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The Gα4 G protein subunit interacts with the MAP kinase ERK2 using a D-motif that regulates developmental morphogenesis in *Dictyostelium*

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ABSTRACT

G protein Gα subunits contribute to the specificity of different signal transduction pathways in *Dictyostelium discoideum* but Gα subunit-effector interactions have not been previously identified. The requirement of the *Dictyostelium* Gα4 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 Gα4 subunit and MAPKs. *In vivo* association of the Gα4 subunit and ERK2 was demonstrated by pull-down and co-immunoprecipitation assays. Alteration of the D-motif reduced Gα4 subunit-ERK2 interactions but only slightly altered MAPK activation in response to folate. Expression of the Gα4 subunit with the altered D-motif in *gα4*$^-$ cells allowed for slug formation but not the morphogenesis associated with culmination. Expression of this mutant Gα4 subunit was sufficient to rescue chemotactic movement to folate. Alteration of the D-motif also reduced the aggregation defect associated with constitutively active Gα4 subunits. These results suggest Gα4 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 (Gαβγ) that disassociate into a Gα subunit and a Gβγ dimer capable of regulating the function of downstream effectors (Hamm, 1998). The Gα subunit provides specificity for receptor coupling and when activated the Gα releases the Gβγ dimer (Conklin et al., 1993). In some signaling pathways, the interaction of the Gα subunit with a specific effector (e.g., adenylyl cyclase, phospholipase C, etc.) has been established but in most pathways the interactions the Gα subunit with downstream effectors remains to be determined (Neer and Clapham, 1988; Neves et al., 2002). Identifying Gα 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 Gα 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 Gα4 subunit is required for chemotaxis to folate and the promotion of spore cell development during multicellular development whereas the closely related Gα5 and Gα2 subunits are required for different or even opposing functions (Hadwiger and Firtel, 1992; Hadwiger et al., 1994, 1996; Kumagai et al., 1991; Natarajan et al., 2000). The Gα2 subunit is required for cAMP chemotaxis and cell aggregation and the Gα5 subunit inhibits folate chemotaxis and promotes prestalk cell development (Hadwiger et al., 1996; Kumagai et al., 1991). The Gα4 subunit and these other Gα subunits are presumed to couple to the same Gβγ dimer because only single genes have been identified for the Gβ and Gγ subunits in the *Dictyostelium* genome and so only the receptor and the Gα subunit are likely to be the determinants of pathway specificity (Lilly et al., 1993; Zhang et al., 2001). A previous study of chimeric Gα subunits suggests that the Gα4 subunit and other Gα subunits can contribute to the specificity of downstream responses, perhaps through interactions with signaling components other than the Gβγ dimer (Hadwiger, 2007). However, Gα 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

Abbreviations: PCR, polymerase chain reaction; MAP kinase, mitogen-activated protein kinase; MAPKK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; CD, common docking site; D-motif, MAPK docking site; SDS, sodium dodecyl sulfate; cAMP, cyclic adenosine monophosphate.

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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., 1994, 1996; 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 Gβγ 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 Gα 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 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 *gα4*$^-$ 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 *gα4*$^-$, Gα4^{HC}, 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 $NaHCO_3$. 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 *Gα4* gene (also designated as *gpaD*) in the vector pBluescriptIISK+ (Stratagene) was previously described (Hadwiger, 2007). Alterations to the Gα4 subunit D-motif, Gα4^{R107E,R108E} subunit (designated Gα4^{d-}), were created from the wild-type *Gα4* 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 *Xho*I site thus allowing the mutant gene to be detected by restriction enzyme digestion analysis. A gene encoding a constitutively active Gα4^{Q200L} subunit (designated Gα4*) was also created from the wild-type *Gα4* gene by PCR mutagenesis using overlapping oligonucleotides (sense strand: 5′-GATTAGATTAAAGA TTGTAGAcGTCGGTGGTCtAAGATCTCAAAGAA-GAAAATGG) and (antisense strand: 5′-CAGAATTTACATTTGATAA-GATTAGATTAAAGATTGTAG). A silent mutation, creating an *Aat*II 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 Gα4^{d-} subunit resulting in a gene encoding a Gα4d$^{-,*}$ subunit. All mutations created in this study were verified by sequence analysis and each mutant *Gα4* gene was then inserted into the *Dictyostelium* expression vector pDXA-GFP2 (*Ddp2*-based plasmid), replacing the *Hin*dIII/*Xba*I 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 p*act15*/*Gα4* gene construct was also transferred as *Sal*I/*Xba*I fragments into the *Ddp1*-based pTX-GFP vector (extra-chromosomal vector) replacing the p*act15*/*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′-gccggcggatccATGAGATTCAAGTGTTTTGGATCAG) and (antisense strand 5′-cggcgctctagaTTAGAAGTGTTCTAATGCTTGAGATAAA-ATTGTTTGTCTAAC). Each amplified *Gα4* gene was inserted at *Bam*HI and *Xba*I sites into a pBluescriptIISK+ vector containing the *Hin*dIII-Myc-*Bam*HI linker created from the oligonucleotides: (sense strand 5′-AGCTTATGGAACAAAAATTATTATCAGAAGAAGATTTAG) and (antisense strand 5′-GATCCTAAATCTTCTTCTGATAATAATTTTTGTTC-CATA). 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 His_6-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′-cgcaagcttggatccctcgagacacaATGT-CATCTGAAGATATAGATAAACATG) 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 His_6-tag (Manstein et al., 1995).

MAPK activation assay

Cells were grown to mid-log phase (approximately $2–3\times10^6$ cells/ml), washed twice in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (5×10^7 cells/ml). The cell suspension was shaken for 1 h 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).