaken for 1 h and then stimulated with 50 μ M folate. Cell extracts were subjected to immunoblot analysis using an α -phospho-p44/p42 MAPK antibody. Coomassie stained gel is provided as a loading control (lower panels). The pMAPK band intensity is indicated in line graph below (symbols indicated above blots). Data represents one of at least two experiments with similar results.

that of wild-type cells and the phosphorylation level of *erk1-erk2*- cells was similar to that of *erk2*- cells. These results suggest ERK1 has very little impact on ERK2 activation in response to folate. No bands were detected in the range of 48 kDa implying ERK1 is not activated in response to folate (data not shown). ERK2 phosphorylation in *Ga5*- cells was similar to that of wild-type, consistent with the *Ga5* subunit not being required for the folate responses.

ERK distribution in response to folate and cAMP stimulation

In many organisms, MAPKs can move from the cytoplasm into the nucleus after cell stimulation (Blackwell et al., 2003; Costa et al., 2006). A previous study of ERK2 using protein blots of *Dictyostelium* lysates indicated that only a very small percentage of ERK2 was associated with nuclear fractions after cAMP stimulation (Knetsch et al., 1996). As an alternative method to assess ERK distribution in the cell, GFP-ERK1 and GFP-ERK2 fusions were created and expressed in cells so that ERK distribution could be monitored in live cells. Both of these fusion proteins were capable of rescuing developmental defects of the respective ERK mutant (Fig. 24A). Both GFP-ERK1 and GFP-ERK2 were found in the cytoplasm and nucleus of cells (Fig. 24B). Upon stimulation of these cells with cAMP or folate no major changes were observed with the distribution of GFP-fusion proteins inside of cells (data for folate stimulation not shown). Although phosphorylated MAPKs can be detected as early as 20 sec after cAMP or folate stimulation, no major changes were observed even up to 3 min after stimulation (data not shown). No major changes in GFP-ERK1 and GFP-ERK2 distribution were observed during chemotaxis to folate on agar plates (data not shown). However, the detection of GFP-ERK1 or GFP-ERK2 does not differentiate between phosphorylated and unphosphorylated forms of the MAPK and so a large pool of inactive GFP-MAPK might possibly mask changes in a smaller pool of activated protein. The analysis of MAPK distribution was also conducted on cells expressing high levels of GFP-ERK1 or GFP-ERK2 as the result of increased drug selection. At the higher expression levels, GFP-MAPK fusions were more concentrated in the nucleus, an observation also found for

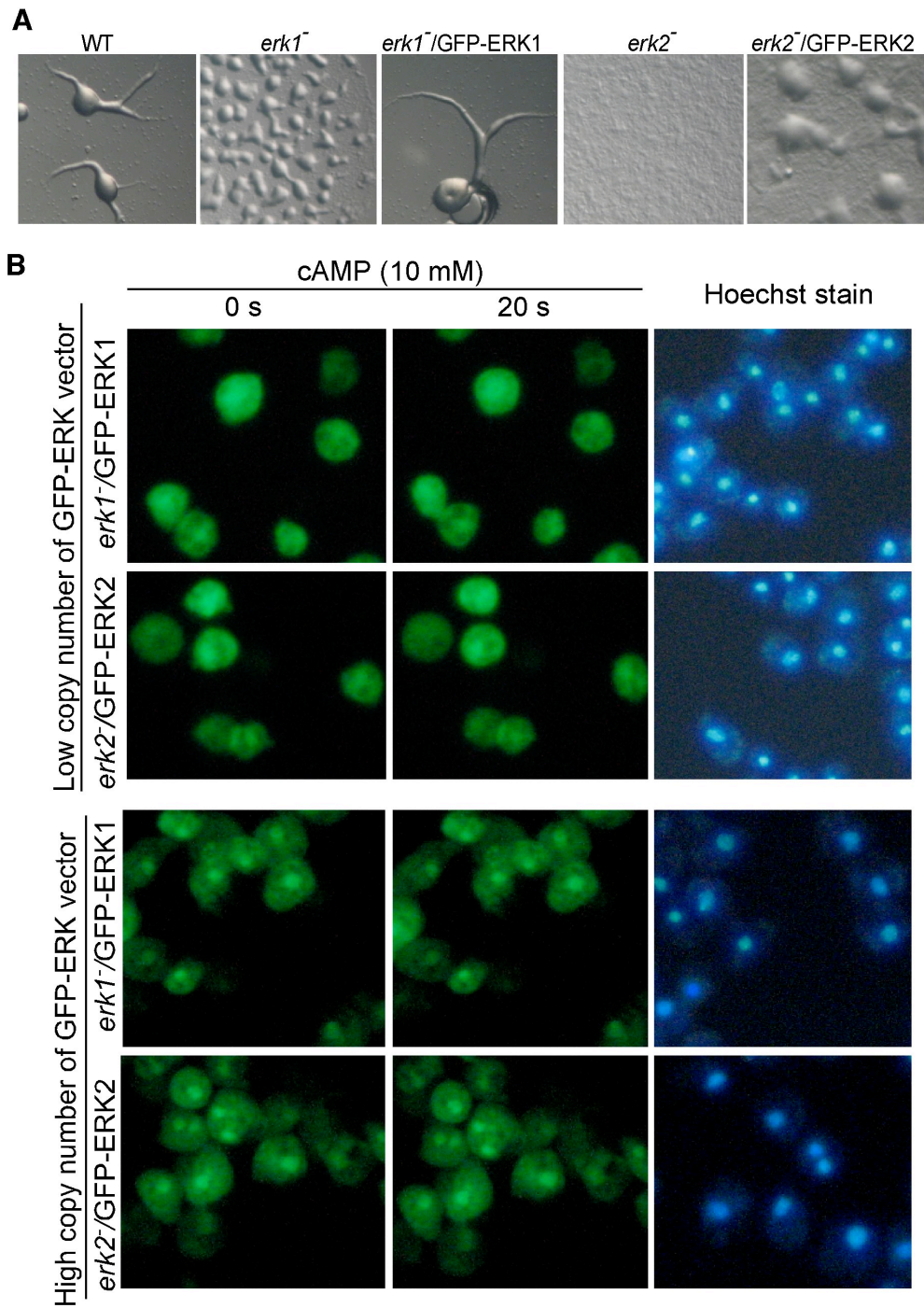


Fig. 24: cAMP-stimulated sub-cellular distribution of GFP-ERK1 and GFP-ERK2. (A) Developmental phenotypes of wild-type, *erk1*⁻ and *erk2*⁻ cell without and with the GFP-ERK expression vectors at 12 h after cell plating. (B) Localization of GFP-ERK1 in *erk1*⁻ cells and GFP-ERK2 in *erk2*⁻ cells selected at low (2 µg/ml) or high (6 µg/ml) drug concentrations. Cells

were prepared for the cAMP stimulation as described in section 2.3 and then allowed to adhere to cover slips for a period of 30 minutes. Cells were stimulated with cAMP 10 mM and GFP fluorescence was photographed at times shown. Each strain was also treated with ethanol (5%) and Hoechst dye to identify nuclei (panels on right).

GFP-MAPKs in mammalian cells (Costa et al., 2006). The stimulation of these cells with cAMP or folate also did not result in any major changes in GFP-ERK1 or GFP-ERK2 distribution (data for folate stimulation not shown).

Discussion

The analysis of ERK1 and ERK2 function in G protein-mediated signaling pathways suggests these ERKs are not redundant or overlapping in function but rather have different roles in different signaling pathways (Fig. 25). The requirement of ERK1 but not ERK2 for the precocious tip development and lethality associated with G α 5 subunit over-expression support a role for ERK1 in G α 5 subunit mediated signal transduction. The requirement for both ERK1 and the G α 5 subunit to inhibit folate chemotaxis is another indication these signaling components function in a common pathway. Previous studies have indicated the importance of ERK2 for G α 4 subunit mediated responses such as chemotaxis to folate even though the *erk2*- cells are only partially defective in this response (Hadwiger et al., 1994; Wang et al., 1998). Although not tested, the G α 4 subunit and ERK2 are also likely to function together in folate-stimulated cAMP accumulation and prespore cell localization and development based on the phenotypes associated with *G α 4*- and *erk2*- cells (Gaskins et al., 1996; Segall et al., 1995). ERK2 rather than ERK1 appears to be the primary MAPK phosphorylated in response to cAMP and the importance of ERK2 in cAMP-mediated aggregation is exemplified by the aggregation defect of *erk2*- cells. Unlike the responses to folate, cAMP-stimulated ERK2 activation does not require the G α 2 or G β subunits. The inability of *erk1*- cells to form large aggregation streams suggests a possible limitation on the

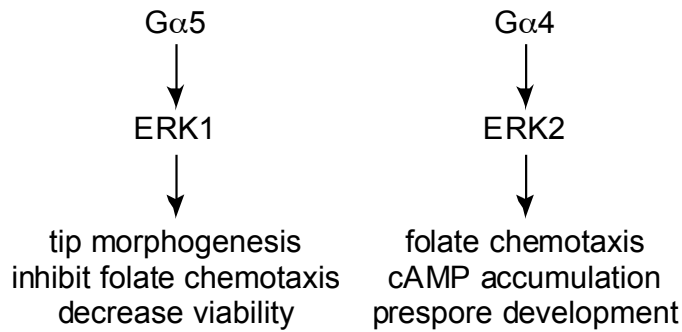


Fig. 25: Model of Gα subunit-ERK pathway specificity. Signals mediated by the Gα5 subunit require ERK1 function to enhance tip formation, inhibit chemotaxis, and reduce cell viability. Folate chemotaxis, cAMP accumulation, and prespore development are responses that can require the Gα4 subunit and ERK2. The Gα4 subunit is necessary for the phosphorylation of ERK2 in response to folate.

aggregation territory that might be attributed to deficiencies in intercellular signaling. However most *erk1*- cells appear to be acutely responsive to cAMP-stimulated chemotaxis or cGMP accumulation.

The distribution of *erk1*- and *erk2*- cells in chimeric organisms implies cell differentiation and/or sorting preferences of these cells is significantly different. The presence of *erk1*- in the prestalk and prespore regions near the anterior of the migratory slug is consistent with the ability of *erk1*- cells to form both spore and stalk cells but inconsistent with the block or delay of tip morphogenesis in clonal populations of *erk1*- cells. While capable of migrating to the tip regions, *erk1*- cells must have defects in intercellular signaling that can be diminished by the presence of wild-type cells. Therefore, ERK1 might possibly mediate the production of signals necessary for tip morphogenesis rather than responding to chemotactic signals such as cAMP that promote localization to the tip (Dormann et al., 2000). The spatial pattern of *erk2*- cells in chimeric organisms reflects the partial deficiencies of these cells to chemotax to cAMP and to become prespore or prestalk A cells as previously described (Gaskins et al., 1994). The *erk1-erk2*- cells

have these same deficiencies as *erk2*- cells except the *erk1-erk2*- cells can migrate to the prestalk A region. This difference is likely due to the absence of ERK1 as indicated by the strong anterior positioning of *erk1*- cells.

The inability to detect phosphorylated ERK1 in response to cAMP or folate suggests very limited if any ERK1 is activated by the typical phosphorylation mechanism in these responses. Possible explanations for the absence of phosphorylated ERK1 might be a limitation on ERK1 expression as suggested by the reduced viability of cells transformed with ERK1 compared to ERK2. Alternatively, ERK1 activation might occur with very different kinetics compared to ERK2. A previous report of ERK1 activation in response to cAMP indicated a relatively rapid peak in ERK1 kinase activity at 15 s. Interestingly, the phosphorylation of mammalian ERKs has been reported as early as 1 min after stimulation by a chemoattractant and the activated state can be maintained for over 15 min after stimulation with a growth factor. Differences in *Dictyostelium* ERK activation might also reflect the specificity of ERK1 or ERK2 involvement in a particular pathway or a subpopulation of developing cells. In mice, the loss of ERK1 can result in increased ERK2 activation suggesting an oppositional relationship between related MAPKs (Mazzucchelli et al., 2002). The similarity of ERK2 activation in wild-type and *erk1*- cells suggests there is no such oppositional relationship between ERK1 and ERK2 function, at least with respect to cAMP- and folate-stimulated MAPK phosphorylation in early development.

Neither ERK1 nor ERK2 is required for cell growth suggesting *Dictyostelium* MAPK function is not an important factor for the proliferation of unicellular eukaryotes. This conclusion was implied by earlier studies showing the viability of yeast *fus3-kss1*- mutants but yeast have other MAPKs that might compensate for the loss of these ERKs (Chen and Thorner, 2007). The requirement of mammalian ERK2 for tumor cell growth and embryonic development suggests that ERK function might be necessary for the proliferation of a specific differentiated cell type or to overcome growth inhibition mechanisms in multicellular organisms (Vantaggiato et al., 2006;

Yao et al., 2003). This concept is consistent with the regulation of cell growth through growth factors activate MAPKs.

CHAPTER V

THE MAP KINASE ERK1 REGULATES THE PHOSPHODIESTERASE REGA AND THE NUCLEAR LOCALIZATION OF THE TRANSCRIPTION FACTOR STATc IN *DICTYOSTELIUM* DEVELOPMENT

Abstract

Mitogen activated protein kinase (MAPK) signaling is important for eukaryotic development but the role of MAPK specificity in gene expression and cell movement remains to be defined in most developmental processes. *Dictyostelium discoideum* expresses only two MAPKs, ERK1 and ERK2, which play different roles in developmental morphogenesis. Using tandem mass spectrometry, proteins associated with ERK1 were identified and these included the cAMP-specific phosphodiesterase RegA and the signal transducer and activator of transcription STATc. Disruption of the *regA* gene in *erk1*- cells rescued tip morphogenesis in clonal aggregates and anterior localization in chimeric organisms implying the absence of RegA function is epistatic to the loss of ERK1. In *erk1*- but not *erk2*- slugs there was an absence of STATc nuclear localization and repression of the *ecmA* gene in pstO cells indicating ERK1 is necessary for STATc regulation in these cells. Similar phenotypes were observed for both *erk1*- and *ga5*- cells, suggesting RegA and STATc act downstream of Gα5 subunit-ERK1 signaling pathways. These findings suggest RegA is negatively regulated by ERK1, in addition to ERK2, and that the regulation of STATc function in pstO cells is ERK1 specific.

Introduction

Mitogen activated protein kinases (MAPKs) function in many eukaryotic signaling pathways to regulate cellular responses to many types of external signals (Lloyd, 2006; Raman *et al.*, 2007). The subgroup of MAPKs known as extracellular signal regulated kinases (ERKs) has been found in a wide range of eukaryotes suggesting these kinases regulate basic cellular processes. These processes include the regulation of cell growth, differentiation and cell movement and therefore these kinases are expected to play important roles in the development of many organisms (Krens *et al.*, 2006; Chen and Thorner, 2007). The activation of ERKs downstream of receptor tyrosine kinases and G protein-coupled receptors typically involves a cascade of kinases resulting in the phosphorylation of conserved residues in the ERK proteins (Caunt *et al.*, 2006). The kinase cascades often include scaffold proteins indicating that ERK activation can occur in complexes that contain other kinases and perhaps other types of signaling proteins (Dhanasekaran *et al.*, 2007). Activated ERKs in turn phosphorylate many different substrates in the cytosol and nucleus and some of the more notable substrates include other protein kinases, phosphodiesterases, and transcription factors (Tanoue and Nishida, 2003; Raman *et al.*, 2007).

The number of ERKs in most organisms is relatively small compared to the number of signaling pathways in which they operate and therefore the specificity of these signaling pathways is likely to be maintained through regulated interactions between ERKs and other signaling proteins (Bogoyevitch and Court, 2004; Morrison and Davis, 2003; Goldberg *et al.*, 2006; Chen and Thorner, 2007; Miranda-Saavedra and Barton, 2007; Krens *et al.*, 2008). In many ERK signaling pathways the use of scaffold proteins is one such mechanism to generate pathway specific complexes (Elion, 2001). This specificity has been demonstrated in the yeast mating response signaling pathway by the ability of the scaffold (Ste5) to enhance the MAPK kinase (Ste7) phosphorylation of a specific ERK (Fus3) (Good *et al.*, 2009). However, less is known about the mechanisms to regulate signaling specificity downstream of ERK activation. The availability and affinity of substrates for specific ERKs are likely to be important factors for

pathway specificity and therefore identifying proteins associated with ERKs is an important step in understanding the role of ERKs in development. Organisms that allow for genetic analysis are particularly valuable in characterizing these protein associations *in vivo* (Chen and Thorner, 2007).

The soil amoebae *Dictyostelium discoideum* contains only two ERKs, ERK1 and ERK2, that share approximately 37% identity with each other (Goldberg *et al.*, 2006; Nguyen *et al.*, 2010). These ERKs regulate different processes during this organism's developmental life cycle as indicated by the phenotypic analysis of ERK mutants (Gaskins *et al.*, 1996; Sobko *et al.*, 2002; Nguyen *et al.*, 2010). ERK2 is required for the down regulation of the cAMP-specific phosphodiesterase, RegA, and the reduced phosphodiesterase activity allows for the generation of a cAMP signal that facilitates the aggregation of individual cells after starvation (Segall *et al.*, 1995; Maeda *et al.*, 2004). ERK2 is also important for prespore gene expression and the development of spores as the multicellular aggregate becomes a migratory slug and then a fruiting body consisting of a mass of spores on top of a stalk (Gaskins *et al.*, 1996). In contrast, ERK1 is important for formation of an anterior tip structure that is composed of prestalk cells and that directs the morphogenesis of the multicellular aggregate into a slug (Sobko *et al.*, 2002; Nguyen *et al.*, 2010; Raisley *et al.*, 2010). ERK1 can function downstream of the Gα5 subunit-mediated signal transduction pathway to regulated tip morphogenesis whereas ERK2 can function downstream of the Gα2 and Gα4 subunit-mediated pathways that respond to external cAMP and folate, respectively (Nguyen and Hadwiger, 2009; Nguyen *et al.*, 2010; Raisley *et al.*, 2010).

The function of ERK1 in tip morphogenesis has not been extensively characterized but many features of tip morphogenesis have been established through the analysis of prestalk gene expression. The anterior prestalk region consists of two major cell types; the pstA cells located at the very anterior and the more posterior pstO cells that separate the pstA cells from the prespore region (Williams, 2006). The prestalk gene *ecmA* is expressed in pstA cells but repressed in pstO

cells in response to prespore cell-released morphogen, DIF-1 (Fukuzawa et al., 2001). The receptor for DIF-1 has not been identified but the activation of a signal transducer and activator of transcription (STAT) protein STATc in pstO cells is required for *ecmA* repression. STATc is localized to the nucleus of pstO and anterior-like cells (posterior cells with prestalk cell characteristics) but not in pstA cells. STATc is one of 4 *Dictyostelium* STAT proteins (STATa-d) with structural and functional similarity with mammalian STAT proteins that regulate gene expression in response to cytokines (Kawata et al., 1997; Leonard and O'Shea, 1998; Zhukovskaya et al., 2004). In contrast to STATc, the *Dictyostelium* STATa protein localizes to the nucleus and represses the expression of the *ecmB* gene in pstA cells (Mohanty et al., 1999). *ecmB* expression occurs primarily in pstAB cells that are localized to the interior core of the tip structure (Williams et al., 1989; Williams, 2006).

ERK1 and ERK2 have different developmental roles in *Dictyostelium* but little is known about how these ERKs maintain pathway specificity (Nguyen *et al.*, 2010). In this study, *ERK1*-associated proteins were identified using tandem mass spectrometry to analyze ERK1 complexes and genetic analysis verified their role in ERK1 signaling pathways. Two of these proteins, RegA and STATc, were examined for function in ERK1 signaling pathways. Loss of RegA function was found to rescue the defects associated with tip morphogenesis in *erk1*- and *ga5*- cells implying the phosphodiesterase is negatively regulated through the Ga5-ERK1 signaling pathway. Both ERK1 and Ga5 subunit function were required for nuclear localization of STATc in pstO cells indicating STATc is also regulated through the Ga5 subunit-ERK1 signaling pathway. These results indicate ERK1 regulates both RegA and STATc to promote proper cell movement and cell differentiation during development.

Materials and Methods

Strains and Recombinant DNA.

All strains were isogenic to the wild-type strain, KAx3, except where noted. The creation of *erk1*-, *erk2*-, and *erk1-erk2*- (double mutant), *ga5*- and *Ga5HC* mutants has been previously described (Nguyen and Hadwiger, 2009; Nguyen *et al.*, 2010; Raisley *et al.*, 2010). Electroporation of *Dictyostelium* was conducted as described (Hadwiger, 2007).

The *regA* gene disruptions were created using a rescued blasticidin resistance-REMI vector with flanking RegA sequences kindly provided and previously described by Adam Kuspa and Gad Shaulsky (Baylor College of Medicine) (Shaulsky *et al.*, 1996). To disrupt RegA in strains already conferring resistance to blasticidin S, a hygromycin resistance vector (pDM371) was co-transformed into cells and transformants were selected for hygromycin resistance. A His₆-tagged ERK1 expression vector was created as previously described for the His₆-ERK2 expression vector (Nguyen and Hadwiger, 2009). The *regA* gene disruptions were verified using PCR amplification analysis of the genomic DNA with the oligonucleotides (sense strand 5'-CTTCAACGTCGACATCACCATCACC-3') and (antisense strand 5'-CGCTTGAACCTAC TGATGCTGCTAATTCTTTGTATTG -3'). GFP:STATc and GFP:STATa reporter constructs were kindly provided by Jeff Williams (U. of Dundee) (Dormann *et al.*, 2001; Fukuzawa *et al.*, 2001). Wild-type and mutant Ga5 subunit expression vectors were previously described (Hadwiger *et al.*, 1996; Raisley *et al.*, 2010). *cotB::GFP*, *ecmA::lacZ*, and *ecmB::lacZ* reporter gene vectors were previously described (Jermyn and Williams, 1991; Nicol *et al.*, 1999).

Development

Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12mM NaH₂PO₄ adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10^8 cells/ml), before spotting on nonnutrient plates (phosphate buffer, 1.5% agar) for development as described (Hadwiger *et al.*, 1996). Cell development was analyzed using a

dissecting microscope or fluorescence microscopy. Staining of cells expressing *lacZ* reporter constructs was conducted as previously described (Haberstroh and Firtel, 1990).

Mass Spectrometry

Cells expressing His₆-ERK1 or His₆-ERK2 and wild-type cells were grown to mid log phase and plated for development as described above. Harvested cells were washed in phosphate buffer and extracts treated with Talon Co²⁺ resin (Clontech) as previously described (Nguyen and Hadwiger, 2009). Eluates were subjected to SDS-PAGE and western blotting using anti-His₆-tag antibody to detect ERK protein as previously described (Nguyen and Hadwiger, 2009). Eluates were also denatured with urea reduced with TCEP, alkylated with IAA, and digested with trypsin, using ammonium bicarbonate to buffer in all solutions prior to MS/MS analysis. Samples were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objectives PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. Protein and peptide identification searches were conducted using SwissProt and NCBI *Dictyostelium* sequences and validated using Scaffold v2.2.00 (Proteome Software) and the PeptideProphet algorithm (Keller et al., 2002). Probability thresholds were greater than 50% probability for protein identifications, based upon at least one peptide identified with 95% certainty. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

RESULTS

ERK1 contributes to the movement of prestalk cells

Previous studies have indicated that *erk1*- aggregates are often delayed or completely deficient in tip morphogenesis even though *erk1*- cells can be found concentrated in the tip of chimeric organisms (Nguyen *et al.*, 2010). However, closer inspection of *erk1*- cells in chimeric aggregates revealed that *erk1*- cells are initially concentrated in the pstO region during the

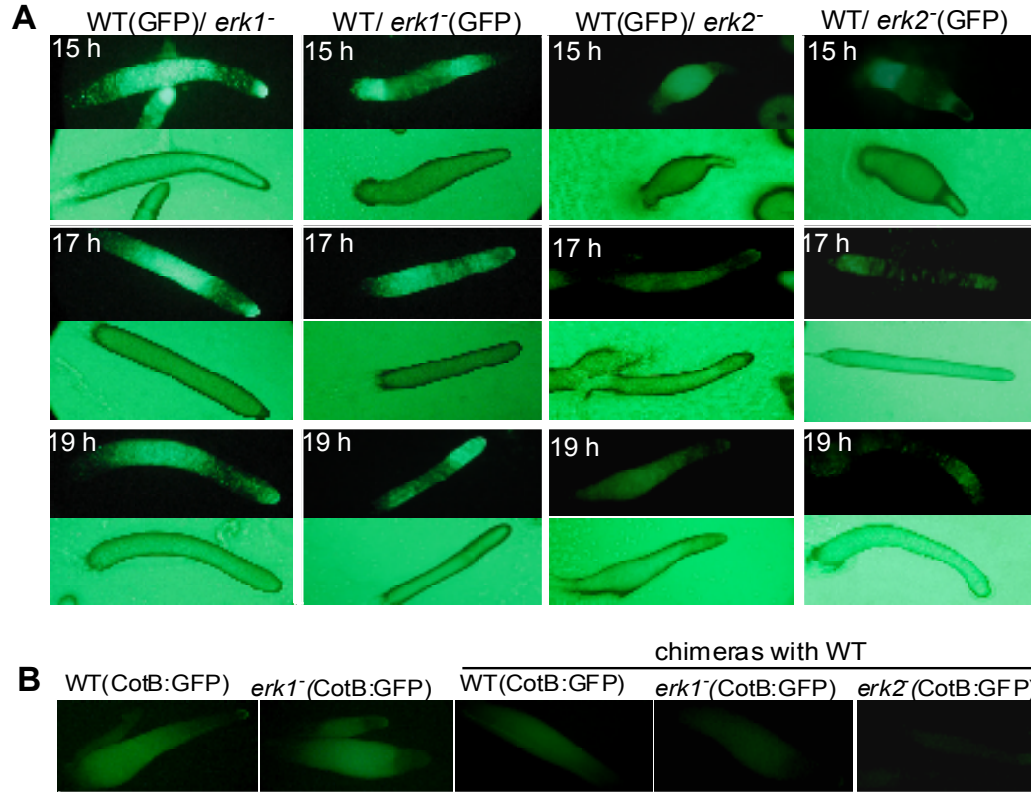


Fig. 26: Localization of *erk1*⁻ and *erk2*⁻ cells in chimeras during the slug stage of development. (A) GFP-expressing wild-type cells were mixed with *erk1*⁻ and *erk2*⁻ cells and GFP-expressing *erk1*⁻ and *erk2*⁻ cells were mixed with wild-type cells in a 1:4 ratio (except for a 1:1 ratio for *erk2*⁻ cells) and the distribution of fluorescent cells were recorded (upper panels) at the times indicated after starvation. Brightfield slug images are shown in the lower panels. Each pattern of cell distribution was representative of several slugs at that time in development. (B) Expression of the prespore reporter CotB:GFP in wild-type and *erk1*⁻ clonal aggregates or chimeric aggregates at early slug stage. The ratio of CotB:GFP expressing cells to wild-type cells was 1:4. Clonal *erk2*⁻ cells expressing the CotB:GFP are not shown because these cells cannot form aggregates.

formation of the slug (Fig. 26A). Later during the slug stage, the *erk1*⁻ cells move forward to occupy the pstA region, in confirmation of the earlier report (Nguyen *et al.*, 2010). This eventual

movement of *erk1*- cells to the pstA region in chimeric organisms suggests there are mechanisms that can bypass the lack of ERK1 function. Such mechanisms could also be responsible for the ability of some *erk1*- clonal aggregates to overcome the block in tip morphogenesis and complete development. In chimeric organisms, the abundance of *erk1*- cells in the posterior region is reduced over time and perhaps some of these cells migrate to more anterior positions. The *erk1*- cell distribution in chimeras is similar to that of *erk2*- cells except that the majority of *erk2*- cells are left behind as the aggregate becomes a migrating slug and the *erk2*- cells retained in the pstO region do not migrate to the pstA region. The sparse distribution of *erk1*- cells in prespore region of chimeras suggests *erk1*- cells might also be deficient in prespore development. However, the prespore reporter protein *cotB::GFP* is easily detected in *erk1*- cells developing as clonal or chimeric aggregates indicating that *erk1*- cells are capable of prespore gene expression and localizing to the prespore region (Fig. 26B). In the case of the *erk2*- cells, prespore specific gene expression is deficient in chimeric organisms consistent with the role of ERK2 in prespore development as previously described (Gaskins *et al.*, 1996). Therefore, *erk1*- cells have clear differences in cell distribution and prespore gene expression compared to *erk2*- cells even though both mutants are initially located in the pstO and extreme posterior regions of chimeric organisms.

Identification of ERK1 associated proteins

One strategy to identify proteins that function in ERK1 signaling pathways is to analyze proteins that co-purify with a His₆-tagged ERK1 protein. A His₆-ERK1 expression vector was introduced into *erk1*- cells and shown to rescue normal aggregate size and tip formation during development indicating the His₆-tag does not significantly diminish ERK1 function (Fig. 27A). The His₆-ERK1 binds effectively to a Co²⁺ resin throughout several washes and then can be eluted as indicated by the detection of this protein in immunoblot analysis of the eluate (Fig.

27B). The eluates of the His₆-ERK1 isolation vegetative and developing cells were also analyzed using tandem mass spectrometry (MS/MS) to identify proteins associated with ERK1. As a

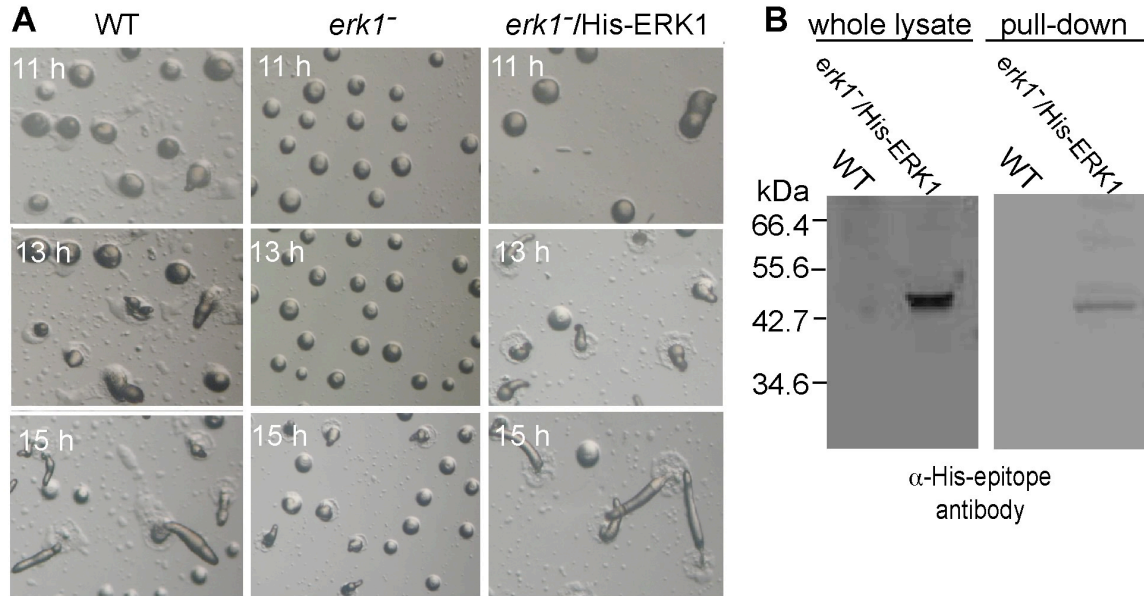


Fig. 27: Expression of His₆-ERK1 in erk1⁻ cells. (A) Wild-type cells and erk1⁻ cells with or without a His₆-ERK1 expression vector were plated on nonnutrient agar and examined for developmental morphogology at the times indicated. (B) Wild-type cells and erk1⁻ cells expressing a His₆-ERK1 expression were lysed and cell extracts were treated with Co²⁺ resin for His₆-ERK1 pull-down isolation. Whole lysates and eluates from the Co²⁺ resin (pull-down) were analyzed by immunoblotting using an anti-His₆-tag rabbit antibody and chemiluminescence detection system. Equivalent levels of initial cell extract were loaded in each lane. Relative positions of marker protein migration (kDa) are indicated.

control, similar Co²⁺-resin extractions were analyzed from *erk2*⁻ cells expressing a His₆-ERK2 expression vector and wild-type cells expressing no His₆-tagged proteins. The MS/MS peptide spectra of all three eluates were compared to identify candidate proteins that might be specifically associated with ERK1 complexes. Several of the proteins identified in the His₆-ERK1 eluates have been previously recognized or characterized as regulators of signal transduction pathways

(Table 1). The transcription factor STATc and the cAMP-specific phosphodiesterase RegA were of particular interest because previous reports have indicated the importance of these proteins in prestalk cell development (Kuspa and Loomis, 1992; Fukuzawa *et al.*, 2001). STATc was

ERK1 associated proteins			
0 h after starvation		6 h after starvation	
Protein	Spectra	Protein	Spectra
ERK1	63	ERK1	32
PakB	6	PakB	8
PTP2A	5	RegA*	2
STATc	4	STATc	1
Dst2	4		

Table 1: ERK1 associated proteins identified through tandem mass spectrometry. Proteins identified by peptide spectra in His₆-ERK1 but not His₆-ERK2 or control pull-down eluates. Pull-down assay and parameters of tandem mass spectrometry analysis are described in the material and methods section. () RegA peptide spectra were also identified in His₆-ERK2 eluates at 6 h after starvation.*

identified specifically in His₆-ERK1 eluates from vegetative and developing cells whereas RegA was identified in both His₆-ERK1 and His₆-ERK2 eluates from developing cells. STATc and RegA were not detected in eluates of wild-type cells that did not express a His₆-tagged ERK. RegA contains a putative D-motif (RRSVVQLIL) at an analogous position to a D-motif in the

human cAMP-specific phosphodiesterase PDE4D3, an enzyme that can be negatively regulated by ERK phosphorylation (MacKenzie et al., 2000). Other signaling proteins specifically associated with His₆-ERK1 eluates included the PP2A phosphatase regulatory subunit (DDB_G0279655) and the protein kinases Dst2 and PakB but only the relationships of ERK1 with RegA and STATc were investigated in this study.

regA gene disruption suppress erk1- defects in development

Previous studies have implicated a down regulation of RegA activity in ERK2 signaling pathways during aggregation (Shaulsky *et al.*, 1996; Maeda *et al.*, 2004). Disruption of the *regA* gene suppresses the defects of aggregation and prespore development of *erk2-* cells by allowing the accumulation of cAMP necessary for intracellular and intercellular signaling. RegA can be inactivated through the phosphorylation of a preferred MAPK phosphorylation site (PVTP) because changing the threonine residue to alanine prevents down regulation in response to extracellular cAMP. The detection of RegA in His₆-ERK1 eluates suggests that RegA might also be regulated in ERK1 signal transduction pathways. Therefore, the *regA* gene was disrupted in *erk1-* cells to examine if the loss of RegA can suppress the defective tip morphogenesis associated with the loss of ERK1. Transformants containing the RegA gene disruption displayed early tip morphogenesis and fruiting body formation at 20 hrs of development (Fig. 28A). The suppression of the *erk1-* tip morphogenesis phenotype indicates the *regA-* mutation is epistatic to the *erk1-* mutation, consistent with the down regulation of RegA function downstream of ERK1. The same *regA* gene disruption was also created in wild-type and *erk2-* cells for comparison. The *regA* gene disruption rescued aggregation and spore development in *erk2-* cells and the disruption accelerated tip morphogenesis in both wild-type and *erk2-* cells as previously reported (Shaulsky *et al.*, 1996).

In chimeric development with wild-type cells, the disruption of the *regA* gene also

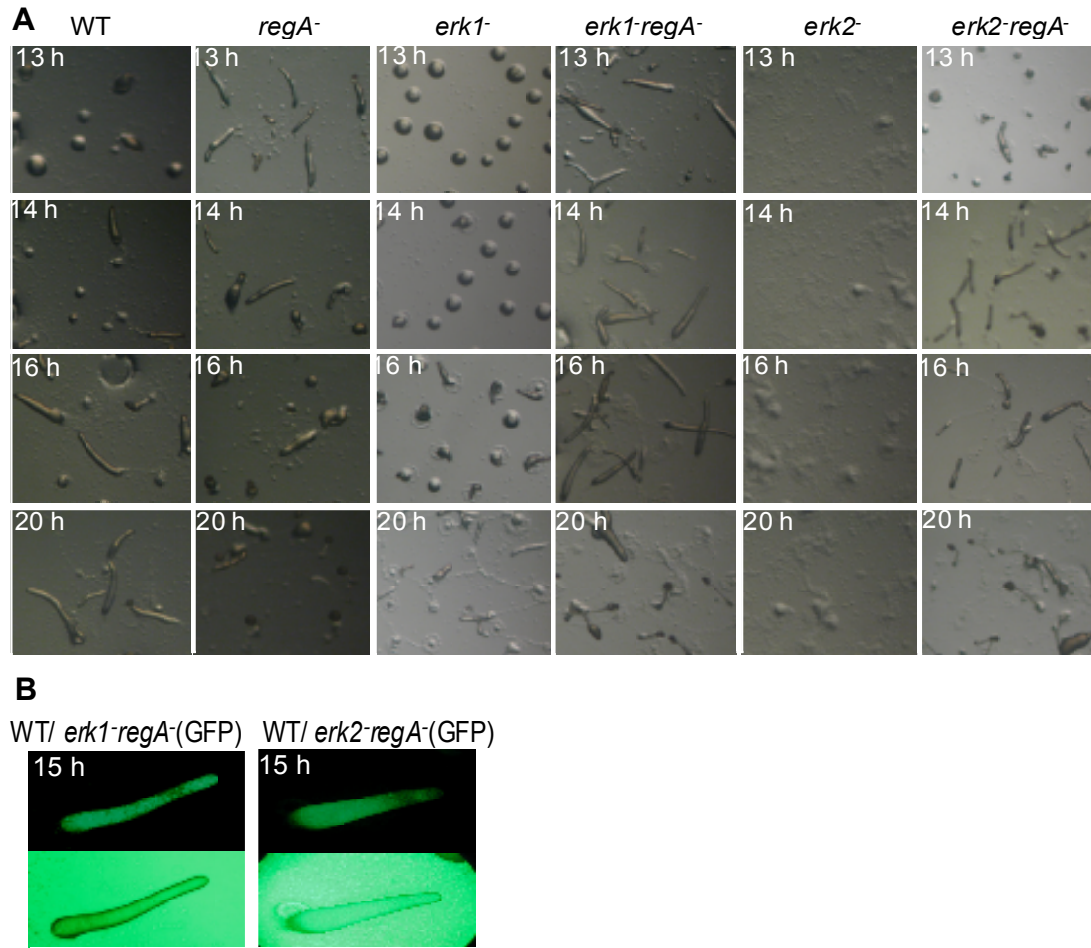


Fig. 28: Development of wild-type, erk1-, and erk2- strains with or without disruption of the regA gene. (A) Wild-type, regA-, erk1-, erk1-regA-, erk2-, and erk2-regA- cells were plated for development on nonnutrient agar plates and examined for developmental morphology at the times indicated. (B) Distribution of GFP expressing erk1-regA- and erk2-regA- cells in chimeras with wild-type cells at 15 hrs in development (upper panels). Mutant strains transformed with a GFP expression vector were mixed with wild-type cells at a 1:4 ratio and plated for development. Brightfield images are shown in the lower panels.

allowed *erk1*⁻ cells to localize to the pstA region as aggregates developed into slugs consistent with the suppression of the tip morphogenesis defects (Fig. 28B). The *erk1-regA*⁻ cells also occupied the middle of the prespore region at this stage of development. Similar shifts to anterior

regions were observed for *erk2*- cells containing the *regA* gene disruption. These changes in cell distribution due to the loss of RegA function suggest intracellular cAMP concentration is a limitation for distribution and/or differentiation of the *erk1*- and *erk2*- mutants. Interestingly, the wild-type cells in both the *erk1-regA*- and the *erk2-regA*- chimeras preferentially occupied the most anterior portion of the prespore region. The significance of this uneven distribution remains to be understood.

ERK1 is required for STATc nuclear localization in pstO cells

The association of STATc with His₆-ERK1 suggests that STATc might function in ERK1 signaling pathways during growth and development. Like other STAT proteins, STATc can be activated by the phosphorylation of a conserved tyrosine residue resulting in dimer formation and localization in the nucleus (Fukuzawa *et al.*, 2001). STATs can also be phosphorylated on other residues (serine/threonine) by ERKs, as reported in mammalian cells, but the importance of this mechanism remains to be completely resolved (Chung *et al.*, 1997). The *Dictyostelium* STATc contains nine putative MAPK phosphorylation sites (SP or TP), including a preferred site (PTSP), suggesting the STATc association with ERK1 could possibly involve STATc regulation through phosphorylation. To determine if STATc regulation depends on ERK1 function the nuclear localization of STATc was examined in *erk1*- cells using a GFP:STATc reporter protein. As previously reported, the GFP:STATc in wild-type cells localized to the nucleus of cells after aggregate formation and the nuclear localization was retained in *pstO* cells and anterior-like cells during slug migration (Fig. 29A) (Fukuzawa *et al.*, 2001). The nuclear localization of GFP:STATc in *erk1*- cells was observed during mound formation and in the slug anterior-like cells but the nuclear localization was greatly reduced or nonexistent in the *pstO* region cells suggesting a requirement for ERK1 in the *pstO* cells. STATc also undergoes nuclear localization in response to hyperosmotic stress as previously reported (Araki *et al.*, 2003). This hyperosmotic

stress response does not require ERK1 function because *erk1*⁻ cells, like wild-type cells, displayed GFP:STATc nuclear localization when exposed to 200 mM sorbitol.

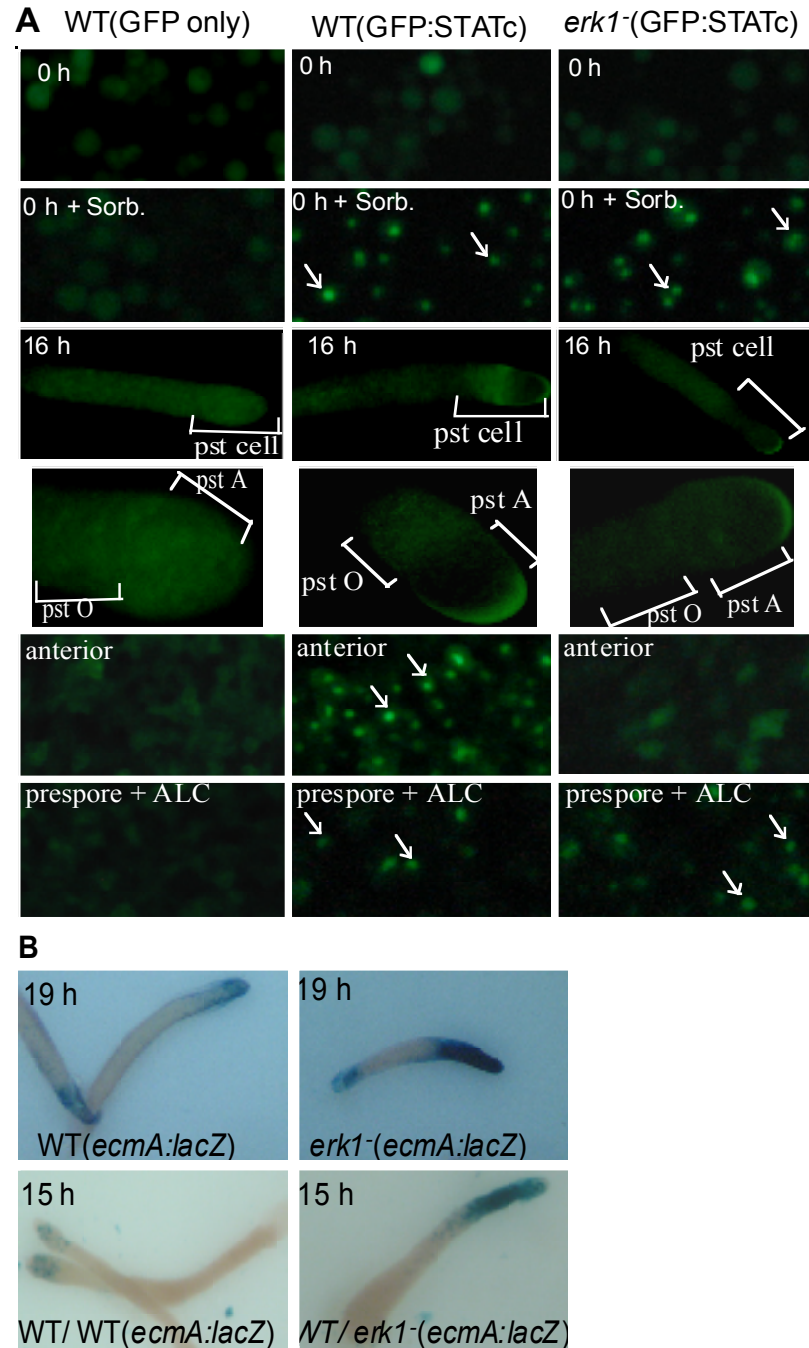


Fig. 29: Expression of GFP:STATc and *ecmA:lacZ* reporter genes in wild-type and *erk1*⁻ cells.

(A) Distribution of GFP:STATc in wild-type and *erk1*⁻ cells at 0 and 16 hrs after starvation. 4x

magnification of *pstO* region at 16 hrs is also provided (thinner panels). Wild-type cells expressing only GFP were used as a control to indicate cytoplasmic fluorescence. Vegetative cells (0 hr) were also incubated in 200 mM sorbitol to induce hyperosmotic stress (second row of panels). Arrows indicate nuclear localization of the GFP:STATc reporter protein. Anterior (*pstO* and *pstA* regions) and prespore/ALC regions of slugs were isolated using an aluminum foil scalpel for dissection and the disaggregated cells were visualized on glass slides with coverslips. Each dissection image represents typical results from several separate dissections from individual slugs. Slugs from several separate clones were analyzed. (B) *ecmA:lacZ* reporter gene expression in wild-type and *erk1*- clonal aggregates (upper panels) and chimeras (lower panels) at times indicated after starvation. Chimeras were created by mixing wild-type or *erk1*- cells expressing the *ecmA:lacZ* reporter gene with wild-type cells in a 1:1 ratio. Patterns of reporter gene expression were representative of multiple slugs from multiple clonal transformants.

STATc has been shown to repress the expression of the prestalk specific marker *ecmA* gene in *pstO* cells of developing aggregates as indicated by the enhanced expression of *ecmA:lacZ* reporter in the *pstO* cells of developing STATc null slugs (Fukuzawa *et al.*, 2001). To determine if ERK1 also regulates *ecmA* expression, an *ecmA:lacZ* reporter gene was expressed and monitored in *erk1*- and wild-type cells. Wild-type slugs displayed *ecmA:lacZ* expression in the *pstA* region but very limited expression in the *pstO* region (Fig. 29B). In contrast, *erk1*- cells had very intense expression of *ecmA* in both the *pstA* and *pstO* regions similar to that reported for STATc null cells. The *erk1*- cells also displayed increased *ecmA* expression near the posterior of the slug. In chimeras, the *erk1*- cells also displayed intense *ecmA* expression in the *pstO* region. The reduced *ecmA* expression in the *pstA* region is likely due to the limited occupancy of *erk1*- cells in this region during early slug stage. The aberrant expression of *ecmA* in the chimeras is cell autonomous and therefore not due to altered intercellular signaling in *erk1*- clonal aggregates.

These results are consistent with an *ERK1*-STATc signaling pathway repressing *ecmA* gene expression in cells located in the pstO region.

ERK2 is not required for STATc nuclear localization in pstO cells

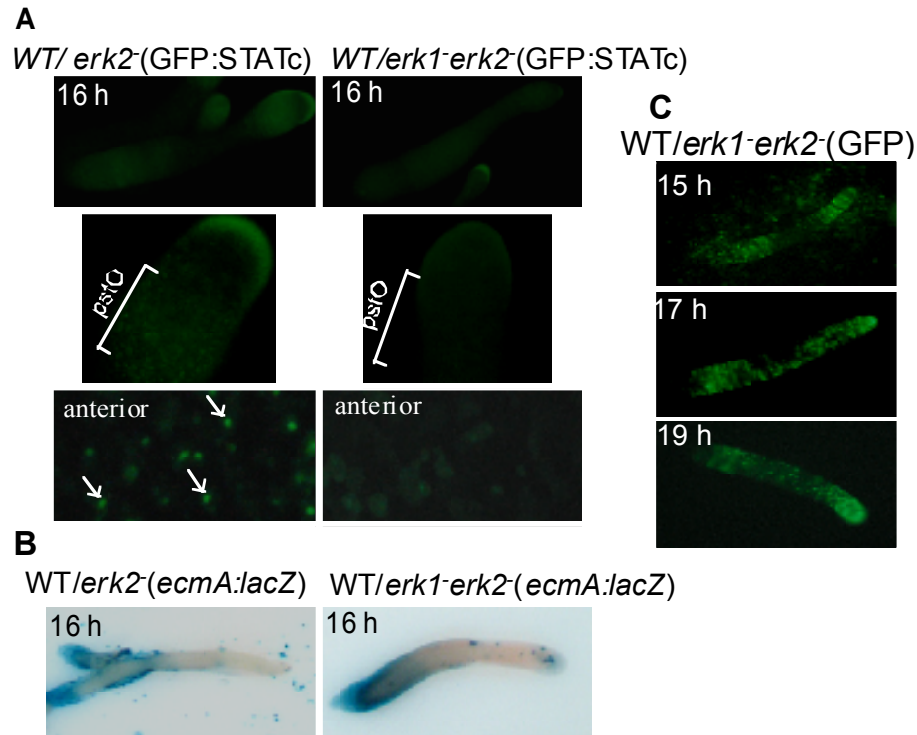


Fig. 30: Expression of GFP:STATc and *ecmA*:lacZ reporter genes in *erk2*⁻ and *erk1*⁻ *erk2*⁻ chimeric slugs. (A) Distribution of GFP:STATc in *erk2*⁻ and *erk1*⁻ *erk2*⁻ chimeric slugs in which the mutant cells were mixed with wild-type cells at a ratio of 1:4. Images were recorded at 16 hrs after starvation. 4x magnification of pstO region at 16 hrs is also provided (thinner panels). Arrows indicate nuclear localization of the GFP:STATc reporter protein. Anterior (pstO and pstA regions) regions of slugs were isolated and observed as described in Figure 4. Each dissection image represents typical results from dissections of multiple slugs from several clonal transformants. (B) *ecmA*:lacZ reporter gene expression in *erk2*⁻ and *erk1*⁻ *erk2*⁻ cells mixed with wild-type cells (ratio 1:1) to create chimeric organisms. Patterns of reporter gene expression were representative of multiple slugs from multiple clonal transformants. (C) GFP-expressing

erk1 erk2 cells were mixed with wild-type cells in a 1:4 ratio and the distribution of fluorescent cells were recorded at the times indicated after starvation. Each pattern of cell distribution was representative of several other slugs at that time in development.

The MS/MS analysis detected the presence of STATc in His₆-ERK1 but not His₆-ERK2 eluates from cells early in development. However, this result does not necessarily exclude the possibility that ERK2 might regulate STATc. To assess this possibility the GFP:STATc reporter protein was expressed and monitored in *erk2*- cells and *erk1-erk2*- double mutant cells during multicellular development. These mutant cells were developed with wild-type cells in chimeric organisms due to aggregation defect of cells lacking ERK2. The *erk2*- cells displayed nuclear localization of the STATc in the cells within the pstO region of the chimeric slugs indicating ERK2 is not a requirement for STATc nuclear localization in these cells (Fig. 30A). However, the *erk1-erk2*- strain did not display nuclear localization of GFP:STATc in pstO cells consistent with the dependency of STATc nuclear localization on ERK1 function. To further verify that ERK2 does not regulate STATc function, the expression of the *ecmA::lacZ* reporter gene was examined in *erk2*- and *erk1-erk2*- cells. The inability to detect reporter gene expression in *erk2*- cells in the pstO region of chimeric slugs further supports the idea that ERK2 is not required for the STATc repression of *ecmA* in pstO cells (Fig. 30B). The lack of reporter gene expression in the pstA region is consistent with the inability of *erk2*- cells to move to this region. The *erk1-erk2*- cells, like *erk1*- cells, displayed expression of *ecmA::lacZ* in both the pstO and pstA regions consistent with the lack of *ecmA* repression in these cells and the ability of *erk1-erk2*- cells to move to the pstA region (Fig 30C). Only a small proportion of the double mutant cells occupy the pstO and pstA regions of chimeras because without ERK2 function many of the mutant cells are left behind during slug development and migration. Interestingly, the *ecmA::lacZ* reporter gene is highly expressed in the cells at the very posterior of both mutants suggesting ERK2 might repress *ecmA* expression in posterior cells.

Gα5 subunit-mediated signaling can function upstream of RegA and STATc function

ERK1 can function in Gα5 subunit-mediated signaling pathways as indicated by a previous study and so RegA and STATc might also function in these pathways (Raisley *et al.*,

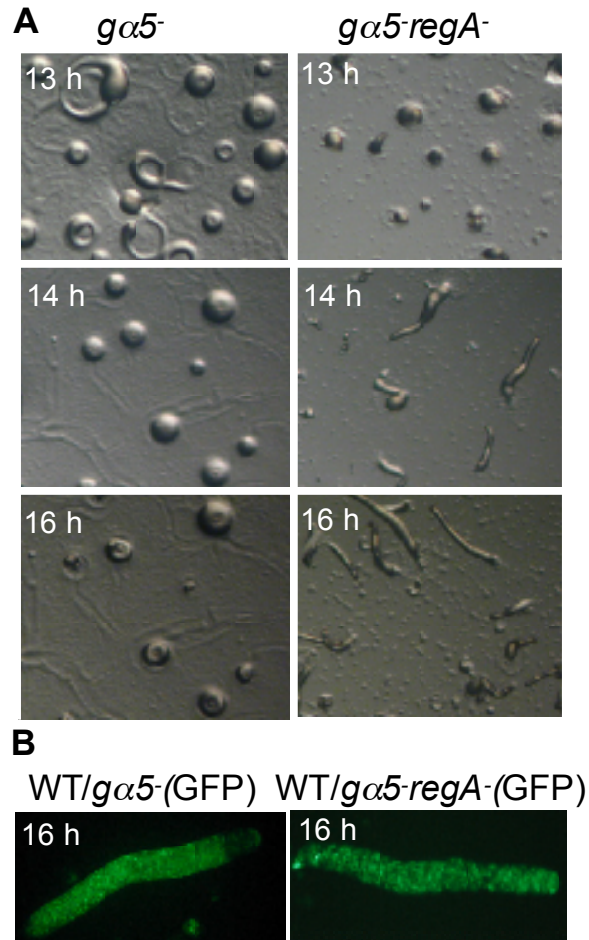


Fig. 31: Developmental morphology and cell distribution of $ga5^-$ and $ga5-regA^-$ cells. (A) $ga5^-$ and $ga5-regA^-$ cells were plated for development on nonnutrient agar plates and examined for developmental morphology at the times indicated. (B) Distribution of GFP expressing $ga5^-$ and $ga5-regA^-$ cells in chimeras containing wild-type cells at 16 hrs in development. Mutant strains transformed with a GFP expression vector were mixed with wild-type cells at a 1:4 ratio and plated for development.

2010). To determine if RegA functions downstream of Gα5 subunit-mediated signaling the *regA* gene disruption was created in *ga5*- cells and the resulting *ga5-regA*- double mutant was examined for the ability to suppress the delayed tip development associated with *ga5*- aggregates. Tip morphogenesis of the *ga5-regA*- aggregates typically occurred much earlier than tip morphogenesis in *ga5*- aggregates suggesting the loss of RegA is epistatic to the loss of the Gα5 subunit (Fig. 31A). The *ga5-regA*- cells were also found distributed to the *pstA* region in chimeric organisms at the early slug stage suggesting RegA activity delays the movement of *ga5*- cells to the *pstA* region of slugs (Fig. 31B). These observations are consistent with the Gα5 subunit activation of ERK1 and the subsequent inactivation of RegA in regulation of tip morphogenesis.

Nuclear localization of STATc was investigated in *ga5*- cells to determine if Gα5 signaling pathways also regulate STATc function. The *ga5*- cells did not show nuclear localization of the GFP:STATc during mound formation or in *pstO* and anterior-like cells during the slug stage of development (Fig. 32A). However, nuclear localization of GFP:STATc did occur in response to hyperosmotic stress (Fig. 32B). The transformation of a Gα5 subunit expression vector (constitutive expression from the *act15* promoter) into these cells resulted in ectopic GFP:STATc nuclear localization in all cells beginning at the aggregation stage of development suggesting that enhanced Gα5 subunit expression promotes STATc nuclear localization. In contrast, the transformation of a Gα5d- subunit (amino terminal D-motif mutant) expression vector into the *ga5*- cells did not rescue the GFP:STATc nuclear localization in *pstO* cells implying that Gα5 subunit-MAPK interactions might be critical to the regulation of STATc. Overexpression of the Gα5d- subunit, due to increased drug selection of the expression vector, did result in ectopic nuclear localization of STATc like that observed for the wild-type Gα5 subunit. The transformation of the GFP:STATc vector into *Gα5HC* cells (contain high copy number of the *Gα5* gene with the *Gα5* promoter) also resulted in ectopic STATc nuclear

localization during development beginning at the aggregation stage (data not shown). In addition to regulating STATc nuclear localization, the Ga5 subunit was also important for the repression of *ecmA* expression in pstO cells (Fig. 32C). The *ecmA:lacZ* reporter gene was strongly expressed in both pstO and pstA regions of *ga5*⁻ slugs.

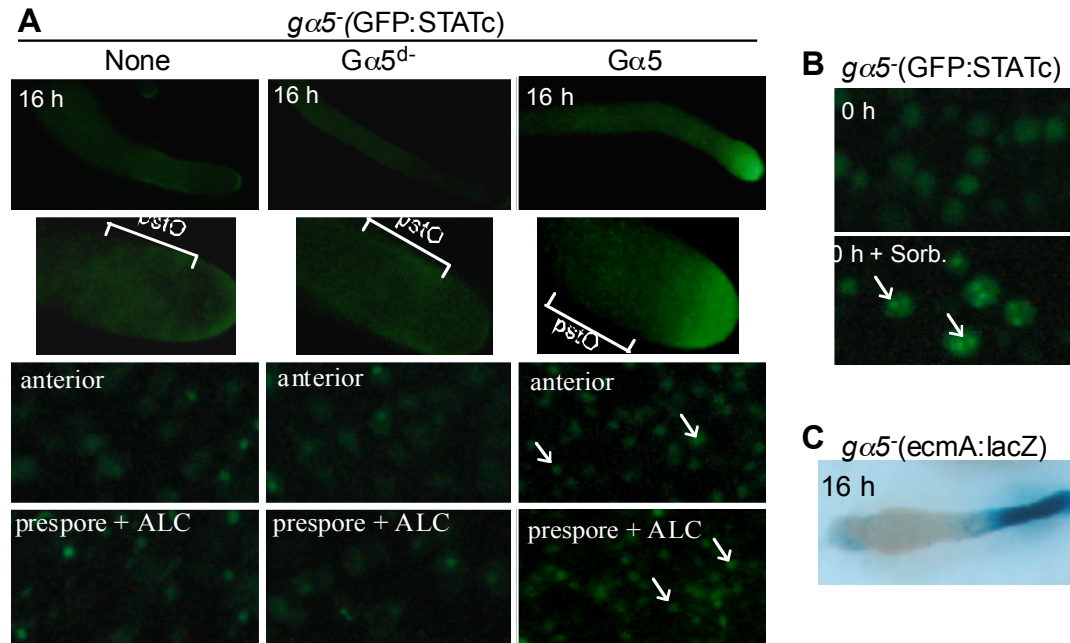


Fig. 32: Expression of GFP:STATc and *ecmA:lacZ* reporter genes in *ga5*⁻ cells with or without *Ga5*^{d-} and *Ga5* expression vectors. (A) Distribution of GFP:STATc in *ga5*⁻ cells with or without *Ga5*^{d-} and *Ga5* expression vectors at 16 hrs after starvation. 4x magnification of pstO region at 16 hrs is also provided (thinner panels). Anterior (pstO and pstA regions) regions of slugs were isolated and observed as described in Figure 4. Each dissection image represents typical results from several separate dissections from individual slugs. Slugs from several separate clones were analyzed. (B) Vegetative *ga5*⁻ cells expressing the GFP:STATc reporter protein (0 hr) were incubated with 200 mM sorbitol to induce hyperosmotic stress (+ Sorb.). Arrows indicate nuclear localization of the GFP:STATc reporter protein. (C) *ecmA:lacZ* reporter gene expression in *Ga5*⁻ clonal aggregates at 16 hr after starvation. Patterns are representative of multiple slugs from multiple clonal transformants.

Gα5 subunit-ERK1 signaling pathway does not directly regulate STATa nuclear localization and ecmB gene expression

The regulation of STATc through an ERK1 signaling pathway suggests other STAT proteins might be regulated downstream of MAPK signaling. The STATa transcription factor also contains a preferred MAPK phosphorylation site and a putative D-motif and therefore STATa might be regulated through MAPK signaling pathways. Nuclear localization of a GFP:STATa reporter protein occurs in many cells after aggregation but this nuclear localization is primarily retained in the extreme anterior region during late slug stage (Dormann *et al.*, 2001). STATa is also a repressor of the prestalk *ecmB* gene in these tip cells (Mohanty *et al.*, 1999). The role of ERK1 and the Gα5 subunit in the regulation of STATa nuclear localization was examined by expressing and monitoring the GFP:STATa reporter protein in wild-type, *erk1*- and *ga5*- cells. STATa nuclear localization was observed in all strains during aggregation but this process was slight delayed in *erk1*- and *ga5*- cells, perhaps due to the delayed development of these mutants (Fig. 33A&B) (Hadwiger *et al.*, 1996; Nguyen *et al.*, 2010). The disruption of RegA in *erk1*- and *ga5*- cells resulted in earlier nuclear localization of GFP:STATa protein consistent with rescued tip morphogenesis in these strains. This result is also expected because STATa nuclear localization is enhanced through increased cAMP levels (Dormann *et al.*, 2001). During the slug stage, the wild-type, *erk1*- and *ga5*- aggregates displayed nuclear localization of the STATa in the extreme anterior region of the slug and a lower level in the prespore region implying the loss of ERK1 or the Gα5 subunit did not impact the pattern of STATa nuclear localization at this stage of development (Fig. 33C). Expression of the *ecmB::lacZ* reporter gene in wild-type, *erk1*- and *ga5*- cells during late slug stage indicated that *erk1*- and *ga5*- slugs have repressed *ecmB* expression in tip cells similar to that observed for wild-type slugs, implying ERK1 and Gα5 function do not repress *ecmB* expression (Fig. 33D). Therefore, aside from the early developmental delays, the nuclear localization of STATa and repression of *ecmB* appear to be relatively normal in *erk1*- and

ga5⁻ slugs, suggesting the Ga5 subunit-ERK1 signaling pathway does not directly regulate STATa repression of *ecmB* expression.

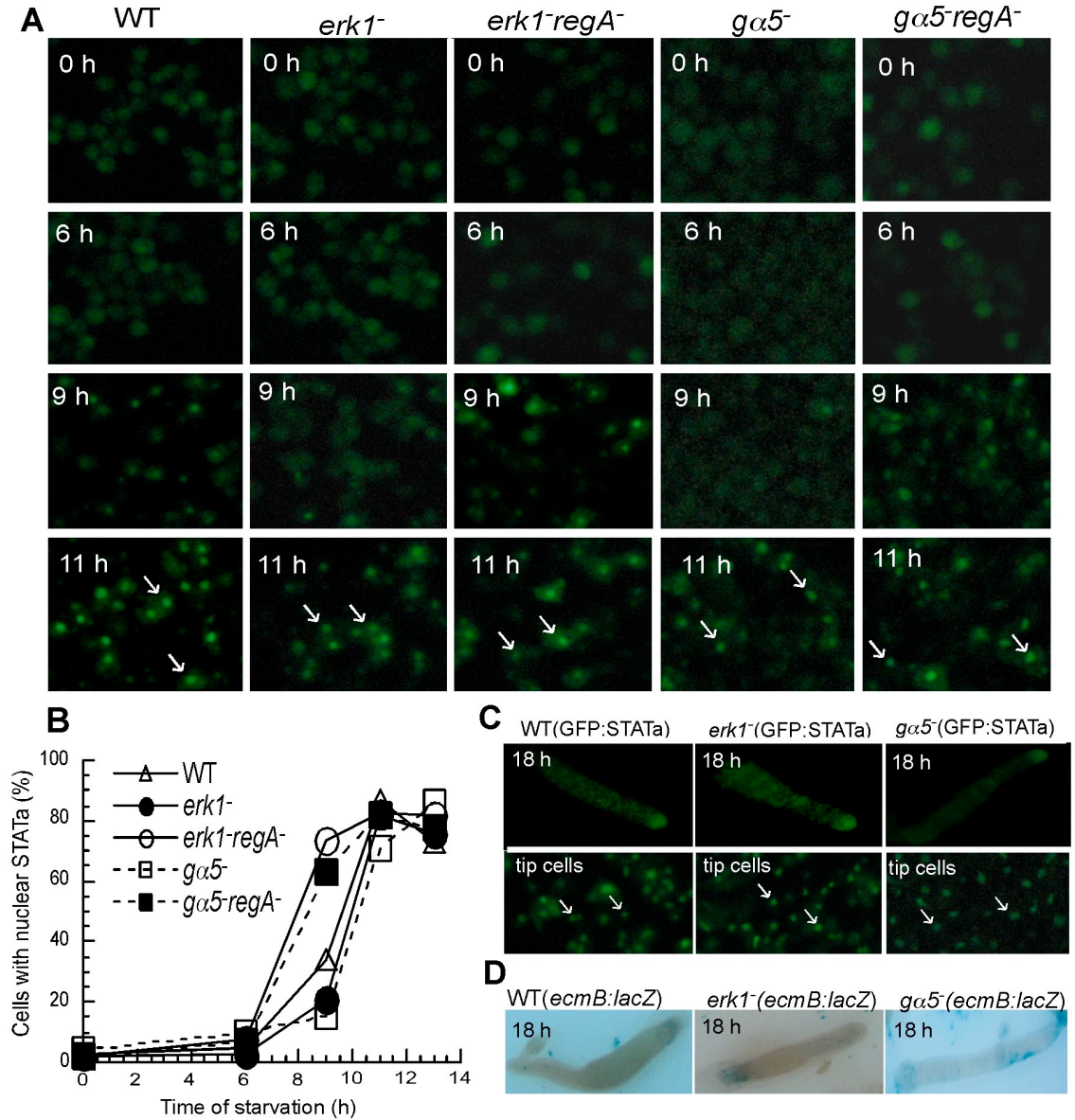


Fig. 33: Expression of GFP:STATa and *ecmB:lacZ* reporter genes in wild-type, *erk1*⁻, and *ga5*⁻ cells. (A) Distribution of GFP:STATa in wild-type, *erk1*⁻, *erk1-regA*⁻, *ga5*⁻, and *ga5-regA*⁻ cells at times indicated after starvation. Cells were developed on nonnutrient agar plates and then transferred to glass slides with coverslips for visualizing STATa localization. (B) Percentage of cells (from part A) with GFP:STATa nuclear localization at times indicated after starvation. Data

represent the analysis of at least 100 cells. (C) Distribution of GFP:STATa in wild-type, erk1⁻ and ga5⁻ slugs and isolated tip cells at 18 hrs after starvation. Arrows indicate nuclear localization of the GFP:STATa reporter protein. Each dissection image represents typical results from dissections from multiple slugs. Slugs from multiple clones were analyzed. (D) ecmB:lacZ reporter gene expression in wild-type, erk1⁻, and ga5⁻ clonal slugs at 18 hr after starvation. Patterns are representative of multiple slugs from multiple clonal transformants.

DISCUSSION

The initial abundance of *erk1*⁻ cells in the pstO and extreme posterior regions of chimeric slugs suggests *erk1*⁻ cells are either attracted to these locations or partially excluded from other regions due to the presence of wild-type cells. Both regions are proximal to the prespore region and can express prestalk genes, implying cell type similarities in spite of the different locations (Devine and Loomis, 1985; Williams, 2006). The delay in *erk1*⁻ cell movement from these regions to more anterior positions in chimeric slugs supports a role for ERK1 in prestalk cell development as previously suspected (Nguyen *et al.*, 2010). The eventual change in *erk1*⁻ cell distribution during slug migration suggests that cells from both regions can move in the direction of the anterior. Previous studies have shown that while *erk1*⁻ cells can chemotax, there might be cell polarity defects that affect directed cell movement (Sobko *et al.*, 2002; Nguyen *et al.*, 2010). Perhaps such deficiencies in directed cell movement contribute to the delay of *erk1*⁻ cell distribution in chimeric slugs.

The restoration of *erk1*⁻ cell distribution in chimeras due to the loss of RegA function suggests ERK1 negatively regulates RegA and that decreased cAMP levels contribute to the aberrant distribution of *erk1*⁻ cells (Fig. 34). The reported degradation of RegA protein after cell aggregation might account for the ability of *erk1*⁻ cells to eventually move toward the anterior. Interestingly, developing *erk1*⁻ cells show relatively normal levels of cAMP production when

stimulated by extracellular cAMP (data not shown) and *erk1*- cells, in contrast to *erk2*- cells, secrete sufficient cAMP to allow for aggregation. Therefore any *erk1*- deficiencies in cAMP

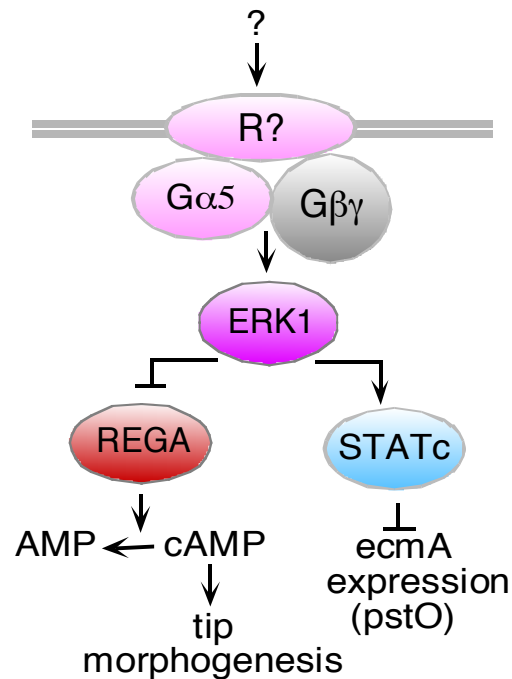


Fig. 34: Proposed model Ga5 subunit-ERK1 pathway regulation of RegA and STATc function in developing cells. Undetermined extracellular signal (?) stimulates Ga5 subunit coupled receptor (R?) resulting in Ga5 subunit mediated ERK1 activation. ERK1 down regulates RegA phosphodiesterase activity resulting in increased cAMP levels and promotion of tip morphogenesis. ERK1 promotes STATc nuclear localization and repression of the ecmA gene in pstO cells.

levels appear to be less critical for overall development than those associated with *erk2*- cells implying that ERK1 and ERK2 contributions to cAMP regulation might be substantially different. In support of this idea, ERK2 expression vectors can suppress *erk1*- tip morphogenesis but ERK1 expression vectors cannot suppress *erk2*- aggregation defects (Nguyen *et al.*, 2010). RegA interactions with ERK1 and ERK2 might also be different because studies in yeast have shown that the D-motif in a downstream MAPK substrate (e.g., Far1) can preferentially interact

with one MAPK over another (Remenyi *et al.*, 2005). The kinetics of ERK activation in *Dictyostelium* might also be a contributing factor because external cAMP stimulates the activation of ERK2 for several minutes whereas ERK1 is activated for less than one minute (Maeda *et al.*, 1996; Sobko *et al.*, 2002). Such differences in activation kinetics could be affected by phosphatase activity and the identification of a PP2A phosphatase subunit in ERK1 but not ERK2 complexes makes this an intriguing possibility (Table 1). Alternatively, ERK1 and ERK2 regulation of RegA might be restricted to different cytoplasmic regions of a cell or different cell types within the developing aggregate. Previous reports have indicated that changes in cAMP concentrations can be localized within individual cells or be varied throughout the different cell types of a multicellular aggregate (Merkle *et al.*, 1984). In support of the latter possibility, ERK2 function appears to be critical for prespore development whereas ERK1 function might preferentially regulate prestalk cell development.

The requirement of ERK1 in STATc nuclear localization is consistent with STATc functioning downstream of ERK1 and the association of STATc with ERK1 complexes (Fig. 9). The role of ERK1 in the regulation of STATc and ultimately the repression of *ecmA* in pstO cells underscores the importance of ERK1 function in prestalk cell development. The inability of ERK2 to regulate STATc nuclear localization and repress *ecmA* expression in pstO cells indicates the importance of ERK1 specificity in this developmental regulation. The actual mechanism of ERK1 regulation of STATc remains to be characterized but the presence of potential MAPK phosphorylation sites on STATc and the association of STATc with ERK1 suggest that STATc could be a potential substrate for ERK1 phosphorylation. In mammals, the ERK phosphorylation of STAT3 alters the regulation of gene expression and possibly contributes to cytoplasmic or mitochondrial functions (Wegrzyn *et al.*, 2009). Although a possible substrate of ERK1, the *Dictyostelium* STATc could also be regulated indirectly by ERK1 through other proteins such as tyrosine kinases, the PTP3 phosphatase, or nuclear import/export regulatory proteins. The

morphogen DIF-1 is thought to stimulate the serine/threonine phosphorylation of PTP3, the phosphatase that dephosphorylates the tyrosine residues of STATc (Araki *et al.*, 2008). ERK1 has been shown to be present in both the cytoplasm and nucleus indicating that ERK1 can potentially regulate transcription factors such as STATc in either location (Nguyen *et al.*, 2010).

The results of this study indicate the Gα5 subunit signaling pathway can regulate both RegA and STATc through ERK1 function providing further support for the function of ERK1 downstream of the Gα5 subunit (Raisley *et al.*, 2010). The regulation of a STAT protein through a G protein-coupled receptor contrasts the canonical regulation of STATs in mammalian systems through receptor tyrosine kinases (Lim and Cao, 2006). The *Dictyostelium* genome does not appear to encode this type of receptor tyrosine kinase and so the regulation of STATs through an alternative mechanism is perhaps not surprising (Williams, 1999). Previous studies in *Dictyostelium* have shown the requirement of cAMP receptors for the activation and nuclear localization of the STATA protein suggesting that G protein signaling might be an ancestral mechanism of STAT regulation. The receptor that activates the Gα5 subunit-ERK1 signaling pathway is not known but the ability of DIF-1 to also regulate STATc and *ecmA* gene expression in pstO cells leaves open the possibility that DIF-1 could activate or modulate this signaling pathway. The requirement for Gα5 subunit but not ERK1 for the nuclear localization of STATc in anterior-like cells suggests not all Gα5 subunit-mediated STATc nuclear localization is dependent on ERK1. The ectopic nuclear localization of STATc in strains overexpressing the Gα5 subunit is consistent with the bias of these strains to promote prestalk rather than prespore development. Many of the developmental phenotypes associated with Gα5 subunit overexpression have also been shown to be dependent on the Gα5 subunit amino terminal D-motif and ERK1 (Raisley *et al.*, 2010).

The approach of using mass spectrometry to identify ERK associated proteins has proven to be a useful strategy in uncovering proteins that function in ERK signaling pathways. The

genetic analysis of STATc and RegA in ERK1 signaling pathways supports the association of these proteins with ERK1 complexes. The apparent specificity of ERK1 and STATc interactions suggests that mechanisms exist for signal transduction pathways to specifically regulate transcription factors and gene expression. In contrast, RegA is associated with both ERK1 and ERK2 indicating not all downstream proteins are regulated by a specific ERK. However, differences in affinities, kinetics or spatial distribution could potentially allow ERK1 and ERK2 to differentially regulate RegA in separate signaling pathways. Although ERKs have been shown to form complexes with proteins that act upstream of ERK activation, the ability to find downstream signaling proteins associated with ERKs suggests that these signaling proteins might also form complexes.

CHAPTER VI

G PROTEIN AND MAPK SIGNALING PATHWAYS REGULATE STATa FUNCTION THROUGH THE PHOSPHODIESTERASE REGA

Abstract

Mitogen activated protein kinases (MAPKs) function in many different signaling pathways but in most organisms the specificity of this function remains to be defined. *Dictyostelium* express only two MAPKs, ERK1 and ERK2 and each plays a different role in the developmental life cycle. In this study, ERK2 but not ERK1 was found to be required for the nuclear localization of the transcription factor STATa in the anterior region of migrating slugs. This requirement is cell autonomous implying ERK2 functions in pstA cells in the prestalk zone of developing aggregates. The regulation of STATa by ERK2 is also required for the repression of the prestalk gene *EcmB* in pstA cells. ERK2 associates with the phosphodiesterase RegA and loss of the RegA gene suppresses ERK2 mutations by allowing STATa nuclear localization and repression of *ecmB*. Both the *Ga2* and *Ga4* signaling pathways can function upstream of ERK2 to regulate STATa nuclear localization. These findings suggest that G proteins regulate ERK2-STATa to control gene expression and prestalk cell development.

Introduction

MAPK signal transduction pathways regulate many aspects of eukaryotic cell growth, differentiation, movement, and survival (Goldsmith and Dhanasekaran, 2007). The presence of these pathways in animals, plants, fungi, and protists supports their role in controlling basic

cellular responses to changes in the environment (Chen and Thorner, 2007). The most conserved subgroup of MAPKs, the extracellular signal-regulated kinases (ERKs), are found throughout eukaryotic kingdom and therefore likely represent the earliest MAPK signaling pathways in evolution (Langenick et al., 2008). ERK signaling pathways can be activated by different types of receptors but many ERK signaling pathways include an upstream cascade of activating kinases (MAPK kinases, MAP2K and MAP2K kinases, MAP3K) that can be organized through scaffolding proteins (Wilkie et al., 1992). Downstream of ERK activation there are many different ERK substrates including other protein kinases, phosphodiesterases, and transcription factors (Raman *et al.*, 2007). Many of the proteins both upstream and downstream that interact with ERKs contain MAPK docking sites (D-motifs) that allow tethered interactions to promote the phosphorylation of or by the ERK. While many ERK-interacting proteins have been identified, many questions remain about ERK specificity in signaling pathways because of phenotypic differences between different ERK mutants (Lloyd, 2006; Nguyen *et al.*, 2010).

The soil amoebae *Dictyostelium discoideum* contains only two MAPKs, ERK1 and ERK2, but both play important roles in cell movement and differentiation during the developmental life cycle that allow nutrient-deprived solitary amoebae to form a multicellular fruiting body (Segall *et al.*, 1995; Gaskins *et al.*, 1996; Sobko *et al.*, 2002; Nguyen *et al.*, 2010). During this developmental process cells differentiate into prestalk and prespore cells that sort out into the anterior and central/posterior regions as the multicellular aggregate forms a migratory slug and then completely differentiate during culmination to form a mass of spores on top of a stalk (Loomis, 1982). ERK1 functions primarily to promote prestalk cell development downstream of the Gα5 G protein-signaling pathway to allow efficient development of the anterior prestalk region (i.e., tip structure) from a multicellular mound (Nguyen *et al.*, 2010; Raisley *et al.*, 2010). This tip structure primarily consists of two prestalk cell types, pstA (located at the extreme anterior) and pstO (located between pstA and the prespore region) (Williams, 2006). Cells

lacking ERK1 or Gα5 are delayed in forming pstA cells and fail to localize the STATc (signal transducer and activator of transcription) to the nucleus in pstO cells, resulting in ectopic expression of the prestalk-specific gene *ecmA* (Nguyen and Hadwiger, unpublished data). The Gα5 subunit-ERK1 pathway also down regulates the phosphodiesterase RegA because loss of RegA rescues the development of *ga5*- and *erk1*- cells. ERK2 functions downstream of the cAMP and folate receptors and respective G protein-mediated signaling pathways (Maeda *et al.*, 1996; Maeda and Firtel, 1997). These pathways utilize the Gα2 and Gα4 subunit containing G proteins for cAMP and folate responses, respectively, but only Gα2 subunit is partially required for ERK2 activation. ERK2 is important for prespore development and also for the pstA cell development (Gaskins *et al.*, 1996; Nguyen *et al.*, 2010). Like ERK1, ERK2 also down regulates RegA but this regulation is necessary to generate sufficient external cAMP for starved cells to undergo aggregation (Maeda *et al.*, 2004).

The recent discovery that STATc nuclear localization in pstO cells requires ERK1 but not ERK2 suggests ERKs might be specific for the regulation of individual STAT proteins (Nguyen and Hadwiger, unpublished data). ERK1 was found not be required for STATa nuclear localization in anterior cells indicating ERK1 does not regulate all prestalk STAT function. Besides STATc, the other well-characterized STAT in *Dictyostelium* is STATa. Loss of STATa results in ectopic *ecmB* expression in cells near the extreme anterior tip indicating STATa represses this gene expression (Mohanty *et al.*, 1999). STATa, like STATc, is activated through a tyrosine phosphorylation and then localizes to the nucleus during aggregate formation (Dormann *et al.*, 2001). However, STATa is retained in the nucleus of cells in the pstA region whereas the STATc is retained in the nucleus of pstO cells. STATa nuclear localization can be induced in prespore cells through stimulation of cAMP receptors or mechanical stress (Dormann *et al.*, 2001). The heterologous expression of an adenylyl cyclase, ACA, can also trigger nuclear localization (Mohanty *et al.*, 1999). The tyrosine kinase for these STATs has not been identified

but serine/threonine kinases can play a role in STAT regulation as indicated by the phosphorylation of STAT3 by ERK2 in mammalian cells (Chung *et al.*, 1997).

In this study, ERK2 was found to be required for STATa nuclear localization in the extreme anterior cells of a developing aggregate. ERK2 was important for cell autonomous signaling and not necessarily for the generation of an extracellular cAMP signal. Disruption of the *regA* gene restores STATa nuclear localization in *erk2*- cells and cell movement. Tandem mass spectrometry analysis of His6-ERK2 pull down assays indicated the association of RegA and the Gα2 subunit but not STATa. These results suggest the Gα2 and Gα4 subunit mediated signaling pathways act through ERK2 to regulate STAT nuclear localization and the repression of the *ecmB* gene.

Materials and Methods

Strains and Recombinant DNA.

All strains were isogenic to the wild-type strain, KAx3, except where noted. The creation of *erk1*-, *erk2*-, and *erk1-erk2*- (double mutant), *ga2*- and *ga4*- mutants has been previously described (Nguyen and Hadwiger, 2009; Nguyen *et al.*, 2010; Raisley *et al.*, 2010). Electroporation of *Dictyostelium* was conducted as described (Hadwiger, 2007).

The *regA* gene disruptions were created using a rescued blasticidin resistance-REMI vector with flanking RegA sequences kindly provided and previously described by Adam Kuspa and Gad Shaulsky (Baylor College of Medicine) (Shaulsky *et al.*, 1996). The His₆-tagged ERK2 expression vector was previously described (Nguyen and Hadwiger, 2009). The *regA* gene disruptions were verified using PCR amplification analysis of the genomic DNA with the oligonucleotides (sense strand 5'-CTTCAACGTCGACATCACCATCACC-3') and (antisense strand 5'-CGCTTGAAGTAC TGATGCTGCTAATTCTTTGTATTG -3'). The GFP:STATa

reporter construct was kindly provided by Jeff Williams (U. of Dundee). The *ecmB:lacZ* reporter gene vector was previously described (Jermyn and Williams, 1991; Nicol *et al.*, 1999).

Development

Cells were grown to mid-log phase (approximately $2-3 \times 10^6$ cells/ml), washed twice in phosphate buffer (12mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10^8 cells/ml), before spotting on nonnutrient plates (phosphate buffer, 1.5% agar) for development as described (Hadwiger *et al.*, 1996). Cell development was analyzed using a dissecting microscope or fluorescence microscopy. Staining of cells expressing *lacZ* reporter constructs was conducted as previously described (Haberstroh and Firtel, 1990).

Mass Spectrometry

Cells expressing His₆-ERK1 or His₆-ERK2 and wild-type cells were grown to mid log phase and plated for development as described above. Harvested cells were washed in phosphate buffer and extracts treated with Talon Co^{2+} resin (Clontech) as previous described (Nguyen and Hadwiger, 2009). Eluates were subjected to SDS-PAGE and western blotting using anti-His₆-tag antibody to detect ERK protein as previously described (Nguyen and Hadwiger, 2009). Eluates were also denatured with urea reduced with TCEP, alkylated with IAA, and digested with trypsin, using ammonium bicarbonate to buffer in all solutions prior to MS/MS analysis. Samples were analyzed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objectives PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. Protein and peptide identification searches were conducted using SwissProt and NCBI *Dictyostelium* sequences and validated using Scaffold v2.2.00 (Proteome Software) and the PeptideProphet algorithm (Keller *et al.*, 2002). Probability thresholds were greater than 50% probability for protein identifications, based upon at least one peptide identified

with 95% certainty. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Results

ERK2 is required for nuclear localization of STATa

The nuclear localization of STATa has been previously shown to occur in many cells upon aggregation and retained in the tip cells of migrating slugs. Increased adenylyl cyclase expression also promotes nuclear localization of STATa suggesting cAMP plays a critical role in STATa regulation. The recent discovery that the ERK1 MAPK can regulate STATc nuclear localization in pstO cells suggests that MAPK function might play a role in STATa nuclear localization. In support of this idea, STATa contains a putative MAPK docking site (D-motif) and several potential MAPK phosphorylation sites (S/T-P) including one preferred site (PTSP). Previous studies have indicated that ERK1 is not required for STATa nuclear localization in the tip cells of migratory slugs and so the role of ERK2 in regulating STATa was investigated by expressing a GFP:STATa reporter protein in *erk2*- mutants. Wild-type cells displayed nuclear localization of GFP:STATa by 11 hrs of starvation but nuclear localization was absent in both *erk2*- and *erk1-erk2*- cells indicating a requirement for ERK2 function (Fig. 35A&B). Since ERK2 is important for cAMP signaling early in development, the absence of STATa nuclear localization might result from a lack of intercellular cAMP signaling. To test this possibility cells expressing the GFP:STATa were treated with exogenous cAMP after 6 hrs of starvation. Wild-type cells displayed nuclear localization of GFP:STATa in response to cAMP but no nuclear localization was observed for *erk2*- or *erk1-erk2*- cells (Fig. 35C). While this result indicates that extracellular cAMP stimulation does not rescue STATa nuclear localization in *erk2*- mutants, other intercellular signaling factors or cell-cell contact might be necessary for *erk2*- mutant to regulate STATa in response to external cAMP.

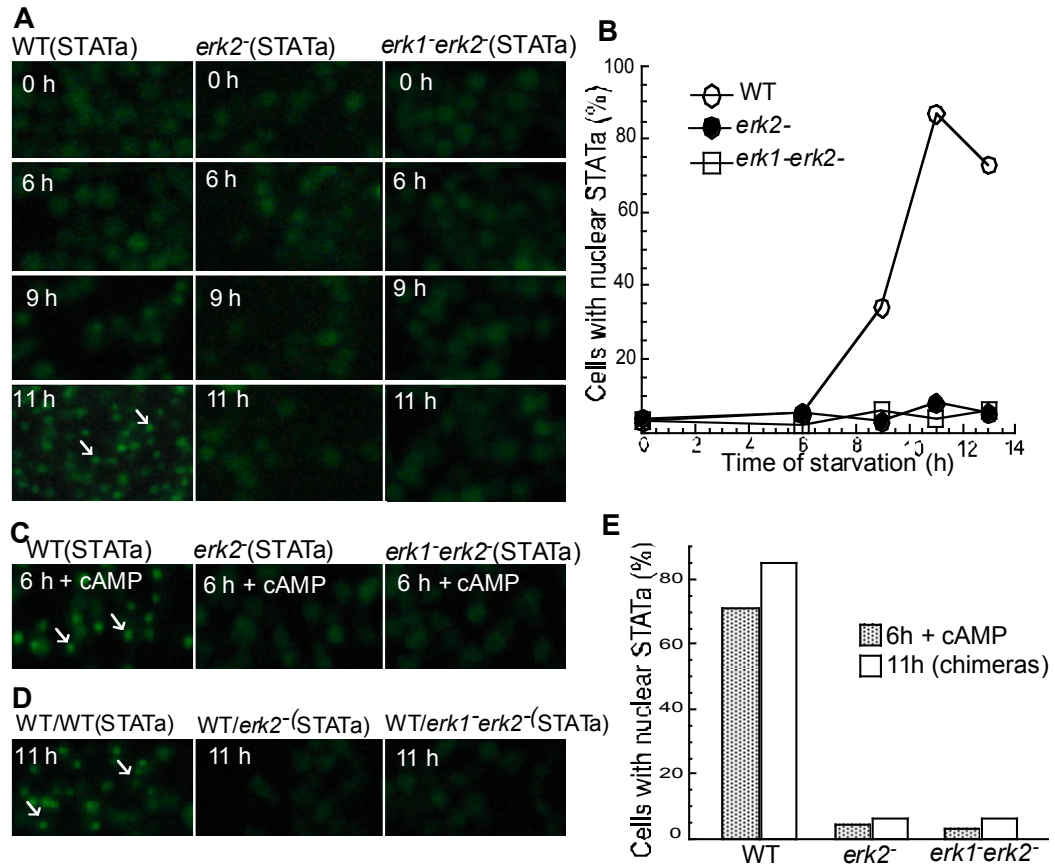


Fig. 35: Nuclear localization of GFP-STATa in wild-type, erk2- and erk1-erk2- strains after starvation. Cells expressing a GFP:STATa expression vector were plated for development as described in the material and methods and then at the indicated times transferred onto a slide for detection of the GFP:STATa protein. (A) Images of GFP:STATa expression in wild-type, erk2- and erk1-erk2- cells at various times after starvation. (B) Graphical representation of the nuclear localization of GFP:STATa in cells used for images in (A). (C) Images of GFP:STATa expression in cells starved for 6 hrs and then treated with 5 mM cAMP for 10 min. (D) Images of GFP:STATa expression in wild-type, erk2-, and erk1-erk2- strains mixed with wild-type cells (with no GFP) and then developed as chimeras for 11 hrs. (E) Graphical representation of GFP:STATa nuclear localization in the experiments described in (C) and (D). Values used for each strain and each time point in both graphs were based on the examination of at least 100 cells.

erk2- cells do not regulate STATa nuclear localization in chimeric organisms

GFP:STATa expressing strains were developed as chimeras with wild-type cells because *erk2-* mutants can co-aggregate with other strains to form multicellular aggregates. The analysis of GFP:STATa in the chimeric mounds indicated that STATa did not localize to the nucleus of *erk2-* or *erk1-erk2-* cells (Fig. 35D&E). However wild-type cells with the GFP:STATa did show nuclear localization. These results indicate that GFP:STATa nuclear localization is not regulated in *erk2-* mutants by the extracellular signaling or aggregation suggesting that ERK2 is a necessary signaling component for the regulation of STATa.

Although *erk2-* cells can form chimeric aggregates with wild-type cells, the *erk2-* cells are concentrated at the extreme posterior and in the pstO regions as the aggregate transforms into a migratory slug (Fig. 36A). In the case of the *erk1-erk2-* double mutant, these cells can migrate from the pstO region to the pstA region during the slug stage of development. Nuclear localization of the GFP:STATa was not observed for *erk2-* or *erk1-erk2-* cells developing in chimeras even though *erk1-erk2-* cells can occupy the extreme anterior tip region (Fig. 36B). The lack of GFP:STATa nuclear localization in *erk1-erk2-* double mutants is not due to the *erk1-* mutation because *erk1-* cells display the nuclear localization in the anterior tip region cells.

ecmB expression is not repressed by STATa in erk2- cells

STATa functions in wild-type cells to repress the expression of the prestalk gene *ecmB* in the anterior tip cells as indicated by the enhanced expression of *ecmB* in STATa null cells (Araki *et al.*, 1999). *EcmB* is typically expressed during late development in pstAB cells that form the internal core of the anterior tips, pstBA cells that localize to the outer region of the pstO region, and the extreme posterior cells. To examine the regulation of *ecmB* in *erk2-* mutants, the *ecmB::lacZ* reporter gene was transformed into *erk2-*, *erk1-*, and *erk1-erk2-* cells. The ERK

mutants expressing this reporter gene were developed as chimeras with wild-type cells because aggregation defect phenotype of *erk2*- strains. At 18 hrs of development wild-type and

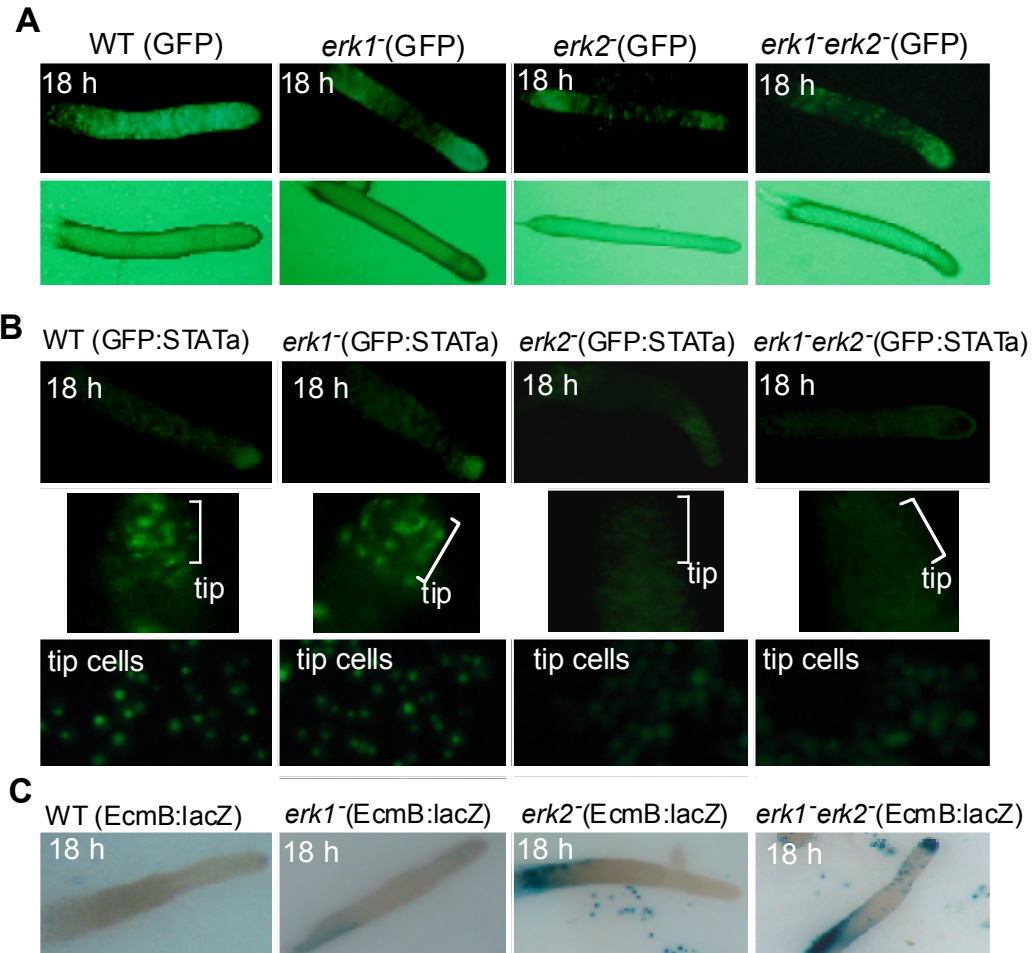


Fig. 36: Cell localization, GFP:STATa localization, and ecmB:lacZ expression of wild-type, erk1-, erk2-, and erk1-erk2- cells at 18 hrs of development in chimeras. Cells expressing reporter proteins were plated for development as described in the materials and methods except that all strains carrying reporter protein expression vectors were mixed with wild-type cells that contained no reporter proteins (1:1 ratio) prior to plating on nonnutrient agar. (A) Images of strains carrying a GFP reporter protein expression vector that were developed as chimeras with wild-type cells (upper panels). Brightfield images of the same slug (lower panels). (B) Images of strains expressing the GFP:STATa reporter protein that were developed with wild-type cells.

Entire slugs (upper panel) and magnification (8X) of an anterior tip (middle panel) and isolated anterior tip cells on a slide (lower panel). Arrows indicate cells with nuclear localization of the GFP:STATa protein. (C) Images of ecmB:lacZ expression in strains mixed with wild-type cells (no reporter) as detected by b-galactosidase activity. All slugs images are oriented with the anterior region on the right.

erk1- cells displayed very little or no *ecmB:lacZ* expression consistent with the repression of *ecmB* until later development (Fig. 36C). Ectopic *ecmB:lacZ* expression was detected in *erk2-* and *erk1-erk2-* cells at 18 hrs of development indicating a requirement of ERK2 function for *ecmB* repression in these cells. The strong expression of the *ecmB:lacZ* gene in the posterior cells of the chimeras is likely to be in part due to the high concentration of *erk2-* or *erk1-erk2-* cells in the posterior. The *erk2-* chimeras exhibited *ecmB:lacZ* expression only in the posterior cells consistent with the inability of *erk2-* cells to migrate to the anterior tip region. However, the *erk1-erk2-* chimeras also displayed *ecmB:lacZ* expression in the anterior tip region, presumably because the *erk1-erk2-* can eventually migrate to this region. This ectopic *ecmB:lacZ* expression in the tip region appears to be different than the pattern typically observed for wild-type cells and this difference might be related to developmental limitations of the *erk1-erk2-* cells.

ERK2 complexes contain the Gα2 subunit, regA but not STATa

An earlier study describing the regulation of STATc in ERK1 signaling pathways has shown an association of STATc with ERK1 using tandem mass spectrometry to detect STATc in His₆-ERK1 pull down assays. A similar association might be expected for STATa and ERK2 because of the regulation of STATa in ERK2 signaling pathways and the existence of a putative D-motif in STATa. Therefore, a His₆-ERK2 expression vector was transformed into *erk2-* cells to allow ERK2 associated proteins to be identified in ERK2 pull down assays. The His₆-ERK2

protein was capable of restoring normal development to *erk2*⁻ cells indicating the His₆-tag does not severely compromise ERK2 function (Fig 37A). The His₆-ERK2 protein also effectively

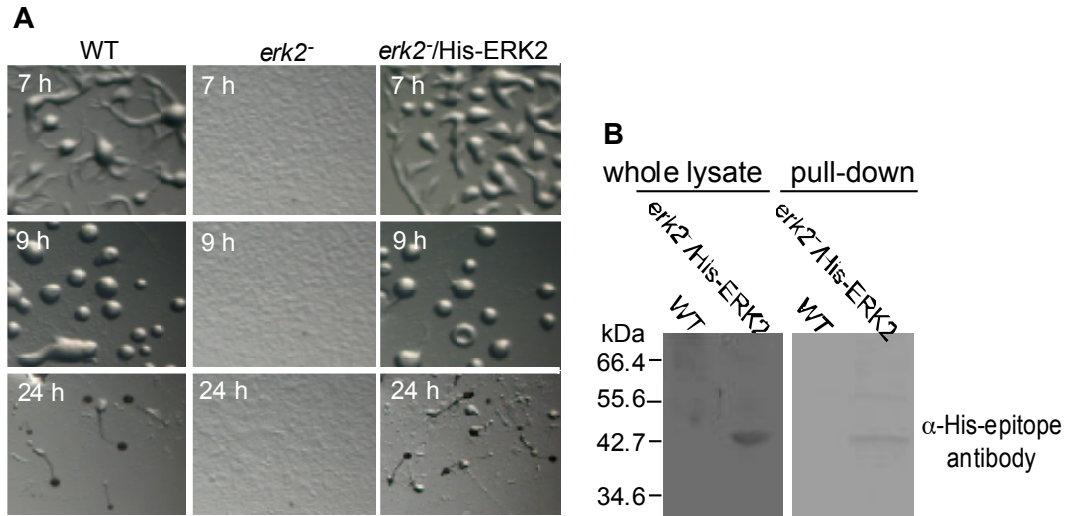


Fig. 37: Expression of His₆-ERK2 in erk2⁻ cells. Wild-type and erk2⁻ cells with or without the His₆-ERK2 expression vector were grown as described in the Materials and methods section. (A) Images of wild-type and erk2⁻ cells with or without His₆-ERK2 expression were developed on nonnutrient agar for the times indicated and examined for morphology. (B) Wild-type cells and erk2⁻ cells expressing the His₆-ERK2 were lysed and cell extracts were treated with Co²⁺ resin for His₆-ERK2 pull-down isolation. Whole cell lysates and eluates from the Co²⁺ resin (pull-down) were analyzed by immunoblotting using anti-His₆-tag rabbit antibody and chemiluminescence detection system. Equivalent levels of initial cell extract were loaded in each lane. Relative positions of marker protein migration (kDa) are indicated.

bound to Co²⁺ resin allowing rapid isolation of ERK2 and associated proteins (Fig. 37B). Eluates of the His₆-ERK2 pull downs were examined using tandem mass spectrometry and compared to the eluates of wild-type cells with no His-tagged proteins and *erk1*⁻ cells expressing a His₆-ERK1 protein. Known signaling proteins associated with His₆-ERK2 in early development included the phosphodiesterase RegA and the G protein subunit Gα2. Both of these proteins have been

previously associated with ERK2 signaling pathways. RegA was also associated with His₆-ERK1 at 6 hrs of starvation consistent with previous reports of RegA regulation by ERK1. The STATc protein was detected in ERK1 pull downs at 6 hrs of starvation as previously reported but STATa protein was not detected in these pull downs (table 2). The inability to detect STATa suggests that STATa regulation through ERK2 does not require a tight association between these two proteins.

ERK2 associated proteins

0 h after starvation		6 h after starvation	
Protein	Spectra	Protein	Spectra
ERK2	25	ERK2	27
RegA	0	RegA*	3
Gα2	0	Gα2	4
STATa	0	STATa	0

Table 2: ERK2 associated proteins identified through tandem mass spectrometry. Proteins identified by peptide spectra in His₆-ERK2 but not His₆-ERK1 or control pull-down eluates. Pull-down assay and parameters of tandem mass spectrometry analysis are described in the material and methods section. () RegA peptide spectra were also identified in His₆-ERK1 eluates at 6 h after starvation.*

STATa nuclear localization in erk2- cells is rescued by RegA gene disruption

The detection of RegA in ERK2 complexes and the previous studies showing RegA knockouts can rescue development of *erk2-* cells suggests that RegA down regulation might be

important for STATa nuclear localization. To test this idea, the GFP:STATa expression vector was introduced into *regA*- and *regA-erk2*- strains. Both strains displayed STATa nuclear localization by 9 hrs of starvation consistent with the precocious development of these strains compared to wild-type cells (Fig 38A&B). This result indicates that the RegA knockout mutation is epistatic to ERK2 mutation in the regulation of STATa. Therefore, ERK2 might regulate STATa through the down regulation of RegA and an increase in cAMP levels.

regA gene disruption restores erk2- STAT regulation in the anterior tip region

Nuclear localization of GFP:STATa was observed in the anterior tip region cells in *regA*- slugs similar to that observed for wild-type slugs (Fig. 38C). Disruption of *regA* in *erk2*- cells also resulted in a normal pattern of GFP:STATa nuclear localization during the slug stage was also observed for *regA-erk2*- cells consistent with the ability of *regA* gene disruption to suppress development effects of *erk2*- mutations. The regulation of *ecmB* expression was also monitored in *regA*- and *regA-erk2*- cells. In both strains, *ecmB::lacZ* expression was repressed in the anterior tip region at 18 hrs of development indicating repression of this reporter gene (Fig. 38D). However, *ecmB::lacZ* expression was observed for posterior cells in both strains suggesting an absence of *ecmB* repression.

Gα2 and Gα4 subunit signaling can regulate RegA and STATa nuclear localization

STATa nuclear localization during early development has been previously shown to require cAMP receptors, cAR/cAR3, suggesting the Gα2 subunit might also be involved with this regulation. The association of the Gα2 subunit with ERK2 pull down assays also supports a role for Gα2 subunit function. Like the *erk2*- phenotype, the starvation of *ga2*- cells did not result in GFP:STATa nuclear localization (Fig. 39A). However, disruption of the *regA* gene in *ga2*- cells allowed nuclear localization of GFP:STATa, consistent with the ability of this gene disruption to restore STATa regulation in *erk2*- cells. Another pathway known to activate ERK2 is the Gα4

subunit-mediated pathway and so STATa nuclear localization was monitored in *ga4-* cells. Nuclear localization of GFP:STATa was observed during the aggregation of *ga4-* aggregated cells but very little if any nuclear localization of GFP:STATa was found in the anterior region of

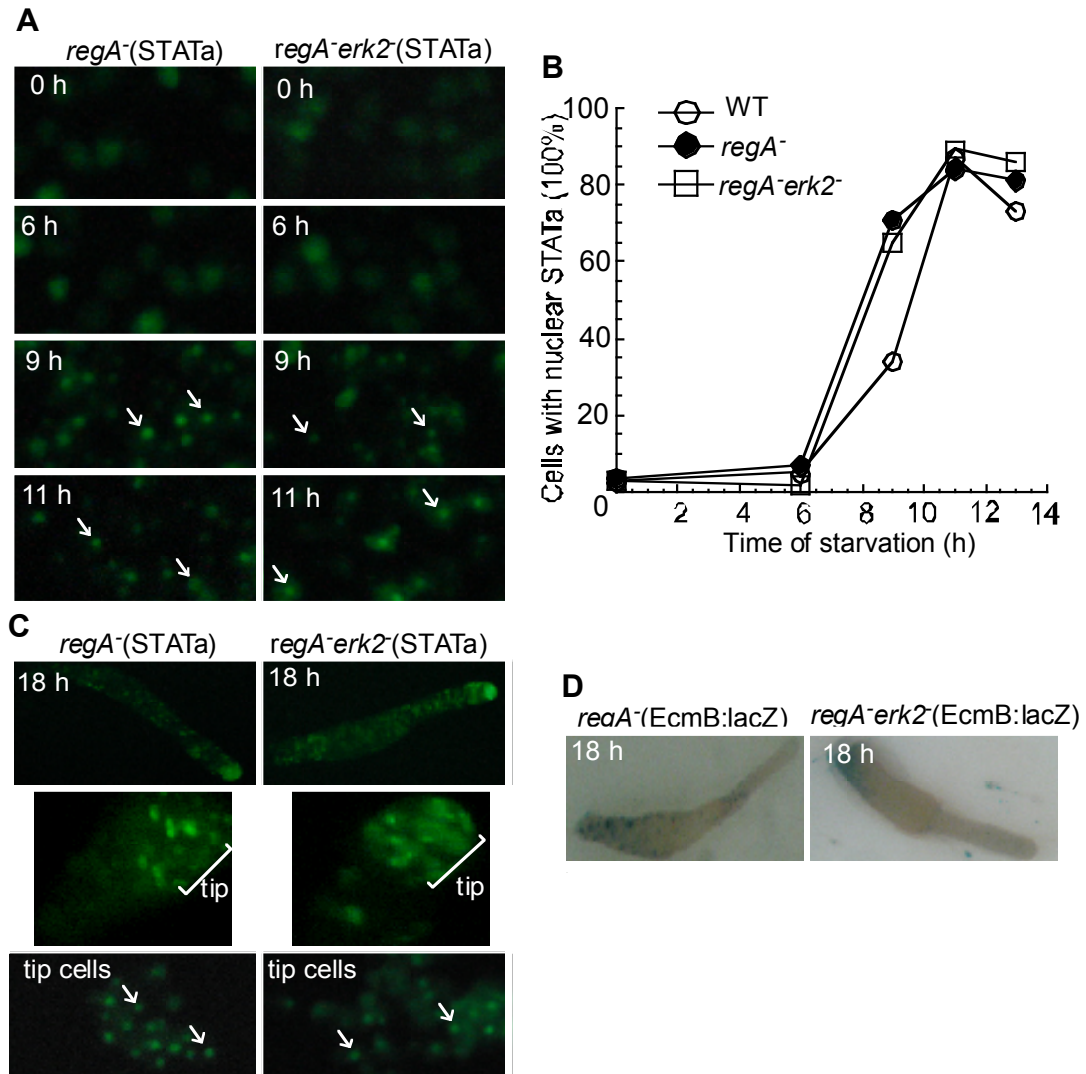


Fig. 38: GFP:STATa nuclear localization and *ecmB:lacZ* expression in *regA*⁻ and *regA-erk2*⁻ strains. (A) Images of GFP:STATa expression in *regA*⁻ and *regA-erk2*⁻ cells starved on nonnutrient agar plates for the times indicated and then transferred to slides. (B) Graphical representation of the experiment in part (A). At least 100 cells were examined for each data point for each strain. (C) Images of GFP:STATa in *regA*⁻ and *regA-erk2*⁻ cells after development on

nonnutrient agar plates. Entire slugs (upper panel) and magnification (8X) of an anterior tip (middle panel) and isolated anterior tip cells on a slide (lower panel). Arrows indicate cells with nuclear localization of the GFP:STATa protein. (D) Images of ecmB:lacZ expression in regA- and regA-erk2- strains as detected through β -galactosidase staining. All slugs images are oriented with the anterior region on the right.

the tips that extended from these mounds (Fig. 39B&C). *ecmB:lacZ* expression was detectable in the slime sheath trailing the extended tips of *G α 4-* aggregates but not in the anterior tip. The presence of *ecmB* expression in this slime sheath might result from the presence of cells in this sheath that have been sloughed off from the tip structure. Given that the tip structures can contain prestalk and some prespore cells, somewhat like a small slug, the cells located in the slime sheath might be analogous to the posterior cells found in larger slugs. If this assumption is correct, then the *ecmB:lacZ* expression in these "posterior" cells might be analogous to the expression in posterior cells of other strains. Interestingly, the loss of RegA function in *ga4-* cells restores STATa nuclear localization to the anterior tip of *ga4-regA-* aggregates indicating RegA functions downstream of *ga4-* signaling pathways. As expected, this restoration of STATa nuclear localization corresponded to *ecmB:lacZ* expression in the pstAB cells.

Discussion

The requirement of ERK2 for STATa nuclear localization and *ecmB* repression in anterior region cells indicates a role for ERK2 signaling pathways in prestalk development. In earlier studies, the role of ERK2 in prestalk cell development was implied from the absence of *erk2-* cells in the pstA region, as well as the prespore region, of chimeric organisms. Also, conditional ERK2 alleles helped demonstrate the importance of ERK2 function in tip development when ERK2 function was restricted soon after aggregation. The dual role of ERK2 signaling in both prestalk and prespore gene expression suggests ERK2 signaling pathways can

function differently in different cell types but some signaling components of these signaling pathways are likely to be conserved. Notably, RegA is likely to be down regulated in both cells types because the disruption of RegA rescues both STATa nuclear localization in *pstA* cells and

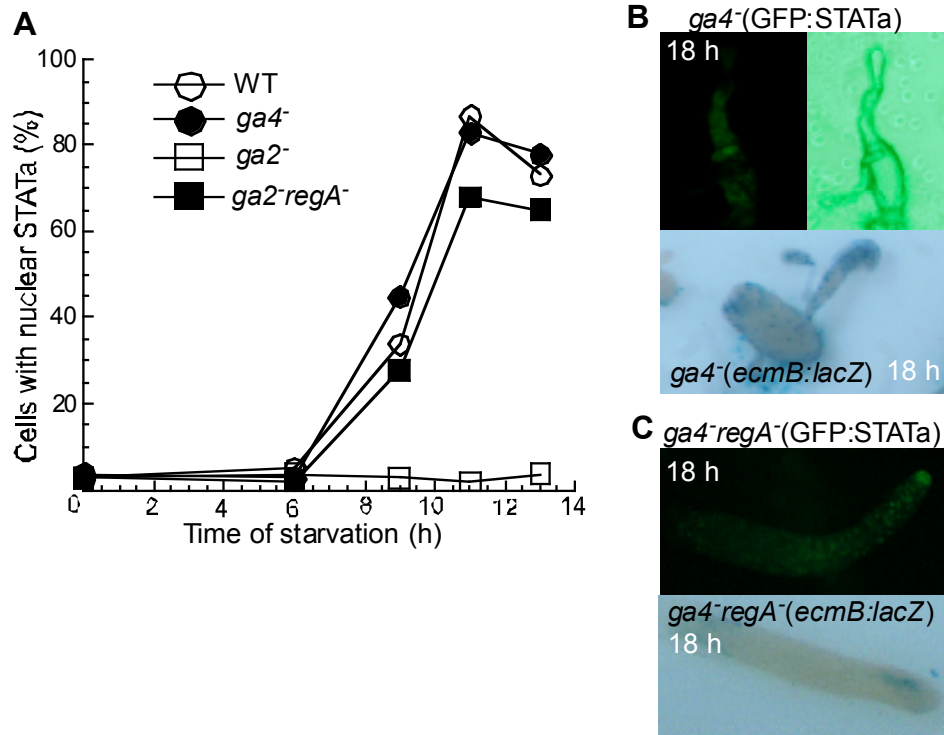


Fig. 39: GFP:STATa nuclear localization and ecmB:lacZ expression in Ga subunit mutants. (A) Graphical representation of the percentage of GFP:STATa nuclear localization in wild-type, ga4-, ga2-, and ga2-regA- strains after indicated times of starvation. At least 100 cells were examined for each strain at each time point. (B) Images of GFP:STATa (upper left panel, brightfield image upper right panel) and EcmB:lacZ expression in ga4- cells after 18 hrs of development. (C) Images of GFP:STATa (upper panel) and EcmB:lacZ expression in ga4-regA- cells after 18 hrs of development.

prespore development. Although some of these rescued processes might result from pleiotropic effects because the loss of regA restores aggregation in *erk2*- clones and *erk2*- cell distribution in chimeric aggregate.

The similarities between ERK2 regulation of STATa and *ecmB* and the ERK1 regulation of STATc and *ecmA* suggest these signaling pathways may have evolved from a common signaling pathway. Both pathways can function downstream of G protein signaling pathways and lead to the repression of prestalk genes. This type of pathway is different than canonical STAT signaling pathways in mammalian cells in which STAT proteins induce gene expression downstream of receptor tyrosine kinases. However, a growing number of studies indicate ERKs can also regulate STAT function in mammals, both in transcriptional regulation and potentially in mitochondrial function. The mechanisms underlying this regulation of mammalian STATs remain to be fully elucidated. Structural comparisons indicate *Dictyostelium* STAT proteins lack a transcriptional activation domain found at the carboxyl terminal region of mammalian STATs suggesting *Dictyostelium* STATs might only serve as repressors rather than inducers of gene expression. Given the similarity of the ERK1 and ERK2 signaling pathways in STAT regulation, the specificity of ERK function is quite interesting. The inability of ERK1 to regulate STATa function in pstA cells and the inability of ERK2 to regulate STATc in pstO cells suggests some mechanism exists for maintaining pathway specificity. This specificity might possibly be determined by the existence of these pathways in different prestalk cell types but other differences might also provide contributions. The specificity of the ERKs and STAT proteins could potentially include differences in direct or indirect physical associations and the presence or absence of D-motifs in the STATs might play a role.

ERK2 function has been previously linked to the G protein-coupled folate and cAMP receptors during early development and now this study demonstrates G protein signal transduction is important for STATa nuclear localization. While nuclear localization of STATa was absent in starved *ga2*- cell, an earlier report has indicated STATa nuclear localization can occur in ~ 45% of *gβ*- cells in shaking culture stimulated with cAMP. The *Gα2* or *Gβ* are not essential for ERK2 activation in response to extracellular cAMP so the difference in the observed

STATa nuclear localization is likely due to the presence or absence of exogenous cAMP treatment because neither strain generates sufficient intercellular cAMP signaling for aggregation. The rescue of STATa nuclear localization in *ga2*- cells with a *regA* gene disruption suggests increased levels of cAMP can bypass the block in upstream signaling. The nuclear localization of STATa in *ga4*- cells during aggregate formation is consistent with the ability of these cells to undergo cAMP signaling but the absence of STATa nuclear localization in the *pstA* region of *ga4*- aggregates indicates this signaling pathway is important for STATa function later in development. A role of *Gα4* subunit function in prestalk cell development has been previously proposed based on the inability of a *Gα4* subunit D-motif mutant to undergo stalk development. This phenotype is very similar to that reported for *stata*- mutants and consistent with the *Gα4* subunit-ERK2-STATa pathway regulating *ecmB* gene expression and *pstA* cell development. This pathway, like the *Gα2* subunit-mediated pathway, probably involves cAMP signaling upstream of STATa function due to the ability of *regA* gene disruptions to rescue STATa function in *ga4*- or *erk2*- mutants.

CHAPTER VII

CONCLUSION

MAPKs are known to regulate many basic cellular activities and MAPK pathways are associated with several human diseases such as cancer, cardiovascular diseases and obesity. However, defining function and signaling specificity of each MAPK in higher eukaryote has been challenging due to the number MAPKs as well as the crosstalk among MAPK pathways. *Dictyostelium* whose genome encodes only two MAPKs ERK1 and ERK2 therefore offers many advantages in studying these pathways. Until recently, very little was known about the other signaling components in *Dictyostelium* ERK pathways, especially the ERK1 pathway. Our purpose in this research was to determine the function and the specificity in signaling pathway of ERK1 and ERK2 through characterizing G protein-mediated pathways and searching for ERK-associated proteins.

Dictyostelium has no RTKs but has over 50 GPCRs, implying that GPCRs are necessary to initiate the ERK pathways. *Dictyostelium* genome encodes 12 different $G\alpha$ subunits but only one common dimer $G\beta\gamma$ suggesting the formation of 12 different heterotrimeric complexes coupled with the GPCRs. Some of the $G\alpha$ subunits including $G\alpha_2$, $G\alpha_4$ and $G\alpha_5$ were known to regulate ERK-associated developmental processes. To ensure specificity of signals from the GPCRs, these $G\alpha$ subunits need to interact with downstream signaling proteins rather than only the $G\beta\gamma$ dimer. However, in most pathways, the interaction of $G\alpha$ subunits with effectors remains to be determined. The sequence analysis of the $G\alpha$ subunits led to the identification of

putative D-motifs that might mediate MAPK docking in Gα2, Gα3, Gα4, Gα5 and Gα11 subunits. This identification led us to the studies on the interactions of ERKs with Gα subunits.

The Gα4 subunit was known to be required for folate-stimulated ERK2 phosphorylation, so the interaction of ERK2 with Gα4 subunit was characterized. Our study showed that ERK2 interacted with Gα4 subunit through the D-motif and this interaction promotes spore production but does not affect folate chemotaxis. Gα4 subunit modeling demonstrated that the Gα4 D-motif is exposed for MAPK interactions even while this subunit associates with its receptor and Gβγ subunits. This observation suggests that Gα4 might help to localize ERK2 to a signaling complex even though the Gα4 D-motif is not required for the activation of ERK2.

The Gα5 subunit contains two D-motifs that located near the amino terminus and at the internal site of the gene (residues 117-134). Demonstrating a physical interaction between the Gα5 subunit and ERK1 was not successful due to the lethal effect of Gα5 resulting in the inadequate expression of the epitope-tagged Gα5 for the interaction assays. However, the genetic analyses suggested that the Gα5 subunit associates with ERK1 through the D-motif at the amino terminus. This interaction reduces cell viability and affects aggregation and tip formation but not for folate chemotaxis. The role of this interaction on ERK1 activation was not characterized yet because the antibodies that detect the phosphorylation in the conserved TEY motif of other ERKs could not detect phosphorylated ERK1. These studies do not exclude the possibility of an interaction between the Gα4 subunit and ERK1 as well as the interaction between the Gα5 subunit and ERK2. The interactions of other Gα subunits with ERKs were not characterized in this study.

In mammals and yeast, a single stimulus simultaneously activates many ERK pathways. This opens the possibility that ERKs might have overlapping or redundant functions. Furthermore, the two MAPKs, ERK1 and ERK2, in *Dictyostelium* are co-expressed during

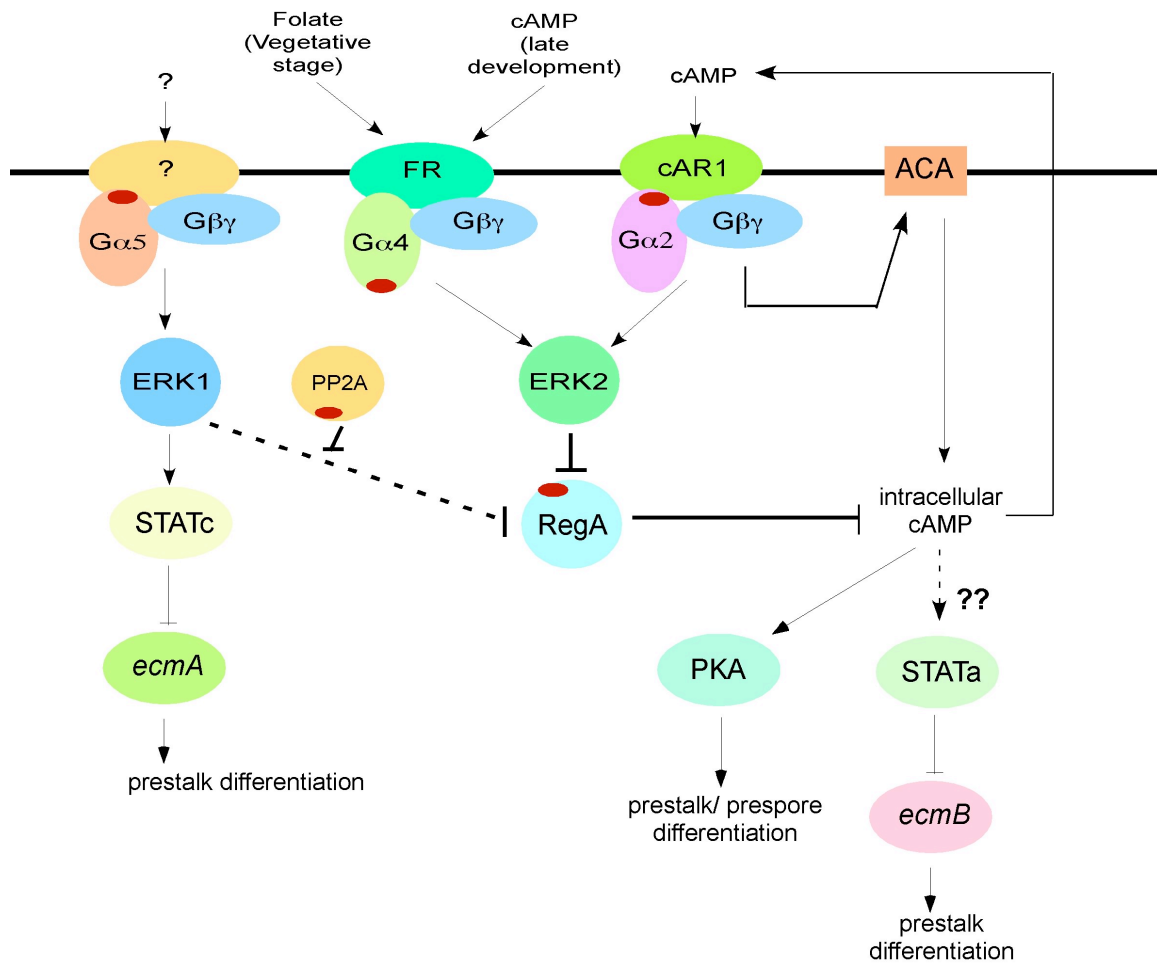


Fig. 40: The suggested model for the signaling pathways of Dictyostelium MAPKs ERK1 and ERK2.

growth and through development, so it was of interest to determine whether these ERKs had redundant or distinct functions. Our results indicated that ERK1 and ERK2 were distributed in both cytoplasm and nucleus but these ERKs are not likely redundant because they do not functionally complement each other. During the developmental process, these ERKs have different roles. ERK1 mostly contributes to the prestalk cells development while ERK2 is mostly required for prespore development. Characterizing the role of ERKs in G protein-mediated pathways showed that the Gα5 subunit-ERK1 pathway regulates tip morphogenesis, lethality and

folate chemotaxis, while the G α 4 subunit-ERK2 pathway regulates prespore development and cAMP accumulation.

In mammalian cells, MAPKs are known to activate a wide range of the substrates such as kinases, transcription factors and phosphodiesterases. However, less is known for ERK substrates in *Dictyostelium*. Previous studies have shown that phosphodiesterase RegA, that hydrolyzes intracellular cAMP, might function downstream of ERK2. To identify other signaling proteins in ERK pathways, we searched for ERK substrates. Proteins in the ERK pathways might be held together by scaffolding proteins and/or by ERK docking sites resulting in the formation of pathway-specific ERK complexes. To test this hypothesis, we used pull-down assays and mass spectrometry to identify ERK1/2-associated proteins. Interestingly, we detected transcription factor STATc in ERK1 complexes but not ERK2 complexes. The genetic analyses indicated that the G α 5 subunit-ERK1 pathway controls nuclear localization of STATc ultimately resulting in repression of a prestalk gene *ecmA*. Although no transcription factors were detected in ERK2 pull-downs, genetic evidence suggests ERK2 regulates the function of another transcription factor STATA and the expression of prestalk gene *ecmB* through down regulating the phosphodiesterase RegA. This role of ERK2 might be connected to the G α 2 subunit-mediated pathway during aggregation and the G α 4 subunit-mediated pathway during development. Interestingly, RegA was detected in both ERK1 and ERK2 complexes and the knockouts of the *regA* gene rescued development of both *erk1*- and *erk2*- cells suggesting that both ERK1 and ERK2 negatively regulate RegA. However, unlike ERK2, ERK1 has only subtle effect on this regulation because ERK1 might be rapidly inactivated by a phosphatase PP2A detected in all ERK1 complexes.

Our study has helped to define the function of ERKs and we have demonstrated the specificity of ERK signaling in the pathways that include G proteins (G α 2, G α 4 and G α 5 subunits), a phosphatase (PP2A), a phosphodiesterase (RegA), transcription factors (STATs) and

cell-type specific genes (*ecmA*, *ecmB*) (Fig. 40). Further studies on these pathways will likely reveal additional signaling proteins that regulate developmental processes.

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Scope and Method of Study: MAPKs are associated with several human diseases.

However, studying MAPK signaling pathways in higher eukaryotes is challenging due to presence of several MAPKs. *Dictyostelium*, whose genome encodes only two MAPKs ERK1 and ERK2, therefore offers many advantages in this respect. Our purpose in this research is to define the function and the specificity in signaling pathway of ERK1 and ERK2 through characterizing G protein-mediated pathways and searching for ERK-associated proteins. We used immunoprecipitation, pull-down assays and genetic analyses to examine the interactions of ERKs with wild-type and mutated $G\alpha$ subunits. We also created ERK mutants and performed the complementation, suppression and epistasis tests to determine role of ERKs in $G\alpha$ subunit-mediated pathways. To search for ERK-associated proteins, His₆-tagged ERK1 and ERK2 complexes were isolated and then subjected to mass spectrometry analysis. Cellular distribution of the transcription factors (STATs), expression of the cell type-specific genes (*ecmA*, *ecmB*) and the knockout of the phosphodiesterase RegA were used to determine the involvement of these ERK-associated proteins in ERK pathways.

Findings and Conclusions: Our results showed that the $G\alpha 4$ subunit interacts with ERK2 and the $G\alpha 5$ subunit associates with ERK1 through the D-motifs. The $G\alpha 4$ -ERK2 interaction contributes to spore development while the $G\alpha 5$ -ERK1 interaction regulates cell viability and tip morphogenesis. We also indicated that ERK1 and ERK2 have different roles in $G\alpha$ subunit-mediated pathways. Genetic analyses of the ERK-associated proteins revealed that both ERK1 and ERK2 negatively regulate RegA; the $G\alpha 5$ subunit-ERK1 pathway controls nuclear localization of STATc ultimately resulting in repression of *ecmA* while the $G\alpha 2/G\alpha 4$ subunits-ERK2 pathways regulate function of STATa and expression of *ecmB* through down regulating RegA.

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