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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.

Keywords

MAP kinase; ERK1; ERK2; G Protein; *Dictyostelium*; 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

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the possibility that ERK paralogs might have overlapping or redundant functions [2,3]. Genetic analysis in mice indicate 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 efficiency 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 [21–23]. 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-mediate 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 $ga4^-$, $ga5^-$, $erk2^-$ 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 $\mu\text{g/ml}$ of the drug G418 or 3 $\mu\text{g/ml}$ of the drug blasticidin S and drug selection was removed several hours prior to analysis. Folate solutions were prepared by neutralizing folic acid with 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'-CGCGGATCCCTCGAGAATTAATGCCACCACCACCAACAAGTG) and (antisense strand: 5'-GCGGTCGACTCTAGATTAATTTTAATTGATTGTTGATTACTTGTG) 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 pBluescriptIISK+ (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 [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 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 *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-*Hind*III 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'-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 $G\alpha 4$ and $G\alpha 5$ subunit expression vectors were previously described [35].

2.3 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 [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 hours 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 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).

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 selection (3 $\mu\text{g}/\text{ml}$ G418), culture plates were treated with increasing selection of drug selection and the number of surviving clones was determined.

2.6 MAPK activation assay

MAPK activation was determined as previously described [28]. 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).

3. Results

3.1 *erk1*[−] cells have altered aggregate morphology and 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 [28]. 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. 1) [15]. 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.

3.2 *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. 2A). However, many *erk1*[−] cells moved in directions other than toward the source of cAMP 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. 2B). 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 [15]. 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 does indicate 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. 2C). 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. 2D). This result also suggests that MEK1 regulates cGMP levels through a pathway that does not require ERK1 function.

3.3 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 [8,40,41]. *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 *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. 3A & 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. 3C). 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 µg/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 µg/ml). The ERK2 expression vector blocked the aggregation of wild-type cells when drug selection was relatively high (10 µg/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.

3.4 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. 4). 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 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 [7,9]. 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 *erk2⁻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.

3.5 ERK mutants are differentially sensitive to Gα subunit over-expression

Other studies have indicated that G protein mediated pathways activate ERK signaling in *Dictyostelium* and in some cases the Gα subunits of these pathways contain D-motifs for MAPK interactions [10,14,28,42]. A D-motif in the Gα5 subunit has been determined to be important for the lethality associated with over-expression or constitutive activation of the

Gα5 subunit suggesting that MAPK interactions are necessary for this phenotype [33]. 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. 5). In contrast, transformation of a Gα4 subunit expression vector 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 [19]. 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.

3.6 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 *ga5*⁻ cells, *erk1*⁻ cells have a slightly enhanced chemotactic response to folate (Fig. 6A). Over-expression of the Gα5 subunit inhibits folate chemotaxis but not in *erk1*⁻ cells suggesting ERK1 is required for this inhibition. Likewise, ERK1 over-expression inhibits folate chemotaxis but not in *ga5*⁻ cells further supporting that the Gα5 subunit and ERK1 function in the same pathway. The over-expression of the Gα4 subunit in wild-type and *erk1*⁻ cells enhances folate chemotaxis suggesting the Gα4 subunit-mediated responses to folate do not require ERK1 function.

Both ERK1 and the Gα5 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 Gα5 subunit and ERK1 were monitored for tip formation. The Gα5 subunit expression vector promotes small aggregate formation and accelerated tip formation in *ga5*⁻ cells even under low drug selections of 2 μg/ml G418 (Fig. 6B). This Gα5 subunit-mediated precocious tip formation is not observed in *erk1*⁻ cells expressing the Gα5 subunit vector even under high drug selection (10 μg/ml) indicating ERK1 is required for this phenotype. However, over-expression of the Gα4 subunit delayed aggregation in *erk1*⁻ cells, as previously described for wild-type cells, indicating this Gα4 subunit-mediated phenotype does not require ERK1 function [18]. 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 Gα5 subunit function.

3.7 Phosphorylation of ERKs in response to folate and cAMP

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 [10,11,42]. 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. 7A). 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 7B). 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 [28,42]. Consistent with earlier studies this phosphorylation requires Gα4 subunit function and ERK2 expression as indicated by the large reduction in the detected protein from *ga4*⁻ and *erk2*⁻ cells (Fig. 8). The phosphorylation of ERK2 in *erk1*⁻ cells was similar to 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 Gα5 subunit not being required for the folate responses.

3.8 ERK distribution in response to folate and cAMP stimulation

In many organisms, MAPKs can move from the cytoplasm into the nucleus after cell stimulation [43,44]. 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 [10]. 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. 9A). Both GFP-ERK1 and GFP-ERK2 were found in the cytoplasm and nucleus of cells (Fig. 9B). 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 GFP-MAPKs in mammalian cells [44]. 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).

4. 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. 10). The requirement of ERK1 but not ERK2 for the precocious tip development and lethality associated with Gα5 subunit over-expression support a role for ERK1 in Gα5 subunit mediated signal transduction. The requirement for both ERK1 and the Gα5 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 Gα4 subunit mediated responses such as chemotaxis to folate even though the *erk2*⁻ cells are only partially defective in this response [13,26]. Although not tested, the Gα4 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 *ga4*⁻ and *erk2*⁻ cells [7,9,18,26]. 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 Gα2 or Gβ subunits [10,11,32]. The inability of *erk1*⁻ cells to form large aggregation streams suggests a possible limitation on the 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 [45]. 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 [5,7]. 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 [46]. 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 [2]. 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 [8,47]. This concept is consistent with the regulation of cell growth through growth factors activate MAPKs.

5. Conclusions

Our results indicate that ERK1 and ERK2 in *Dictyostelium* function in different G protein-mediated signaling pathways rather than providing redundant functions in a common pathway. ERK1 is necessary for signaling in Gα5 subunit-mediated pathways and ERK2 is necessary for signaling in Gα4 subunit-mediated pathways. While ERK1 and ERK2 could possibly perform similar functions in different pathways, these pathways clearly regulate different developmental processes as indicated by the phenotypic analysis of *erk1*[−] and *erk2*[−] mutants.

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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Figure 1.

ERK1 gene disruption and developmental phenotypes. A) Disruption of the ERK1 locus at the *Bcl*I site with the blasticidin S resistance gene (*Bs^r*). 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.

Figure 2.

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) Wildtype (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.

Figure 3.

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 2–5 × 10⁵ 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 μ g/ml or 10 μ g/ml (HC) G418.

Figure 4. 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.

Figure 5. Viable transformants expressing Gα 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 μg/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 μg/

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).

Figure 6.

Folate chemotaxis and development of ERK and $G\alpha$ subunit mutants. (A) Folate chemotaxis distances traveled by wild-type, *erk1*⁻, *ga5*⁻ and *ga4*⁻ cells with or without $G\alpha$ 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 μ g/ml G418 except for $G\alpha5$ expression vector in *ga5*⁻ cells (2 μ g/ml

G418) because of the lethal effects of $G\alpha 5$ subunit expression. Inset of $g\alpha 5^-/G\alpha 5$ subunit 12 hr photograph is a 3X magnification of one section displaying a tipped aggregate (indicated by arrow).

Figure 7. 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.

Figure 8. 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.

Figure 9. 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 μ g/ml) or high (6 μ g/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).

Figure 10.

Model of $G\alpha$ subunit-ERK pathway specificity. Signals mediated by the $G\alpha 5$ 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 $G\alpha 4$ subunit and ERK2. The $G\alpha 4$ subunit is necessary for the phosphorylation of ERK2 in response to folate.