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MAP kinases have different functions in *Dictyostelium* G protein-mediated signaling

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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.

1. 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 [1–4]. 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 [5–7]. The simultaneous activation of multiple ERKs in response to a single stimulus opens the possibility that ERK paralogs might have overlapping or redundant functions [2,3]. Genetic analysis in mice indicates the closely related ERK1 and ERK2 proteins have different roles in development [3]. 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 [8]. 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 [2]. 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 [5–7,9]. 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 [10–12]. *erk2*$^-$ cells can chemotax to cAMP, although with low efficiently at high concentrations, but clonal populations of these cells do not form aggregates when starved [7,13]. ERK2 is also important for the prespore cell specific gene expression and differentiation [9]. ERK2 is also activated in response to folate and *erk2*$^-$ cells exhibit a slight reduction in folate chemotaxis [13,14]. 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 [15]. ERK1 can be activated in response to cAMP and this activation is mediated by the MAP2K, MEK1 [15]. 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 [16–20]. Responses to cAMP are mediated through cAMP receptors and the G protein containing the Gα2 subunit [21–

Abbreviations: MAPK, MAP kinase or mitogen-activated protein kinase; MAP2K, MAP kinase kinase; ERK, extracellular-signal regulated kinase; SDS, sodium dodecyl sulfate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate.

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23]. cAMP stimulation is responsible for the aggregation phase of development and aids in the establishment of prespore and prestalk cell zones in the aggregate [24,25]. Response to folate or related pterin compounds is mediated through a pathway using the Gα4 subunit and this pathway allows cells to chemotax to bacterial food sources [26]. This Gα4 subunit-mediated pathway is also important for the localization and development of prespore cells in multicellular aggregates and the morphogenesis associated with fruiting body formation [18,27,28]. Responses to folate are inhibited by another G protein pathway using the Gα5 subunit and the signal activating this pathway is unknown [29]. The Gα5 subunit helps to regulate cell size, growth, and the rate of morphogenesis after aggregate formation [19]. All three of these Gα subunits presumably couple to a common Gβγ dimer and function in pathways that affect ERK function [30,31]. All three Gα subunits also contain known or putative MAPK docking sites (D-motifs) [28]. The Gα4 subunit is required for folate stimulated ERK2 activation and recently the Gα4 subunit has been shown to associate with ERK2 [10,14,28]. The lethality associated with Gα5 subunit over-expression requires a MAPK docking motif and ERK1 function and the Gα2 subunit is not required for ERK2 activation in response to cAMP [10,32,33].

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 Gα 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.

2. Materials and methods

2.1. Strains and media

All *Dictyostelium* strains used in the study were derived from the wild-type strain KAx3. The *gα4*$^-$, *gα5*$^-$, *erk2*$^-$ strains were previously described [18,19,28]. 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* [34]. DNA vectors were electroporated into cells as previously described [35]. 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 $NaHCO_3$.

2.2. 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 [36,37]. The open reading frame was amplified using PCR with oligonucleotides (sense strand: 5′-CGCGGATCCCTCGAGAATTAATGCCACCACCACCAAC AAGTG) and (antisense strand: 5′-GCGGTCGACTCTAGATTAATTTTTAA TTGATTGTTGATTTACTTGTTG) 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 *Hin*dIII/*Sal*I digested PCR product was inserted into the same sites of the vector pBluescriptIISK+ (Stratagene) and verified by sequence analysis. To create an ERK1 expression vector, the *ERK1* gene construct was transferred as a *Sal*I/*Xba*I fragment into the Ddp2-based vector pDXA-3H [38]. 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 [39]. Similar results were obtained using either the Ddp1- and Ddp2-based vectors but only data from Dpd1 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′ *Hin*dIII fragment was deleted from the *ERK1* gene and a *Bam*HI fragment containing the *Bs*^{r} 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-*Hin*dIII fragment and electroporated into cells. PCR verification of the *ERK1* gene disruption used oligonucleotides specific to the *Bs*^{r} gene (antisense strand: 5′-CTGCAATACCAATCGCAATGGCTTCTGCAC) and a region of the *ERK1* gene outside the sequence used in the disruption construct (antisense strand: 5′-GTATGGTGCCTGTGGATCTTCAG-GATG). 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 *Hin*dIII/*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 [35].

2.3. Development and chemotaxis assays

Cells were grown to mid-log phase (approximately 2-3 × 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 [19]. Cell development was analyzed using a dissecting microscope or fluorescence microscopy. Above-agar assays were used to measure chemotaxis as previously described [19]. Briefly, 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 cAMP chemotaxis assay, cells were shaken for 4 h with 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.

2.4. Cyclic GMP assays

cGMP concentration in *Dictyostelium* was determined using a radioimmunoassay as previously described [20]. 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 h 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).

2.5. 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