The *Dictyostelium* MAPK ERK1 is phosphorylated in a secondary response to early developmental signaling

David J. Schwebs and **Jeffrey A. Hadwiger**[*]

Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK 74078-3020

Abstract

Previous reports have suggested that the two mitogen-activated protein kinases (MAPKs) in *Dictyostelium discoideum*, ERK1 and ERK2, can be directly activated in response to external cAMP even though these MAPKs play different roles in the developmental life cycle. To better characterize MAPK regulation, the levels of phosphorylated MAPKs were analyzed in response to external signals. Only ERK2 was rapidly phosphorylated in response to the chemoattractants, cAMP and folate. In contrast, the phosphorylation of ERK1 occurred as a secondary or indirect response to these stimuli and this phosphorylation was enhanced by cell-cell interactions, suggesting that other external signals can activate ERK1. The phosphorylation of ERK1 or ERK2 did not require the function of the other MAPK in these responses. Folate stimulation of a chimeric population of *erk1⁻* and *gα4⁻* cells revealed that the phosphorylation of ERK1 could be mediated through an intercellular signal other than folate. Loss of ERK1 function suppressed the developmental delay and the deficiency in anterior cell localization associated with *gα5⁻* mutants suggesting that ERK1 function can be down regulated through Gα5 subunit-mediated signaling. However, no major changes in the phosphorylation of ERK1 were observed in *gα5⁻* cells suggesting that the Gα5 subunit signaling pathway does not regulate the phosphorylation of ERK1. These findings suggest that the activation of ERK1 occurs as a secondary response to chemoattractants and that other cell-cell signaling mechanisms contribute to this activation. Gα5 subunit signaling can down regulate ERK1 function to promote prestalk cell development but not through major changes to the level of phosphorylated ERK1.

Keywords

MAPK; G protein signaling; Dictyostelium; phosphorylation; development; chemotaxis

[*]Corresponding Author: Jeffrey A. Hadwiger, Department of Microbiology and Molecular Genetics, Oklahoma State University, 307 Life Sciences East, Stillwater, Oklahoma 74078-3020, Tel. 405-744-9771; Fax. 405 744-6790; jeff.hadwiger@okstate.edu.

1. Introduction

Mitogen-activated protein kinases (MAPKs) are important regulatory proteins in many different signaling pathways [1–5]. MAPKs have been associated with the regulation of cell growth and differentiation and therefore play critical roles in the eukaryotic development [6–8]. Extracellular signal regulated protein kinases (ERKs) are the most evolutionary conserved subgroup of MAPKs and these proteins are activated by MAPK kinases (MAP2Ks) that phosphorylate a threonine and a tyrosine residue within a conserved motif (TXY) located within the catalytic region [1, 4, 5, 9]. Antibodies directed against this phosphorylated motif have been useful for detecting the activation of MAPKs in many different organisms and this characterization of MAPK regulation has led to many insights about MAPK function [10]. Some closely related MAPKs, such as mammalian ERK1 and ERK2 (86% primary sequence identity) are co-activated and appear to share redundant roles [9, 11]. However, ERKs with less sequence identity, such as the budding yeast FUS3 and KSS1 (54% primary sequence identity), can also be co-activated by a common MAP2K but yet have very different roles in cell fate [2]. In this latter case, MAPK interactions with scaffolding proteins can provide pathway specificity and the predominant activation of one of the MAPKs [12, 13]. The specificity of MAPK activation and function is of general interest because of the important roles MAPKs play in developmental signaling pathways in a wide range of eukaryotes.

The soil amoebae *Dictyostelium discoideum* has only two MAPKs, ERK1 and ERK2 (39% primary sequence identity), and both play important roles in the developmental life cycle that allows solitary cells to aggregate and develop into a fruiting body structure consisting of a stalk and a mass of spores [3]. During this multicellular development, intercellular signaling mediated through G protein coupled receptors regulates the differentiation and sorting of prespore and prestalk cells within the aggregate and some of these signaling pathways involve MAPK activity. ERK2 function is essential for the cell aggregation process in which cells respond to and produce an extracellular cAMP signal that allows cells to chemotax toward each other [14–16]. The stimulation of cell surface cAMP receptors leads to ERK2 activation but interestingly this activation does not require the Gα2 and Gβ subunits of the G protein that couples with cAMP receptors [17, 18]. Genetic analysis has indicated that ERK2 down regulates the cAMP-specific phosphodiesterase RegA and thereby allows the accumulation of cAMP for internal signaling and the relay of external cAMP signaling [19]. A loss in ERK2 function results in an aggregation defective developmental phenotype because insufficient cAMP accumulates to relay the cAMP signal to other cells [15]. This aggregation deficiency can be corrected by developing *erk2⁻* cells in chimeric populations with wild-type cells that produce sufficient extracellular cAMP signaling but *erk2⁻* cells do not sort properly within these chimeric aggregates [14, 20, 21]. The aggregation deficiency of *erk2⁻* cells can also be suppressed by disrupting the *regA* gene to reduce cAMP phosphodiesterase activity [15, 19]. ERK2 is also activated in response to extracellular folate, another chemoattractant, and this activation does require the Gα4 and Gβ subunit that couple to folate receptors [22, 23]. *Dictyostelium* use this Gα4-mediated signaling pathway to forage for new bacterial food sources [24].

The activation and function of ERK1 in *Dictyostelium* development has not been characterized as well as the developmental role of ERK2. Earlier reports have suggested that ERK1 can be activated in response to external cAMP signaling and that this activation can be mediated by MEK1, the only *Dictyostelium* MAP2K identified by sequence similarity to other eukaryotic MAP2Ks [25, 26]. Loss of MEK or ERK1 function results in small aggregate formation and accelerated development consistent with both kinases functioning in the same pathway. A major challenge in characterizing ERK1 function has been the inability to detect phosphorylated ERK1 using antibodies that recognize the phosphorylated TXY motif of other MAPKs, such as ERK2 [21]. While the analysis of ERK1 activation has been quite limited, the phenotypic analysis of erk1- mutants suggests that ERK1 plays an important role in determining aggregate size and the rate of development. Developmental signaling pathways mediated by the Gα5 subunit can also regulate aggregate size and rate of development suggesting a possible connection between Gα5 and ERK1 function [27].

The phenotypic analyses of *erk1⁻* and *erk2⁻* strains indicate the two *Dictyostelium* MAPKs have different roles in development but some studies have suggested these MAPKs are co-activated in response to cAMP. In this report we identified an antibody that can detect the phosphorylated ERK1 protein and demonstrate that the phosphorylation of ERK1 does not occur synchronously with the phosphorylation of ERK2 when cells are stimulated by cAMP. The phosphorylation of ERK1 in response to folate was also examined because MAP2K activity is present to activate ERK2. The phosphorylation of ERK1 or ERK2 was examined in cells deficient in the other MAPK to evaluate whether or not the activation of one MAPK was dependent on the other. Finally, the role of ERK1 function in Gα5 signaling pathways was examined through an epistasis analysis of *erk1⁻* and *gα5⁻* mutations and an analysis of phosphorylated ERK1 in *gα5⁻* mutants. The results of this study suggest different mechanisms exist for the regulation of ERK1 and ERK2 function and that these differences might reflect the different contributions these MAPKs provide in development.

2. Materials and methods

2.1. Strains and Media

All *Dictyostelium* strains were isogenic with the wild-type strain KAx-3 except for the noted mutations. The creation of the *erk1⁻*, *erk2⁻*, *gα4⁻*, and *gα5⁻* strains has been previously reported [21, 23, 28, 29]. New *erk1⁻* strains in KAx-3 and JH10 backgrounds were created, using the previously described gene disruption construct and strategy because the initial *erk1⁻* strain did not exhibit the accelerated development phenotype described in other reports [25], possibly the result of secondary mutations. The erk1 gene was also disrupted in a *gα5⁻* strain to give a *gα5⁻erk1⁻* double mutant. Verification of the *erk1* gene disruptions was conducted by PCR analysis using the oligonucleotides (sense strand) 5′-cgcggatccctcgagAATTAATGCCACCACCACCAACAAGTG -3′ and (antisense strand) 5′-GTATGGTGCCTGTGGATCTTCAGGATG -3′ (lower case nucleotides differ from those in the template DNA) (Supplementary Figure 1). Electroporation of *Dictyostelium* was performed as previously described [30]. GFP labeling of strains was accomplished by using the pTX-GFP vector as previously described [31]. Cells were grown axenically in HL-5 medium [32].

2.2. Analysis of phosphorylated MAPKs

Cells were grown in shaking cultures to mid log phase (~3×10^6 cells/ml) and then harvested by centrifugation. Cells were washed once in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH) and suspended at 1×10^8 cells/ml. Starved cells were shaken in a conical tube for 3–5 hours with pulses of 100 nM cAMP every 15 min except for the 15 min prior to an assay. For analysis of cAMP stimulation, cells were stimulated with 10 μM (high dose) or 100 nM (low dose) cAMP with or without vortexing and cell samples were collected and lysed at the indicated time by mixing with SDS-PAGE loading buffer on ice. Cell extracts were subjected to immunoblot analysis using a rabbit monoclonal antibody phospho-p42/44 MAPK (#4370, Cell Signaling Technology). For folate stimulation cell suspensions were shaken for 1 h prior to stimulation. Starved cells were also plated on Whatman filters (No. 50) at a density of 6×10^7 cells per filter (42.5 mm diameter) and then placed on nonnutrient plates (15% agar in phosphate buffer) for the analysis of MAPKs during development on a solid surface. The biotinylated mitochondrial 3-methylcrotonyl-CoA carboxylase α (MCCC1) was used as a gel loading control and this protein was detected using HRP-Streptavidin as previously described [33].

2.3. Developmental morphology analysis

Cells were grown to mid-log phase (~3×10^6 cells/ml) in shaking cultures or collected from cultures grown on petri dishes that had a recent medium change (within past 24 hrs). Cells were pelleted by centrifugation and washed in phosphate buffer. Cells were plated on nonnutrient plates from suspensions of 1×10^8 or less. For chimeric development, clonal populations were mixed at indicated ratios prior to plating cells on nonnutrient plates. Fluorescent images were detected and recorded using fluorescence microscopy.

3. Results

3.1. ERK1 is phosphorylated during early development

Earlier attempts to detect phosphorylated ERK1 in *Dictyostelium* extracts with polyclonal antibodies directed against the conserved phosphorylated TXY motif have been unsuccessful even though these antibodies recognize phosphorylated ERK2 [21, 23, 34]. These observations led to speculation that the *Dictyostelium* ERK1 might not be activated in a canonical manner or that amino residue differences surrounding the conserved motif might prevent antibody binding. However, the use of a monoclonal antibody directed against the same phosphorylated motif did allow for the detection of a protein in cell extracts that has a size consistent with that predicted for the ERK1 protein (61 kDa) (Figure 1A). The absence of this band in *erk1⁻* strains indicated ERK1 specificity and this specificity was further supported by the presence of a higher MW species in *erk1⁻* cells that express a GFP:ERK1 fusion protein (predicted 88 kDa). The phosphorylation of the GFP:ERK1 fusion protein was expected because this protein can functionally complement *erk1⁻* mutations [21]. The phosphorylated ERK1 band was significantly larger than the phosphorylated ERK2 band that can also be detected in the same blots. The relative intensities of the ERK1 and ERK2 bands suggests that the basal level of phosphorylated ERK1 might be greater than that of ERK2, at least after 1 h of starvation with no exogenous stimulation.

3.2. Basal level of phosphorylated ERK1 during growth and early development

To determine if ERK1 is activated during early development the phosphorylation of ERK1 was examined in starved cells developing on nonnutrient agar plates. Phosphorylated ERK1 was detected throughout 15 h of development whereas phosphorylated ERK2 was detected primarily between the 6 and 9 h time points that correspond to the aggregation phase of development (Figure 1B). The phosphorylation of ERK1 was also examined in shaking cultures of starved cells that were pulsed with exogenous cAMP (100 nM) every 15 min after the 3 h time point to help facilitate early developmental progression under these conditions. In this analysis, basal levels of phosphorylated MAPKs were analyzed in cell extracts obtained 15 min after the last cAMP pulse. The levels of phosphorylated ERK1 were easily detected throughout the 5 h time course but phosphorylated ERK2 was not detected (Figure 1C). These results indicate different patterns of phosphorylation for ERK1 and ERK2 during early development and suggest that the phosphorylation of ERK1 is not as dependent on cAMP signaling as is the phosphorylation of ERK2. In some immunoblots phosphorylated ERK1 bands of lower size can be detected suggesting these bands might be possible degradation products. ERK1 contains an atypical amino terminal region with long runs of glutamine residues that are not found in other MAPKs and so this region might be subjected to unusual processing or degradation resulting in the smaller ERK1 species.

3.3. Phosphorylation of ERK1 is sensitive to disruption of cell-cell interactions

A previous study has suggested that ERK1 might be transiently activated in response to the stimulation of cell surface cAMP receptors [25]. Therefore, the level of phosphorylated ERK1 was analyzed immediately after exogenous cAMP stimulation. Cells stimulated with a high dose of exogenous cAMP (10 μM) with repeated vortexing displayed a slight rise in the level of phosphorylated ERK1 that peaked around 2 min. This pattern was different than the rapid rise in phosphorylated ERK2 that occurred within 1 min of cAMP stimulation. The rapid phosphorylation of ERK2 response was typical of that observed in earlier studies. The repeated vortexing during the cAMP stimulation disrupted the small aggregates that formed in the shaking suspension (data not shown) and this disruption might possibly affect endogenous signaling between cells. To determine if the mechanical disruption of cell aggregates affects the basal level of phosphorylated ERK1, cells were vortexed without exogenous cAMP stimulation. Under this condition the basal level of phosphorylated ERK1 gradually decreased over time suggesting that the phosphorylation of ERK1 might depend on cell-cell interactions (Figure 2B, C). These observations indicate that cell-cell interactions are important for maintaining levels of phosphorylated ERK1 during starvation and early development in the absence of exogenous cAMP signaling.

3.4. Phosphorylation of ERK1 is a secondary response to cAMP signaling

The stimulation of cell suspensions with cAMP but without the mechanical disruption of cell-cell interactions (i.e., no vortexing treatment) resulted in a rapid increase of phosphorylated ERK2 and then a robust increase in the level of phosphorylated ERK1 as the level of phosphorylated ERK2 decreased (Figure 3A, D). The late rise in phosphorylated ERK1 under these conditions suggests that the phosphorylation of ERK1 might be a secondary response to cAMP that requires cell-cell interactions. Interestingly, the level of

phosphorylated ERK1 persisted for a much longer period than the level of phosphorylated ERK2 in response to the cAMP stimulation. An earlier study has shown a correlation in the duration of phosphorylated ERK2 levels and the levels of extracellular cAMP [17]. Therefore, phosphorylated ERK1 levels were also examined after cells were stimulated with a low dose of cAMP (100 nM) stimulation to determine if this treatment shortens the duration of the phosphorylated ERK1 response. Under these conditions the level of phosphorylated ERK1 decreased by the 8 min time point indicating the concentration of the exogenous cAMP impacts the duration of the phosphorylate ERK1 response (Figure 3B, D). The rise in phosphorylated ERK1 did not occur in a mock experiment that omitted the cAMP stimulation indicating that this response is dependent on the initial stimulation with exogenous cAMP (Figure 3C). These results suggest that cAMP stimulation of developing cells can induce the phosphorylation of ERK1 as a secondary or indirect response after an initial rise in phosphorylated ERK2 and that this response is dependent on cell-cell interactions and the dosage of the exogenous signal.

3.5. Phosphorylation of ERK1 does not require ERK2 function

The increase in the level of phosphorylated ERK1 as the level of phosphorylated ERK2 decreases suggests a possible coordination between these responses, such that ERK1 phosphorylation response might depend on ERK2 function. Therefore, the level of phosphorylated ERK1 was examined in an *erk2⁻* strain to explore a possible role of ERK2 function in this response. It is important to note that the *erk2⁻* strain used in this analysis, like the *erk2⁻* strains used in previous *Dictyostelium* studies, has reduced but not absent ERK2 expression due to an insertion mutation (a recapitulated REMI mutation) located near the *erk2* gene [15]. Using *erk2⁻* cells with this allele, treatment with a high or low dose of cAMP resulted in a subtle rise in phosphorylated ERK2 but a robust secondary rise in phosphorylated ERK1 with approximately the same kinetics as previously observed with wild-type cells (Figure 4A, B, D). The variability of this response to the low dose of cAMP was greater than that observed at the high dose but the basis of this variability has not been determined (Figure 4D). The level of phosphorylated ERK1 after cell stimulation with the high dose of cAMP did not persist as long as that observed for wild-type cells suggesting that the duration of the phosphorylated ERK1 response might not be as extensive in the *erk2⁻* cells. The dependency of the phosphorylated ERK2 response on ERK1 function was also examined. The stimulation of *erk1⁻* cells with a low dose of cAMP resulted in an abundant level of phosphorylated ERK2 with no major changes in the temporal pattern of this response (Figure 4C). These results indicate that the phosphorylation of ERK1 or ERK2 does not require the function of the other MAPK.

3.6. Folate signaling results in a secondary rise in phosphorylated ERK1

The stimulation of starved cells with folate leads to the rapid phosphorylation and activation of ERK2 and so the level of ERK1 phosphorylation was also examined under these conditions. Cells starved for 1 h and then treated with folate without mechanical disruption (i.e., without vortexing) displayed a rapid increase in phosphorylated ERK2, as expected from previous studies (Fig. 5A, C). This response was followed by a rise in phosphorylated ERK1 indicating that folate, like cAMP, can stimulate a secondary response that leads to the phosphorylation of ERK1. The phosphorylation of ERK1 was also observed after folate

stimulation of *erk2⁻* cells indicating the phosphorylation of ERK1 was not dependent on ERK2 function under these conditions as well (Figure 5B, C). The abundance of the lower band of phosphorylated ERK1 was quite variable in the folate stimulated *erk2⁻* cells. These findings suggest that the phosphorylated ERK1 responses to folate are similar to the responses observed after cAMP stimulation.

3.7. Phosphorylation of ERK1 can be mediated by intercellular signaling

The late rise in phosphorylated ERK1 in response to cAMP and folate stimulation possibly indicates the existence of a secondary signaling mechanism that is triggered by some component of the initial response. Stimulated cells might produce other external signals that stimulate an ERK1-specific signaling pathway. To test this latter possibility, the phosphorylation of ERK1 was analyzed in a chimeric population of *erk1⁻* and *gα4⁻* cells stimulated by folate. Only the *erk1⁻* cells in this population can respond to folate because the Gα4 subunit is required for most, if not all, folate responses, including the activation of ERK2 [22, 23]. However, only the *gα4⁻* cells express ERK1 in this population and so the phosphorylation of ERK1 can only occur if the *gα4⁻* cells are stimulated by a signal originating from the folate-stimulated *erk1⁻* cells. An equal mixture of *erk1⁻* and *gα4⁻* cells was stimulated with folate and the expected rise in the phosphorylation of ERK2 was observed within the first 2 min (Figure 6A, D). A low but reproducible rise in phosphorylated ERK1 was observed soon after suggesting that the folate stimulated *erk1⁻* cells produce an intercellular signal that leads to the phosphorylation of ERK1 in *gα4⁻* cells. The low intensity of this secondary response suggests that mechanisms other than intercellular signaling contribute to the activation of ERK1. In control experiments, folate-stimulated clonal *gα4⁻* cells did not exhibit a rise in phosphorylated ERK1 and folate-stimulated *erk1⁻* cells did not display any phosphorylated ERK1 (Fig. 6B, C, D). However, ERK2 was phosphorylated in *erk1⁻* cells stimulated with folate providing additional evidence that the phosphorylation of ERK2 is not dependent on ERK1 function.

3.7. Loss of ERK1 accelerates the developmental delay of *gα5⁻* cells

The loss of ERK1 or the overexpression of the Gα5 subunit both result in small aggregate formation and accelerated development whereas the over-expression of ERK1 or the loss of Gα5 subunit both result in delayed development. The similarity of these phenotypes suggests that the Gα5 signaling pathway might possibly include the regulation of ERK1 function. To examine the epistatic relationship between the *gα5⁻* and *erk1⁻* mutations, a double *gα5⁻erk1⁻* mutant was created and examined for developmental morphology. When starved, the double mutant formed small aggregates that progressed rapidly through development (Figure 7). Although slightly larger than the *erk1⁻* aggregates, *gα5⁻erk1⁻* aggregates were significantly smaller than *gα5⁻* and wild-type aggregates and the accelerated developmental progression of the double mutant was more closely matched to that of the *erk1⁻* aggregates. The *gα5⁻erk1⁻* phenotype indicates that the *erk1⁻* mutation is epistatic to the *gα5⁻* mutation with respect to temporal and morphological development. This finding suggests that the developmental defects associated with the loss of Gα5 function might result from the inability to down regulate ERK1 function.

3.8 Loss of ERK1 corrects the anterior localization defect of *ga5⁻* cells

When developed with an excess of wild-type cells, *ga5⁻* cells are abundant in most areas of the chimeric aggregate except in the extreme anterior region (Figure 8). This anterior region is referred to as the prestalk A (pst A) region and this region is populated by prestalk cells that express the *ecmA* gene. In contrast, *erk1⁻* cells are very abundant in the anterior and posterior regions of chimeric aggregates indicating that the localization of *erk1⁻* cells differs from that of the *ga5⁻* cells. The *ga5⁻erk1⁻* double mutant cells were found in all regions of chimeric aggregates, including the pstA region, suggesting that the *erk1⁻* mutation masks the cell localization defect associated with the *ga5⁻* mutation. The ability of the *erk1⁻* mutation to correct the cell localization defect associated with the *ga5⁻* mutation suggests that the defect in the ability of *ga5⁻* cells to develop as pst A prestalk cells might be attributed to unregulated ERK1 function. These results are consistent with the Gα5 subunit functioning in the development of prestalk cells through the down regulation of ERK1 function.

3.9. Gα5 function does not affect the pattern of phosphorylated ERK1

A possible mechanism for Gα5 signaling to down regulate ERK1 function is to reduce the abundance of phosphorylated ERK1. The external signal that stimulates the Gα5-mediated signaling pathway remains to be determined but the loss of Gα5 function enhances folate chemotaxis and increases aggregate size during development suggesting Gα5 signaling indirectly affects folate and cAMP signaling. The stimulation of *ga5⁻* cells with cAMP or folate resulted in a typical secondary response of phosphorylated ERK1 as compared to that in wild-type cells (Figure 9). Interestingly, the folate-stimulated *ga5⁻* cells displayed a higher level of phosphorylated ERK2 than cells stimulated with cAMP. This difference might be attributed to the delayed development of *ga5⁻* cells that could possibly affect the onset of cAMP responsiveness. The similar patterns in the level of phosphorylated ERK1 in *ga5⁻* cells compared to wild-type cells suggest that Gα5 signaling pathway does not down regulate the function of ERK1 through reducing the level of phosphorylation of ERK1. These observations do not exclude the possibility that other signals, perhaps ones that specifically activate the Gα5 pathway, might down regulate the phosphorylation of ERK1.

4. Discussion

The increase in phosphorylated ERK1 after the level of phosphorylated ERK2 peaked in cAMP stimulated cells was unexpected because an earlier study had suggested that ERK1 activity peaked within 30 s after cAMP stimulation [25]. However, this earlier study had examined *in vitro* kinase activity associated with an immunoprecipitated complex of an epitope-tagged ERK1 and so the detected protein kinase activity might have been associated with other kinases present in that immune complex. The detection of phosphorylated ERK1 is expected to be a better determinant of ERK1 activation because of the strong correlation that exists between the phosphorylation of the TXY motif and MAPK activation in eukaryotic signaling pathways [9]. The temporal difference in the phosphorylation of ERK1 and ERK2 after chemoattractant stimulation and the different patterns in the basal levels of phosphorylated ERK1 and ERK2 are consistent with these MAPKs playing different roles in

early *Dictyostelium* development. These different roles are also supported by the phenotypic differences observed for *erk1⁻* and *erk2⁻* cells as they progress through development.

The rise in phosphorylated ERK1 as a secondary response to external cAMP or folate stimulation could potentially contribute to the adaptation of cells to these signals. The endogenous cAMP signaling between aggregating cells occurs as a pulsatile signal approximately every 6 min and during this period cells chemotax, adapt, and re-sensitize so that they can respond to the next pulse of cAMP. Folate stimulation does not involve a pulsatile signal but the response is likely to require an adaptation process, typically found in other eukaryotic chemotactic responses [35]. Stimulation of *Dictyostelium* with either cAMP or folate results in a rapid accumulation of cGMP that peaks within 15 s and a slightly slower accumulation of cAMP that peaks about 2 min after stimulation [36–38]. The accumulation of cAMP is likely to be regulated in part by the down regulation of the cAMP-specific phosphodiesterase RegA by active ERK2 and the cessation of cAMP accumulation could possibly be attributed to the loss of ERK2 activity and a gain of RegA activity [19]. However, the rise in phosphorylated ERK1 at this time opens the possibility that ERK1 function might be in part responsible for reducing the accumulation of cAMP through the regulation of phosphodiesterase or adenylyl cyclase activity. The presence of multiple putative MAPK phosphorylation sites on RegA offers the possibility of differential regulation by the two MAPKs. The accelerated development of *erk1⁻* cells is similar to the phenotypes observed for mutants that have elevated cAMP signaling. For example, the loss of RegA phosphodiesterase activity or the overexpression of the PKA catalytic subunit, PKA-C, both result in precocious development. [25, 39–41]. This correlation between the rate of development and cAMP signaling suggest that ERK1 function might prevent precocious development through the limitation of cAMP signaling.

The contribution of cell-cell interactions to the phosphorylation of ERK1 suggests that signals other than cAMP and folate can regulate this response as illustrated in our model of ERK1 regulation (Figure 10). *Dictyostelium* has over fifty G protein-coupled receptors and possibly other types of receptors that might function in the pathway leading to the phosphorylation of ERK1 [42, 43]. Several external stimulants of *Dictyostelium* besides cAMP and folate have been identified but none have been examined for possible roles in ERK1 activation [44, 45]. Intercellular signaling presumably becomes more efficient and important as cells begin to adhere to each other during the aggregation stage of development. The onset of cell-cell adhesion occurs as cells become responsive to cAMP and this adhesion allows cells form streams as they aggregate into mounds [46]. Chemotactic responses to folate do not facilitate cell-cell adhesion but groups of cells can chemotax to folate while in contact with each other (unpublished results). The relatively weak phosphorylation of ERK1 in *gα4⁻* cells stimulated by an external signal other than folate suggests that cell autonomous signaling also contributes a significant role in ERK1 activation. The mechanism by which this cell autonomous signaling occurs remains to be identified.

The function of ERK1 to increase aggregate size and prevent precocious cell differentiation acts in opposition to the Gα5 subunit-mediated signaling pathway that promotes rapid development and small aggregate size. The Gα5 subunit promotes the development of the

anterior prestalk region as indicated by Gα5 dependent changes in anterior tip formation and prestalk gene (i.e., *ecmA*) expression. The suppression of *ga5⁻* developmental phenotypes through the loss of ERK1 argues that Gα5 subunit-mediated signaling pathways down regulate ERK1 function. Based on the analysis of phosphorylated ERK1 in *ga5⁻* mutants this down regulation does not appear to affect levels of activated ERK1. However, ERK1 function could be regulated through other mechanisms such as sequestering active ERK1 from target substrates. MAPK binding sites (D-motifs) such as those found on Gα5 subunit or other proteins could potentially regulate ERK1 function through limiting the cellular distribution of ERK1. In yeast, a D-motif in the mating response Gα subunit has been proposed to down regulate MAPK function through altering the cytoplasmic/nuclear localization of the MAPK [47, 48]. The D-motif of the *Dictyostelium* Gα5 subunit is required for the precocious development of small aggregates associated with Gα5 subunit overexpression suggesting that Gα5 function might be mediated through MAPK interactions [27].

5. Conclusion

The temporal differences in the phosphorylation of ERK1 and ERK2 are consistent with these MAPKs providing different functions in developmental and chemotactic signaling. Previous studies have indicated that ERK2 function is important for increased cAMP signaling. The timing of ERK1 activation suggests this MAPK might contribute to a decrease in cAMP signaling, perhaps as part of an adaptation process. The limited sequence similarity between these MAPKs also supports the idea that each MAPK has a different role in development but does not rule out the possibility of competitive interactions with other signaling components in their signaling pathways. Results of the genetic analysis suggest that the Gα5 subunit signaling pathway down regulates ERK1 function but the specific mechanism does not appear to involve a reduction in activated ERK1. The ability to detect both phosphorylated ERK1 and ERK2 is an important tool for characterizing the specificity of MAPK signaling in *Dictyostelium* and further analysis of ERK1 and ERK2 regulation will likely provide important insight into the interactions of these MAPKs with other signaling components during chemotaxis and development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

MAPK   mitogen activated protein kinase

ERK   extracellular-signal regulate kinase

| **cAMP** | cyclic adenosine monophosphate |
| **Pst A** | prestalk A |
| **MCCC1** | mitochondrial 3-methylcrotonyl-CoA carboxylase α |

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Highlights

- ERK1 is phosphorylated as a secondary response to cAMP and folate.

- Cell-cell interactions contribute to the phosphoryation of ERK1.

- Loss of ERK1 suppresses the developmental delays of $ga5^-$ mutants.
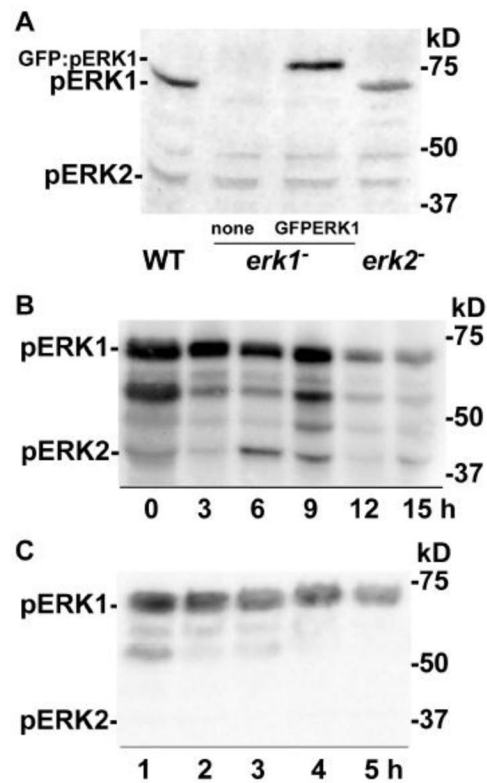
Figure 1.
Detection of phosphorylated ERK1 protein. *Dictyostelium* strains were grown in shaking cultures and harvested as described in the Materials and methods. After starvation in shaking cultures or on filters cells were harvested and cell extracts were subjected to immunoblot analysis using the anti-phospho-MAPK antibody that detects phospho-ERK1 (pERK1, 61 kDa), GFP:phospho-ERK1 (GFP:pERK1, 88 kDa), and phospho-ERK2 (pERK2, 42 kDa). Extracts from approximately 1×10^6 cells were loaded in each lane. (A) Cells shaken in phosphate buffer for 1 h before immunoblot analysis. Lanes correspond to extracts from wild-type cells (WT), *erk1⁻* cells, and *erk1⁻* cells expressing a GFP:ERK1 fusion protein (GFP:ERK1). Positions of protein molecular weight standards are indicated on the right side of the blot. (B) Cells were developed on filters as described in the Materials and methods and harvested at times indicated. (C) Cells were shaken in phosphate buffer without exogenous stimulation and collected at the times indicated.
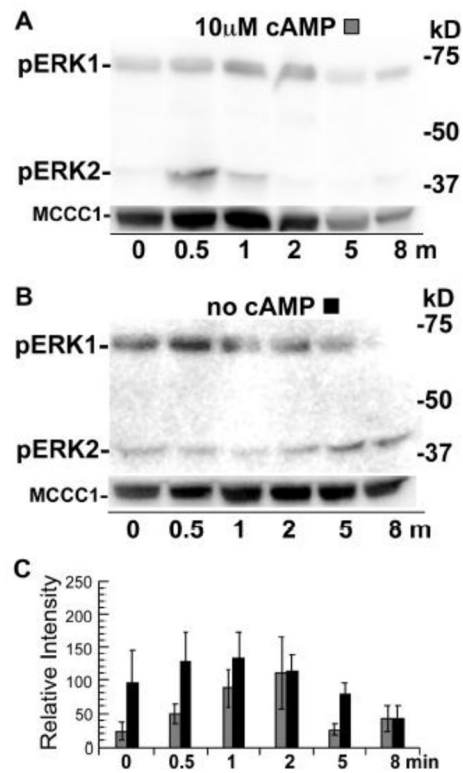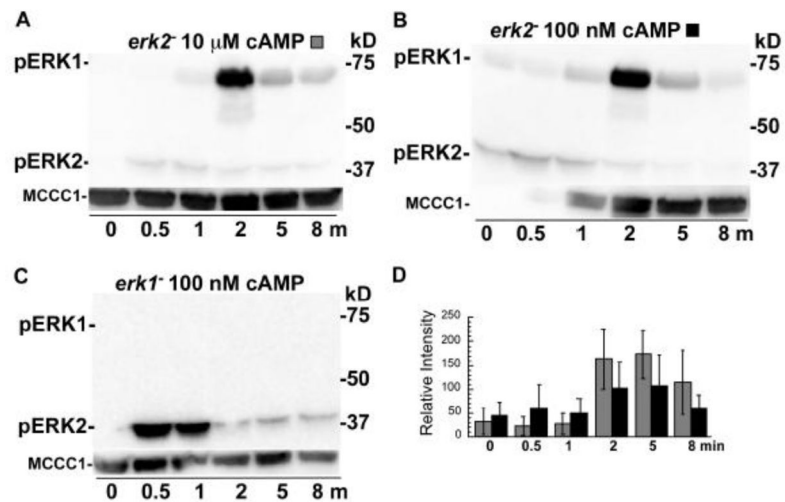
Figure 2.
Phosphorylation of ERKs in wild-type cells treated with cAMP and vortexing. Cells were starved for 5 hours in shaking cultures and pulsed with cAMP for the last 2 hours to induce cAMP responsiveness as described in the Materials and methods section. Cells were harvested at times indicated after repeated vortexing with or without cAMP stimulation and then the resulting extracts were subjected to immunoblot analysis. Upper panels are immunoblots using the anti-phospho-MAPK antibody and lower panels are immunoblots that detect the loading control protein MCCC1. Each lane contained extracts from approximately 1×10^6 cells. Exposure times varied for the chemiluminescent detection of different blots. (A) Cells were treated with 10 μM cAMP and repeated vortexing. (B) Cells were vortexed repeatedly without cAMP treatment. (C) Graph of the mean pixel intensity of the phosphorylated ERK1 bands of three immunoblots of multiple experiments. Gray and black shaded columns correspond to treatments (A) and (B), respectively. Error bars represent the standard deviation.
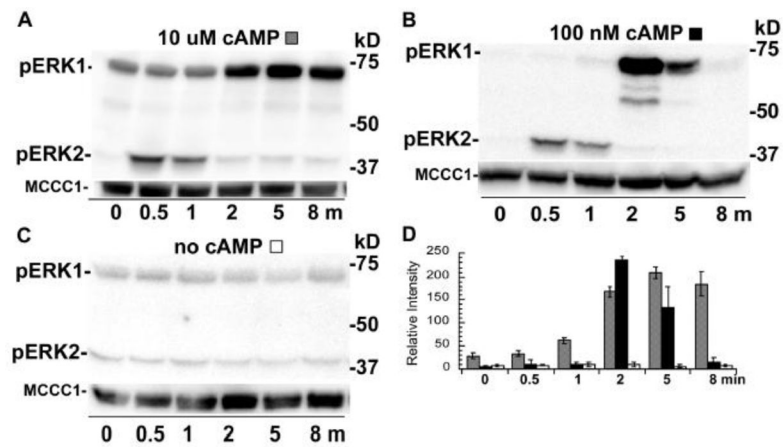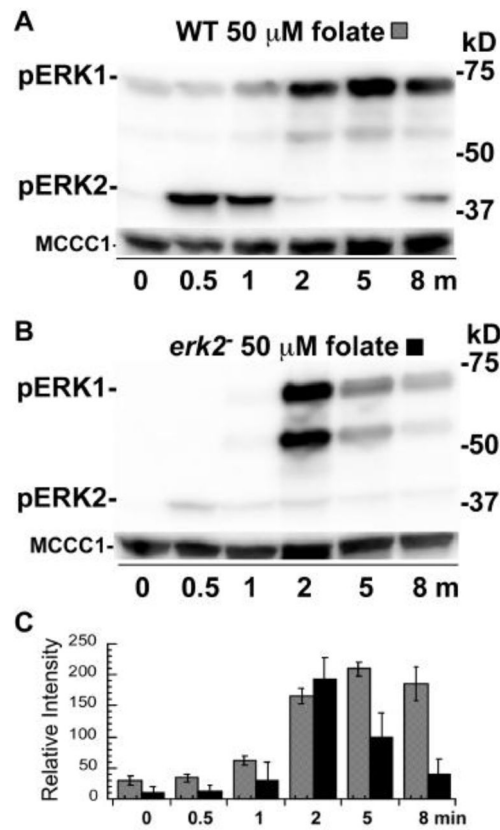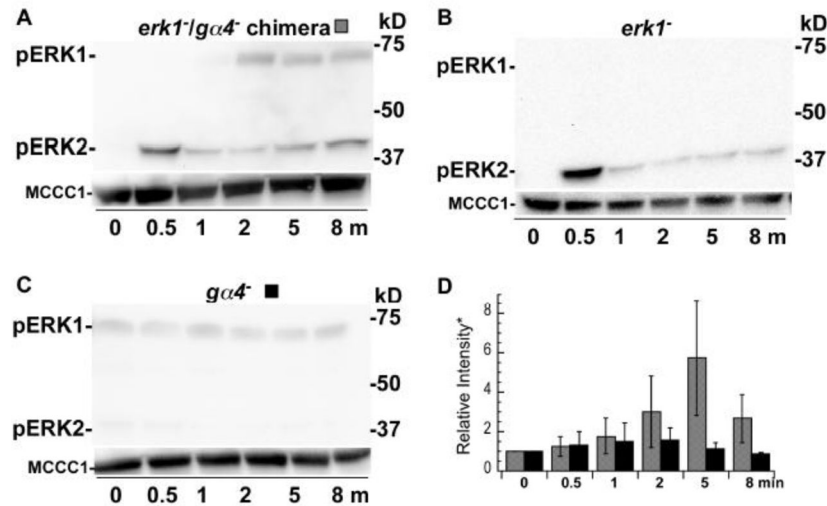
Figure 3.
Phosphorylation of ERKs in wild-type cells stimulated with cAMP but without vortexing. Cells were prepared, treated, and analyzed as described in Figure 2 except that cAMP stimulation was conducted while cells were continuously shaken at 200 rpm in conical tubes (no vortexing). (A) Wild-type cells stimulated with 10 μM cAMP. (B) Wild-type cells stimulated with 100 nM cAMP. (C) Wild-type cells not stimulated with cAMP. (D) Graph of the mean pixel intensity of the phosphorylated ERK1 bands of three immunoblots from multiple experiments. Gray and black shaded and open columns correspond to treatments (A), (B), and (C), respectively. Error bars represent the standard deviation.
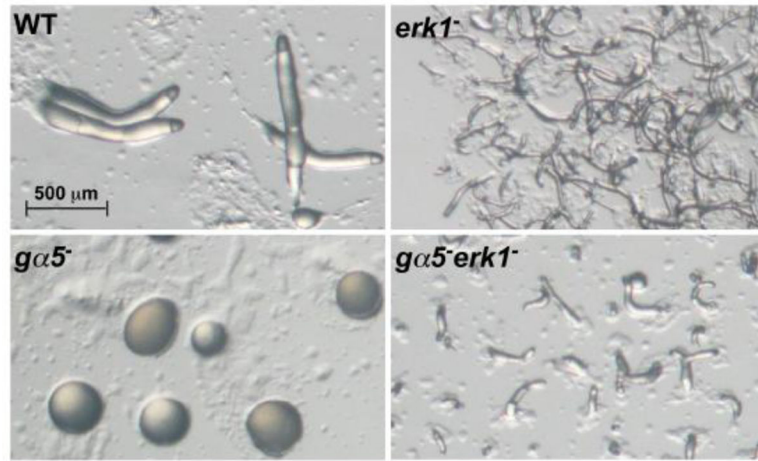
Figure 4.
Phosphorylation of ERKs in *erk2⁻* cells stimulated with cAMP. Cells were prepared, treated, and analyzed as described in Figure 3. (A) *erk2⁻* cells stimulated with 10 μM cAMP. (B) *erk2⁻* cells stimulated with 100 nM cAMP. Uneven detection of MCCC1 is responsible for low band intensity in the early time points of the control blot (coomassie staining of blotted gel displayed equal loading) (C) *erk1⁻* cells stimulated with 100 nM cAMP. (D) Graph of the mean pixel intensity of the phosphorylated ERK1 bands of three immunoblots of multiple experiments. Gray and black shaded columns correspond to treatments (A) and (B), respectively. Error bars represent the standard deviation.
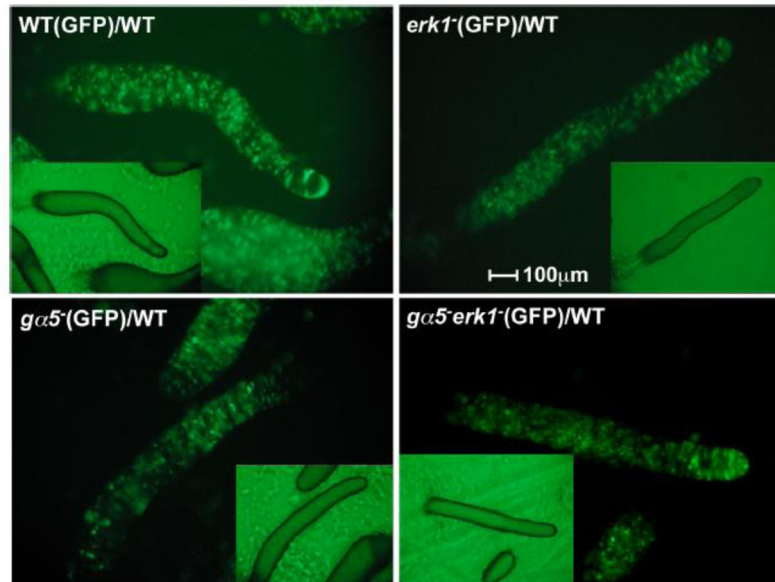
Figure 5.
Phosphorylation of ERKs in wild-type and *erk2⁻* cells stimulated with folate. Cells were starved in phosphate buffer for 1 hour and folate stimulation was conducted while cells were continuously shaken at 200 rpm in conical tubes (no vortexing). (A) Wild-type cells stimulated with 50 μM folate. (B) *erk2⁻* cells stimulated with 50 μM folate. (C) Graph of the mean pixel intensity of the phosphorylated ERK1 bands of three immunoblots of multiple experiments. Gray and black shaded columns correspond to treatments (A) and (B), respectively. Error bars represent the standard deviation.
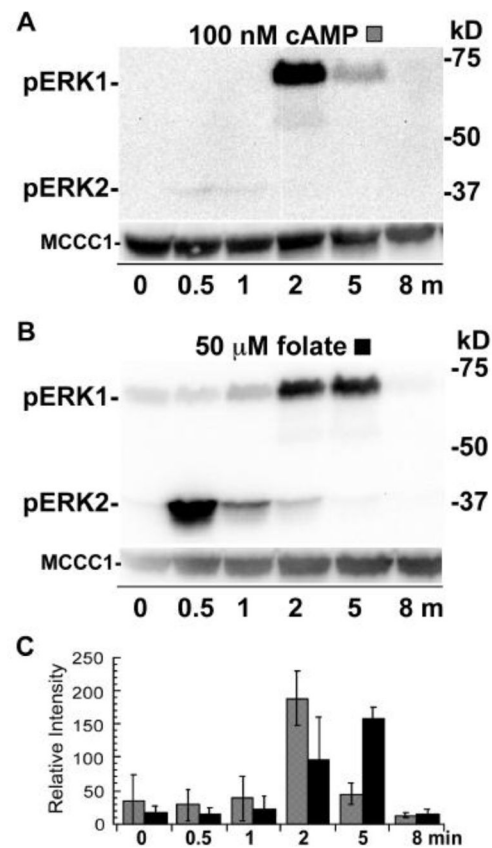
Figure 6.
Phosphorylation of ERKs in *erk1⁻*, *gα4⁻* chimeric and clonal cell populations stimulated with folate. Cells were prepared and stimulated with folate as described in figure 5. (A) Chimeric population of *erk1⁻* and *gα4⁻* cells mixed in a 1:1 ratio and stimulated with 50 μM folate. (B) *erk1⁻* cells only stimulated with 50 μM folate. (C) *gα4⁻* cell only stimulated with 50 μM folate. (D) Graph of the mean pixel intensity of the phosphorylated ERK1 bands of three or more immunoblots of multiple experiments (*values have been normalized to the 0 min time mean intensity). Gray and black shaded columns correspond to treatments (A) and (C), respectively. Error bars represent the standard deviation.
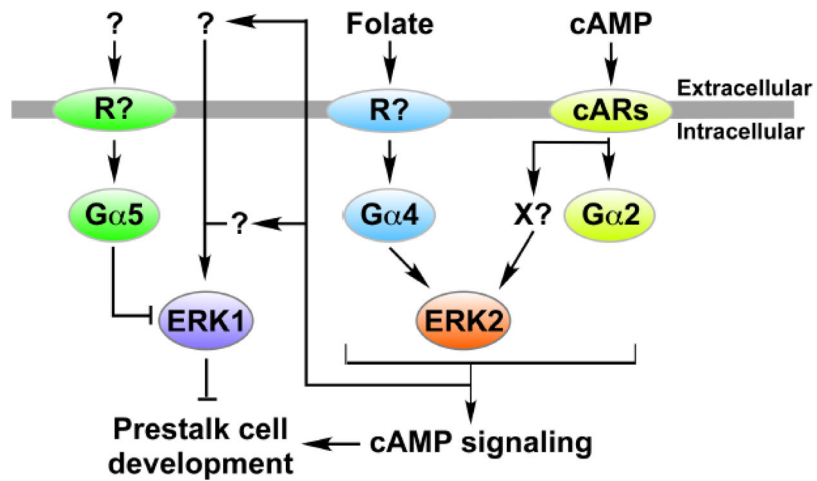
Figure 7.
Developmental morphology of wild-type (WT), *erk1⁻*, *gα5⁻*, and *gα5⁻erk1⁻* aggregates. Strains were grown and plated for development on nonnutrient plates as described in the Materials and methods. Images of developmental morphology were recorded at 13 h post starvation. All images are shown at the same magnification.

Figure 8.
Spatial distribution of *erk1⁻* and *gα5⁻* mutants in chimeras with wild-type cells. Wild-type (WT), *erk1⁻*, *gα5⁻*, and *gα5⁻erk1⁻* cells were labeled with a GFP expression vector and mixed with an excess of unlabeled wild-type cells (1:10 ratio) before plating for development. GFP fluorescence in migratory slugs was recorded at 16 h of development. Insets show brightfield images (2 fold size reduction). The central slug in each image is positioned with the anterior on the right side of the image.

Figure 9.
Phosphorylation of ERKs in $ga5^-$ cells stimulated with cAMP or folate. (A) $ga5^-$ cells were prepared and treated with 100 nM cAMP as described in Figure 3. (B) $ga5^-$ were prepared and treated with 50 μM folate as described in Figure 5. (C) Graph of the mean pixel intensity of the phosphoERK1 bands of three immunoblots of multiple experiments. Gray and black shaded and open columns correspond to treatments (A) and (B), respectively. Error bars represent the standard deviation.

Figure 10.
Model of ERK1 activation with respect to G protein signaling pathways. Signals mediated by G protein signaling pathways in response to cAMP and folate activate ERK2 but then later lead to the activation of ERK1 in a secondary response. ERK1 can also be activated through intercellular signaling. The Gα5-mediated signaling pathway can down regulate ERK1 function but not through changing the phosphorylated state of ERK1.