The *Dictyostelium* MAP kinase ERK2 regulates multiple, independent developmental pathways

Chris Gaskins,1 Alexandra M. Clark,1 Laurence Aubry,1 Jeffrey E. Segall,2 and Richard A. Firtel1,3

1Department of Biology, Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634 USA; 2Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461 USA

We showed previously that the MAP kinase ERK2 is essential for aggregation. *erk2* null cells lack cAMP stimulation of adenyl cyclase and thus cannot relay the cAMP chemotactic signal, although the cells chemotax to cAMP [Segall et al. 1995]. In this paper we have examined the role of ERK2 in controlling developmental gene expression and morphogenesis during the multicellular stages, making use of a temperature-sensitive ERK2 mutation. Using suspension assays, we show that ERK2 is not essential for aggregation-stage, cAMP pulse-induced gene expression, or for the expression of postaggregative genes, which are induced at the onset of mound formation in response to cAMP in wild-type cells. In contrast, the prespore-specific gene *SP60* is not induced and the prestalk-specific gene *ecmA* is induced but at a significantly reduced level. Chimeric organisms, comprised of wild-type and *erk2* null cells expressing the prestalk-specific *ecmA/lacZ* reporter, show an abnormal spatial patterning, in which *Erk2ts/erk2* cells are excluded from the very anterior prestalk A region. To further examine the function of ERK2 during the multicellular stages, we bypassed the requirement of ERK2 for aggregation by creating an ERK2 temperature-sensitive mutant. *erk2* null cells expressing the ERK2ts mutant develop normally at 20°C and express cell-type-specific genes but do not aggregate at temperatures above 25°C. Using temperature shift experiments, we showed that ERK2 is essential for proper morphogenesis and for the induction and maintenance of prespore but not prestalk gene expression. Our results indicate that ERK2 functions at independent stages during *Dictyostelium* development to control distinct developmental programs: during aggregation, ERK2 is required for the activation of adenyl cyclase and during multicellular development, ERK2 is essential for morphogenesis and cell-type-specific gene expression. Analysis of these results and others supports the conclusion that the requirement of ERK2 for cell-type differentiation is independent of its role in the activation of adenyl cyclase.

[Key Words: Dictyostelium discoideum; signal transduction; MAP kinase]

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Upon starvation, *Dictyostelium* amoebae initiate a developmental process that leads to the formation of a multicellular organism comprised of up to $10^6$ cells that forms a fruiting body containing a sorus of spores on a slender stalk held up by a basal disk [Loomis 1982]. The formation of the multicellular organism is mediated by the chemotactic responses to extracellular cAMP that binds to cell surface receptors and activates intracellular signaling pathways [Devreotes 1994; Firtel 1995]. These pathways include (1) the activation of adenyl cyclase, leading to the secretion of cAMP and the relay of the chemotactic signal; (2) the activation of guanylyl cyclase, which is important for chemotaxis; and (3) the expression of genes (including the cAMP receptor cAR1 and the coupled Go2 subunit) essential for the aggregation process. These pathways are regulated by oscillatory pulses of cAMP, which control the sequential activation and adaptation of these pathways, whereas a continuous flux of cAMP results in cells that are continually adapted [Van Haastert 1991; Devreotes 1994; Firtel 1995]. Upon the formation of the mound, rising levels of cAMP activate a second signaling pathway that results in the expression and activation of the transcription factor GBF and the induction of postaggregative gene expression and the regulatory cascade that leads to the expression of prestalk- and prespore-specific genes, cell-type differentiation, morphogenesis, and the repression of aggregation-stage gene expression [Abe and Yanagisawa 1983; Schnitzler et al. 1994, 1995; Firtel 1995]. In contrast to the cAMP-mediated responses during aggregation, postaggregative gene expression and cell-type differentiation require a high, continuous level of cAMP.

MAP kinases (ERKs) are components of signaling cascades found in all eukaryotes that regulate a diverse set
of pathways, including mating and osmoregulation in yeast, stress-responses in mammalian cells, and cellular and developmental responses to a diverse set of ligands in higher eukaryotes through both seven-span/serpentine and tyrosine kinase receptors [Davis 1993; Errede and Levin 1993; Blumer and Johnson 1994; Hsu and Perrimon 1994; Hunter 1995; Karin 1995; Marshall 1995]. In many cases, these involve the activation of transcription factors [Errede and Levin 1993; Hunter 1995; Karin 1995; Marshall 1995]. Two MAP kinases, ERK1 and ERK2, have been identified in Dictyostelium. ERK1 is developmentally regulated, being expressed from one promoter during growth and a second promoter that is induced upon the onset of multicellular development and is preferentially expressed in cells showing characteristics of the anterior-like cell [ALC] population [Gaskins et al. 1994]. ERK1 is essential for growth and also plays an important role in regulating multicellular development. ERK2 was identified by a molecular genetic screen for mutants that are unable to aggregate, using a procedure for insertional mutagenesis [REMIL] and independently by a PCR screen for genes encoding MAP kinases [Segall et al. 1995]. erk2 null cells do not aggregate and lack the ability to activate adenyl cyclase in response to extracellular cAMP. erk2 null cells are, however, capable of forming coaggregates with wild-type cells, but spores are not formed from the erk2 null cells.

In this paper we report the creation of a temperature-sensitive mutation of ERK2 and its expression in erk2 null cells. This strain was used, in combination with cell suspension assays and chimeras containing wild-type and erk2 null cells, to examine the role of ERK2 in gene expression, cell-type differentiation, and morphogenesis during the multicellular stages. Our results indicate that ERK2 is required for proper morphogenesis and prespore cell differentiation. Expression of the prestalk marker ecmA is reduced and erk2 null cells expressing ecmA/lacZ show an abnormal spatial distribution in chimeras. Because prespore genes can be induced in cells lacking the adenyl cyclase ACA [Pitt et al. 1993], which is not activated in erk2 null cells, our results indicate that ERK2 has multiple, possibly independent functions, including the regulation of signal relay during aggregation, morphogenesis, and cell-type differentiation.

Results

Examination of gene expression in erk2 null cells

The regulation of expression of genes induced during aggregation and multicellular stages of Dictyostelium development can be examined in suspension cultures [Mehdy and Firtel 1985; Mann and Firtel 1987; Berks and Kay 1990]. These assays have the advantage that the role of extracellular morphogens and cell-cell contact on gene expression can be directly examined. To determine whether ERK2 was part of the signaling pathway required for the induction of aggregation-stage gene expression in response to pulses of cAMP, we assayed the expression of the gene encoding the cell surface adhesion molecule contact sites A [csA/gp80], which has previously been shown to be induced in response to nanomolar pulses of cAMP [Noegel et al. 1986; Mann and Firtel 1989]. As shown in Figure 1, csA expression in both wild-type and erk2 null cells was induced in response to cAMP pulses, but was only minimally induced when the cells were not pulsed with exogenous cAMP. The level of csA expression was slightly reduced in the erk2 null cells when compared to that of the wild-type strain that is isogenic with the erk2 null cells except for the disruption of the ERK2 gene.

Postaggregative genes, which are induced as the mound forms, and prestalk- and prespore-specific genes, can be induced in suspension culture in response to high [μM] continuous levels of cAMP rather than the nM pulses that induce many aggregation-stage genes [Firtel 1995]. Postaggregative genes, such as the ones encoding the transcription factor GBF and genes whose expression is enriched in prestalk cells [e.g., pst-cath/CP2 and rasD], are induced in fast-shake suspension culture in the presence of EDTA to help eliminate cell-cell contacts [Mehdy and Firtel 1985; Dynes et al. 1994]. Expression of prespore and prestalk cell-type-specific genes [e.g., SP60/CotC and ecmA, respectively] requires regulatory signals such as stable cell-cell contacts, the LagC gene product, and DIF, in addition to the cAMP-mediated signaling pathway, and can be assayed in suspension culture under slow-shake conditions [Mehdy and Firtel 1985; Williams et al. 1987, Berks and Kay 1990, Haberstroh and Firtel 1990, Dynes et al. 1994; Schnitzler et al. 1994, 1995; Firtel 1995].

As shown previously for wild-type cells, the postaggregative genes GBF, CP2, and rasD were induced in the presence but not the absence of high cAMP levels under fast-shake conditions [Fig. 2A]. GBF showed a starvation-dependent, low-level induction in the absence of high cAMP observed in 6-hr starved cells, as described previously [Schnitzler et al. 1994]. When examined in erk2 null cells, GBF and rasD were also induced, but the level of expression was reduced. CP2 was only minimally expressed under fast-shake conditions, but as in wild-type

Figure 1. Regulation of the pulse-induced gene contact sites A (csA) in wild-type and erk2 null cells. Vegetative cells were harvested, washed, and placed in suspension culture at 5×10⁶ cells/ml in MES-PDF buffer [Mann and Firtel 1987]. The cells were either pulsed (as designated by P) or not pulsed for the time periods indicated. RNA was isolated, size-fractionated in denaturing gels, and probed for csA. Methods are as described previously [Mann and Firtel 1987, 1989].

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cells, this expression was dependent on exogenous cAMP. Under slow-shake conditions, which permit cell-cell contacts and in which small cell agglomerates are formed, GFB and CP1 were induced to similar levels in erk2 null and wild-type cells in the presence of exogenous cAMP. In wild-type cells, this induction was independent of exogenous cAMP, as cAMP is produced in the agglomerates to a sufficient level to induce these genes (Mehdy and Firtel 1985). As in other strains unable to induce the activation of adenyl cyclase, such as goa2 null cells (Kumagai et al. 1991; Schnitzler et al. 1995), there was no induction of CP2 nor any increase in the level of GFB expression in erk2 null cells in the absence of exogenous cAMP. In wild-type cells, rasD expresses both a 1.2- and a 1.0-kb transcript in slow-shake conditions in the presence of cAMP, with the 1.0-kb transcript being specific for the multicellular stages and the expression level of the 1.2-kb transcript being lower than that observed in fast-shake conditions (Reymond et al. 1984; Esch and Firtel 1991; Esch et al. 1992). In erk2 null cells, the 1.2-kb transcript was expressed at a slightly higher intensity than in wild-type cells, whereas the 1.0-kb transcript was expressed at a reduced level [Fig. 2A]. In both erk2 null cells and wild-type cells constitutively expressing GFB from the Act15 promoter (Schnitzler et al. 1995), CP2, rasD, and LagC gene expression was induced to a similar level at the onset of development in response to cAMP [data not shown; Schnitzler et al. 1995]. The results described here indicate that under conditions that allow cell-cell contacts and in the presence of high cAMP, ERK2 activity was not essential for this class of postaggregative genes.

We also examined the expression of a prestalk- and a prespore-specific gene, ecmA and SP60, respectively, under slow-shake suspension assays. As seen in Figure 2B, ecmA was expressed in the erk2 null cells, but at a significantly reduced level, whereas SP60 was not detectably expressed, even on exposures that are 10× longer than the one shown [data not shown].

**Figure 2.** Regulation of postaggregative and cell-type-specific genes in wild-type and erk2 null cells. (A) Vegetative cells were washed and placed in suspension culture at 5x10⁶ cells/ml and shaken for 6 hr at 150 rpm. Cells were then split and shaken at 250 rpm after the addition of 2 mM EDTA (pH 7.2), either with or without the addition of 300 mM cAMP added every 2 hr. For slow-shake (S), cells were shaken at 125 rpm in the absence of added EDTA with the same additions of cAMP. Cells were harvested after an additional 6 hr of fast (F)- or slow-shake condition. RNA blot hybridization was used to determine the relative expression levels of the RNA. (B) Slow-shake conditions as described in A were used to examine the expression of the prestalk-specific gene ecmA and the prespore-specific gene SP60/CotC.

**Regulation of late gene expression in erk2 null cells in chimeras with wild-type cells: ERK2 function is cell autonomous**

erk2 null cells, as with many aggregation-deficient strains that lack the ability to activate adenyl cyclase, will coaggregate, and in some cases, proceed through development when mixed with wild-type cells (Darmon et al. 1975; Pereira da Silva et al. 1975; Mann and Firtel 1991, Pitt et al. 1993; Insall et al. 1994a, Soede et al. 1994; Segall et al. 1995). By marking the mutant cells with lacZ reporter constructs, one is able to determine if the mutant cells can respond to signals present in coaggregates with wild-type cells and induce cell-type-specific gene expression and if the mutant cells show a normal or abnormal spatial pattern. This method is very sensitive, permitting the detection of expression in a limited number of cells within the population. erk2 null cells carrying the ecmA/lacZ, rasD/lacZ, SP60/lacZ, or the stalk cell-specific ecmBΔ9/lacZ reporter construct were mixed with wild-type cells in a ratio of 1:3 (erk2 null: wild-type cells). Chimeric organisms were then stained for β-gal expression at different times in development. The anterior ~15% of the slug comprises the prestalk domain and is composed of three compartments as determined by the expression pattern of the two prestalk-specific, DIF-inducible genes ecmA and ecmB (Jermyn et al. 1989; Early et al. 1993; Williams et al. 1993a,b). In wild-type strains, the very anterior of the slug contains a core of prestalk AB cells surrounded by prestalk A cells. Slightly more posterior, but within the anterior 15% of the slug, is the prestalk O compartment, in which the ecmA gene is expressed at a significantly lower level than in prestalk A cells [Early et al. 1993]. In addition, both ecmA and ecmB are expressed in the ALC, a regulatory cell population that is found scattered throughout the slug (Jermyn and Williams 1991; Williams et al. 1993a,b, Hadwiger et al. 1994; Firtel 1995). For a comparison with the results using the mu-
tant erk2 null cells described below, a cartoon showing the spatial organization at the slug stage and during culmination is presented in Figure 3 (left).

As shown in Figure 3 (right), ecmA/lacZ-expressing cells showed a pattern of localization that comprised a subset of cells seen in wild-type strains (Williams et al. 1989, 1993a). Staining was concentrated in the posterior of the developing fingers (possibly rear guard cells) and scattered throughout the body, with a distribution similar to that of anterior-like cells. Strong staining was also observed in the anterior of the second finger. However, this staining appeared to be restricted to a band of cells with a pattern similar to that of prestalk O cells, with no visible staining cells in the more anterior prestalk A domain, suggesting erk2 null cells cannot form prestalk A cells or enter the very anterior of the slug. During culmination, staining was observed in the upper and lower caps, stalk tube, and basal disk in mature fruiting bodies. To examine prestalk/stalk cell differentiation further, we determined the spatial pattern of expression of ecmBΔ89, a deletion of the prestalk-specific ecmB promoter, which is expressed during stalk cell differentiation during culmination (Ceccarelli et al. 1991). As can be seen in Figure 3, erk2 null cells expressing this construct were localized to the stalk, basal disc, and lower cup. In some sorocarps, cells comprising the very papillar region were also stained. When SP60/lacZ was examined, no detectable β-gal expression was seen, consistent with the cell suspension assays (data not shown).

In wild-type cells, expression of the lacZ reporter from the rasD promoter during the multicellular stages is first induced in the late aggregation streams (Esch and Firtel 1991). The β-gal staining cells then sort to the tip and are present in the prestalk A/O region and ALCs during the slug and culminant stages. In the erk2 null cell:wild-type chimeras, the pattern of staining was very similar to that observed for ecmA (data not shown).

The above staining and suspension culture assay results indicated that ERK2 was essential for prespore gene expression and also affected either the distribution or sorting of prestalk A/O and anterior-like cells that express prestalk-specific (ecmA) and prestalk-enriched (rasD) genes. Previously, the total distribution of erk2 null cells in chimeras was examined by loading erk2 null cells with the fluorescent marker CMFDA (Segall et al. 1995). When these cells were mixed with wild-type cells in a 1:10 ratio, a preferential localization of the erk2 null cells was observed in the anterior of the slug, with very

Figure 3. Spatial patterning of erk2 null cells in chimeras with wild-type cells. (Left) Cartoon showing the organization in wild-type organisms. [Redrawn from Firtel (1995) and taken from data as referenced in the text.] The drawings were, in part, a gift from J. Williams, (University College, London, UK). rasD (data not shown in B) is expressed in prestalk A, prestalk O, and ALCs (Esch and Firtel 1991). (Right) erk2 null cells carrying either the ecmA/lacZ reporter or the ecmBΔ89/lacZ reporter were mixed with three parts wild-type cells, allowed to form coaggregates, and then histochemically stained for β-gal expression as described previously. (A,B) ecmA/lacZ staining fingers; (C,D) ecmA staining during mid (C) and late (D) culmination; (E,F) ecmBΔ89 staining during mid (E) and late (F) culmination. Labeled arrowheads mark the very anterior prestalk A domain (a), the slightly more posterior prestalk O domain (b), and the rear guard domain (c).
few cells in the posterior, prespore domain. Because of our finding that ecma/lacZ-expressing cells localized to the boundary of the preprostak/prespore domains and the posterior one-third of the finger, we examined the location of erk2 null cells within chimeras using an Act15/lacZ reporter. In the mound and slug stages, the erk2 null cells expressing Act15/lacZ showed a more uniform distribution than observed previously: cells were present in the posterior as well as anterior of the slug, although the very anterior of the slug had few erk2 null cells (data not shown). These data are consistent with the distribution of ecma/lacZ-expressing cells in different domains of the aggregate in chimeras between wild-type cells and erk null cells expressing ecma/lacZ. It was also clear from these experiments, and consistent with previous results, that many of the erk2 null cells do not effectively participate in the initial formation of the multicellular aggregate. Thus, although erk2 null cells can chemotax to cAMP (Segall et al. 1995), they did not efficiently coaggregate with wild-type cells. When mixed in a ratio of one part erk2 null cells to three parts wild-type cells, an estimated 30%–40% of the erk2 null cells were not found in the multicellular aggregates and remained as free amoebae. In the mature fruiting body, most of the erk2 null cells were localized in the stalk, upper and lower cups, and the apical papilla of the sporocarp. Few staining cells were located in the sorus, consistent with previous observations of the absence of mature erk2 null spores in the chimeras (Segall et al. 1995).

**Examination of an ERK2-conditional mutation identifies a developmental function for ERK2 during the multicellular stages**

To further examine possible functions of ERK2 during the multicellular stages, we needed to bypass the inability of erk2 null cells to aggregate. To do this, we created a temperature-sensitive [ts] mutation of ERK2 based on the strategy of Hsu and Perrimon (1994). Carr et al. (1989) showed that a temperature-sensitive mutation in the Schizosaccharomyces pombe Cdc2 protein resulted from the change of a highly conserved proline residue to a serine. Hsu and Perrimon (1994) created a temperature-sensitive mutation in the Drosophila MAP kinase kinase [MEK] by changing the homologous proline to a serine. We created the same mutation in ERK2 (see Table 1) and expressed it in erk2 null cells (Erk2tS/erk2 cells).

The Erk2tS/erk2 cells aggregated and formed mature fruiting bodies indistinguishable from wild-type fruiting bodies at temperatures below 23°C, whereas at temperatures above 25°C, the cells did not aggregate (Fig. 4, left). At 20°C, the timing of development of the Erk2tS/erk2 cells was delayed, with loose aggregates forming at 11 hr compared to 9 hr for the wild-type cells and mature fruiting bodies forming at 30 hr rather than 26 hr. When cells were developed at 19°C, we also observed a developmental delay, although less than at 20°C. These results suggest that the Erk2ts protein may not have full activity at physiologically relevant temperatures for this organism (data not shown). When cell-type-specific gene expression was examined in Erk2*/*erk2 cells plated for development at 19°C, both ecma and SP60 were expressed, although SP60 was expressed at a slightly reduced level and with kinetics that were ~3 hr delayed compared to those in wild-type cells (Fig. 5). When the expression vector carrying the wild-type ERK2 was transformed into erk2 null cells or wild-type cells, the cells developed normally and produced wild-type fruiting bodies at the temperatures tested (18°C–27°C). Transformation of this vector into wild-type or erk2 null cells resulted in a several-fold increase in the level of ERK2 protein as determined by Western blot analysis (data not shown). The level of overexpression of ERK2 in wild-type cells observed did not cause an observable phenotype.

We have shown that ERK2 activity is stimulated in response to cAMP using in-gel assays in which the protein is denatured in SDS and then renatured using guanidine hydrochloride (M. Maeda, L. Aubry, R. Insall, C. Gastins, P. Devreotes, and R.A. Firtel, in prep.). When Erk2tS/erk2 cells were similarly assayed under standard conditions (assay performed at 20°C; Gaskins et al. 1994), no ERK2 activity was observed (data not shown), indicating that the protein is very labile and may be unable to refold properly after severe denaturation.

To determine whether ERK2 function is required for later development, Erk2*/*erk2 cells were plated for development at 20°C until they formed loose aggregates and were then shifted to 27°C. As is seen in Figure 4A, the development became very abnormal, indicating a specific role for ERK2 in proper morphogenesis. We assayed for the presence of mature heat- and detergent-stable spores and none were found (<0.1% of wild-type). Wild-type cells showed completely normal development at 27°C, forming fruiting bodies that were indistinguishable from those of wild-type cells and having the same number of mature spores in 24–26 hr (data not shown).

To determine whether ERK2 was required at specific stages in development, we plated cells on 12 mM Na/KPO4 [pH 6.2] agar plates and allowed them to develop for varying times at 19°C. The plates were then shifted to 27°C and either left at 27°C or returned to 19°C after 5 hr. When cells were shifted from 19°C to 27°C at times between early aggregate formation (9 hr) and tipped aggregate (13 hr) for 5 hr and then back to 19°C, cells formed normal fruiting bodies with spores, indicating that the

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**Table 1. The domain containing the conserved proline residue in S. pombe cdc2, Drosophila MEK, and Dictyostelium ERK2**

<table>
<thead>
<tr>
<th>Temperature sensitive mutations</th>
<th>cdc2</th>
<th>Dm-MEK</th>
<th>Dd-ERK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts s</td>
<td>HRDLKFDLGD</td>
<td>HRDLKFSNLVVN</td>
<td>HRDLKFSNLV1N</td>
</tr>
</tbody>
</table>

See text for details and references.
MAP kinase function in *Dictyostelium*

**Figure 4.** Phenotypes of ERK2<sup>ts</sup> mutant. **(Left)** Individual panels are labeled appropriately. In the top three panels, wild-type or erk2 null cells were allowed to develop at either 20°C or 27°C. In the bottom panels, erk2 null cells were first allowed to develop at 20°C. **(Bottom left panel)** cells form mounds at this stage; **(bottom right panels)** cells were shifted from 20°C to 27°C at the time of loose aggregate formation and allowed to develop. Various morphological phenotypes were observed. Two representative phenotypes are shown. **(Right)** Developmental phenotypes of cells shifted from permissive to restrictive to permissive temperatures. Cells were plated on Na/KPO<sub>4</sub> agar plates and allowed to develop at either 19°C or 22°C to the times indicated. Cells were then shifted to 27°C and either left there for the indicated times or shifted back to the starting temperature (19°C or 22°C) after 5 hr. Photographs show representative phenotypes. **(A)** cells developed at 19°C for 17 hr; **(B)** cells developed at 19°C for 17 hr and transferred to 27°C for 5 hr; **(C)** cells developed at 19°C for 17 hr and transferred to 27°C for 5 hr and then shifted to 19°C (photograph taken after an additional 12 hr); **(D)** cells developed at 19°C for 17 hr and transferred to 27°C (photograph taken after an additional 17 hr); **(E)** cells developed at 22°C for 17 hr and transferred to 27°C for 5 hr; **(F)** cells developed at 22°C for 17 hr and transferred to 27°C; **(G)** cells developed at 22°C for 17 hr and transferred to 27°C for 5 hr; **(H)** cells developed at 19°C for 21 hr; **(I)** cells developed at 19°C for 21 hr and transferred to 27°C for 5 hr; **(J)** cells developed at 19°C for 21 hr and transferred to 27°C; **(K)** cells developed at 19°C for 9 hr and transferred to 27°C for 5 hr and then shifted to 19°C (photograph taken after an additional 17 hr).

cells could recover completely for proper morphogenesis and spore formation (K in Fig. 4, right; data for 13 hr not shown). When cells were shifted from 19°C to 27°C at the first finger (17 hr; A in Fig. 4, right) or second finger stage (21 hr; H in Fig. 4, right), morphogenesis was slightly abnormal after 5 hr [Band I, respectively, in Fig. 4, right]. If the cells remained at 27°C very aberrant fruiting body-like structures were formed. If the 17- or 21-hr cells were then shifted back to 19°C after 5 hr at 27°C, they formed almost normal fruiting bodies with mature spores, showing only some abnormal stalk morphogenesis [C in Fig. 4, right; data for 21 hr not shown]. If the cells are first developed at 22°C rather than 19°C before shifting to 27°C, morphogenesis is significantly more abnormal either after 5 hr at the elevated temperature [e.g., Fig. 4, right, B–G vs. B–H], after extended incubation at 27°C [Fig. 4, right, B–E vs. B–D], or after being shifted back to 19° or 22°C after 5 hr [Fig. 4, right, B–F vs. B–C]. This suggests that the presence of some inactive ERK2 or having ERK2 with a lower activity is sufficient to further impair development, and suggests a continual requirement of ERK2 activity for proper morphogenesis.

**ERK2 is required for prespore but not prestalk cell-type differentiation**

The temperature-sensitive mutation allowed us to deter-
were taken at various time points, RNA isolated, size-fraction-hybridization. For wild-type cells, mature fruiting bodies were formed at 26-28 hr in these experiments.

and wild-type cells, Figure genes in the KAx-3) were plated for development on filters at 20°C. Filters indicated the relative level of expression of ERK2ts/erk2.

Comparison.

Wild-type RNA were loaded on the same gel (21WT) for comparison.

showed mature fruiting bodies at -30-32 hr. To obtain a better indication of the relative level of expression of ermA and SP60 genes in the erk2 null cells expressing ERK2ts, 21-hr samples of wild-type RNA were loaded on the same gel (21WT) for comparison.

mine whether ERK2 was required at the time of cell-type-specific gene expression or for some early developmental process that was subsequently required for cell-type-specific gene expression. Because the induction of cell-type-specific gene expression is controlled by extracellular cAMP interacting with cell surface receptors and because erk2 null cells lack cAMP-mediated activation of adenylyl cyclase, it was necessary to perform these studies in suspension, which allowed us to add exogenous cAMP. Erk2ts/erk2 and wild-type cells were allowed to develop to the loose aggregate stage at 19°C on Na/KPO4 agar plates, dissociated into single cells, and then stimulated with cAMP at either 19°C or 27°C under slow-shake suspension conditions. As shown in Figure 6, neither strain showed detectable expression of the prespore (SP60) or the prestalk (ermA) gene at the time of aggregate dissociation. In the presence of exogenous cAMP, Erk2ts/erk2 cells induced ermA to a level similar at 19°C or 27°C to that in wild-type cells. At the nonpermissive temperature, ermA expression was completely dependent on exogenous cAMP at the restrictive temperature and no expression was observed in the samples lacking cAMP. The requirement for exogenous cAMP in the Erk2ts/erk2 strain at the nonpermissive temperature is consistent with the requirement of ERK2 for the activation of adenylyl cyclase. Thus, although the cells formed agglomerates in suspension conditions that allowed wild-type cells to produce cAMP endogenously, the ERK2ts protein was not active at the nonpermissive temperature and thus adenylyl cyclase was not activated. In contrast, SP60 expression was induced in Erk2ts/erk2 cells at 19°C but not at 27°C, whereas it was induced at both temperatures in wild-type cells. The level of expression at the permissive conditions was lower than that of wild-type cells under the same conditions. These results indicate ERK2 is required for the induction of prespore gene expression and, in effect, distinguishes the effect of ERK2 on adenylyl cyclase and prespore gene expression, suggesting that the temperature sensitivity of cell-type-specific gene expression is restricted to the prespore pathway.

To determine whether ERK2 activity was required for continued expression of prespore genes, Erk2ts/erk2 cells were developed on agar until the mound stage at 19°C, when SP60 was induced, and then shifted to 27°C. Time points were taken and SP60 and ermA RNA levels were assayed (Fig. 7). At this stage, SP60 transcripts were present at levels approaching those of wild-type cells at early culmination (18 hr), whereas the level of ermA transcripts was still increasing. After being shifted to the restrictive temperature, SP60 RNA remained high at the 4-hr time point and then fell rapidly. In contrast, ermA transcripts continued to increase for at least 8 hr before decreasing. This subsequent decrease may be attributable to an inhibition in the ability to activate adenylyl cyclase and produce cAMP. The results showed that ERK2 activity is required continuously for SP60 expression. The difference in the kinetics of decline of ermA and SP60 suggested that ERK2 activity is required continually for SP60 expression. When wild-type cells were shifted from 19°C to 27°C at the aggregate stage, prior to

Figure 5. Regulation of cell-type-specific genes in Erk2ts/erk2 and wild-type cells. erk2 null cells expressing the ERK2ts cells (top two panels, Erk2ts/erk2) or wild-type cells (bottom panels, KAx-3) were plated for development on filters at 20°C. Filters were taken at various time points, RNA isolated, size-fractionated, blotted, and probed for expression of ermA or SP60 by blot hybridization. For wild-type cells, mature fruiting bodies were formed at 26-28 hr in these experiments. Erk2ts/erk2 cells showed mature fruiting bodies at -30-32 hr. To obtain a better indication of the relative level of expression of ermA and SP60 genes in the erk2 null cells expressing ERK2ts, 21-hr samples of wild-type RNA were loaded on the same gel (21WT) for comparison.

Figure 6. Regulation of cell-type-specific gene expression in Erk2ts/erk2 cells under restrictive and permissive conditions. Erk2ts/erk2 cells and wild-type cells were allowed to differentiate at 19°C on agar containing 12 mM Na/K phosphate (pH 6.2). Cells were harvested at the loose mound stage for each strain. The cells were dissociated and split into four aliquots, which were assayed under slow-shake conditions as described in the legend to Fig. 2. Two aliquots each were assayed at 19°C and 27°C. Exogenous cAMP was added to one of these aliquots. RNA was isolated at 6 hr after cells had been placed in suspension.
induction of ecmA and SP60, both genes were induced normally and continued to be expressed (Fig. 7). The level of ecmA was higher than that of cells developed at 20°C, whereas SP60 was similar, indicating that induction and maintenance of cell-type-specific gene expression was not negatively affected at 27°C.

Discussion

MAP kinases have been shown to control multiple regulatory functions mediated through signaling cascades as outlined above. Previously, we showed that erk2 null cells could not effectively activate adenylyl cyclase and thus were unable to produce and relay the cAMP signal [Segall et al. 1995]. However, erk2 null cells activate guanylyl cyclase, chemotax to cAMP, and form coaggregates with wild-type cells, although we estimate that only two-thirds of the erk2 null cells participate, suggesting that chemotaxis is not fully type B. This paper examined the functions of the MAP kinase ERK2 in controlling Dictyostelium development and showed that ERK2 has a specific cell-autonomous function for cell-type differentiation. Using a temperature-sensitive mutant, we show that ERK2 is required at multiple stages for proper Dictyostelium development, including morphogenesis and cell-type-specific gene expression. Furthermore, ERK2 was not essential for the regulation of pulse-induced gene expression during aggregation, suggesting that ERK2 does not function by directly regulating transcription during this stage of development. Other strains that are also unable to activate adenylyl cyclase, including strains in which the gene encoding adenylyl cyclase ACA, CRAC (a regulator of adenylyl cyclase), or the catalytic subunit of PKA has been disrupted, are aggregation deficient. These strains are unable to produce and relay cAMP and, like erk2 null cells, can chemotax to cAMP, coaggregate with wild-type cells, and express pulse-induced genes in response to cAMP [Mann and Firtel 1991, Pitt et al. 1993; Insall et al. 1994a,b]. aca null cells and cells lacking the Ga subunit Ga2, which is required for the activation of adenylyl and guanylyl cyclases through cAMP receptors (ga2 null cells), induce prestalk- and prespore-specific genes in response to cAMP and form aberrant structures that contain stalk cells and spores after cAMP treatment and plating on non-nutrient agar [Pitt et al. 1993; Schnitzler et al. 1995].

These results suggest that the ability to activate adenylyl cyclase and produce endogenous cAMP is not essential for cell-type differentiation or the expression of prestalk and prespore genes under these conditions [Kumagai et al. 1989, 1991; Pitt et al. 1993; Firtel 1995; Schnitzler et al. 1995]. By using chimeric organisms and suspension cultures, we bypassed the inability of erk2 null cells to activate adenylyl cyclase to examine its possible function in controlling gene expression and cell-type differentiation. We showed that the ability of erk2 null cells to express cell-type-specific genes was very different from that of aca null cells. We therefore conclude that inability of erk2 null cells to express prespore genes is probably unrelated to the inability of the erk2 null cells to activate adenylyl cyclase. In addition, we showed that ERK2 activity is required for the continued expression of the prespore gene SP60 within developing aggregates.

We demonstrated that the postaggregative genes (including GFB, CP2, and rasD) and the prestalk-specific gene ecmA, but not the prespore gene SP60, were expressed in erk2 null cells in suspension culture in response to cAMP or when the erk2 null cells formed a coaggregate with wild-type cells. The level of expression of some of these genes was reduced compared to wild-type cells in suspension cultures in the absence of cell-cell contact, but in slow-shake culture in which cell agglomerates were formed, the level of expression of these genes was similar to that seen in wild-type cells. A reduced level of expression was also seen for rasD and CP2 in strains in which LagC, a postaggregative gene whose expression is required for cell-type-specific gene expression, is deleted. It is possible that ERK2 may also have a broad role in modulating postaggregative gene expression but is not absolutely essential, as it is for prespore gene expression. The reduced level of expression of the postaggregative genes in fast-shake conditions (no sustained cell-cell contact) is also seen in cells lacking the putative cell surface protein LagC and may be attributable to either a pleiotropic effect of the ERK2 deficiency or the fact that ERK2 is not essential for induction of postaggregative gene expression but is required for full expression.

Examination of the localization of the ecmA-expressing erk2 null cells in chimeras and the level of induction of ecmA in erk2 null cells in suspension culture showed that ERK2 also plays a role in prestalk gene expression and cell patterning. The erk2 null cells expressing ecmA are localized in a domain expected for prestalk O cells as
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well as being found in the posterior prespore domain in an ALC pattern. The absence of erk2 null/ecmA-expressing cells within the prestalk A domain in the finger may be a function of the inability to induce the prestalk A component of the ecmA promoter at this stage or the exclusion of erk2 null cells from the anterior by wild-type cells at this stage. The very anterior cells are thought to function as the cAMP oscillator and organizer (for review, see Schaap 1991; Firtel 1995). The inability of erk2 null cells to activate adenylyl cyclase could thus exclude them from this central organizing function and restrict them to a more posterior domain in the slug. The ecmBΔ89-expressing cells in the very anterior of developing culminants [Fig. 3E,F] may be attributable to the localization of anterior-like cells to this region during culmination. Interestingly, erk2 null cells did express the stalk-specific promoter ecmBΔ89, although it is expected that these cells cannot produce endogenous cAMP.

In contrast, no prespore-specific gene expression was observed under any condition. The use of chimeric organisms comprised of various ratios of wild-type and mutant cells allows one to determine whether a mutant is cell nonautonomous. Under such conditions, wild-type cells would supply any required cell-surface or extracellular signals that are lacking in the mutant cells. The inability of erk2 null cells to express the prespore-specific reporter SP60/lacZ in the chimera or the endogenous SP60 gene in suspension assays suggests that the requirement of ERK2 function is cell autonomous. This is consistent with the cell suspension assays showing that Erk2+/erk2 can induce gene SP60 under the permissive but not the restrictive conditions after cells are first allowed to form aggregates. Our results with the suspension assays using Erk2+/erk2 also distinguished between the ERK2 requirement for the activation of adenylyl cyclase and the induction of prespore gene expression. ecmA gene expression required exogenous cAMP to be induced under nonpermissive conditions, in which the ERK2 protein was presumably inactive and thus adenylyl cyclase was not activated, whereas SP60 was not induced with or without exogenous cAMP. We presume that ERK2 is present in all cells, at least initially, because during aggregation it is required in all cells for cAMP-mediated activation of adenylyl cyclase and it is expressed during vegetative growth. Our data therefore suggest that ERK2 may be required for the phosphorylation of a protein necessary for the induction of prespore genes but not for the expression of the prestalk marker ecmA in at least some prestalk cells.

The morphological abnormalities observed when Erk2+/erk2 cells were shifted to the restrictive temperature after mounds formed are very distinct from those observed for other strains in which the cAMP signaling pathways are affected. aca null cells, in which the gene encoding adenylyl cyclase ACA is disrupted, and ga2 null cells, in which the gene encoding the Gα subunit known to be required for cAMP stimulation of adenylyl cyclase in vivo is disrupted, form small, wild-type-looking fruiting bodies if the cells are first given extracellular cAMP [Pitt et al. 1993; Schnitzler et al. 1995]. Moreover, it has been shown for both mutants that extracellular cAMP can stimulate cell-type-specific gene expression and, in the case of aca null cells, the cAMP analog 2′,3′cAMP, which binds very weakly to the PKA-R subunit and thus cannot activate PKA, can also effectively induce development and cell-type-specific gene expression [Pitt et al. 1993; Schnitzler et al. 1995]. This analysis suggests that the abnormal morphogenesis observed for Erk2+/erk2 cells when shifted to the restrictive temperature may very well be distinct from the ability to efficiently activate adenylyl cyclase. This argues that the mechanism by which ERK2 regulates late gene expression and morphogenesis is, at least in part, distinct from its function in controlling aggregation. Using temperature shift experiments, we show that Erk2+/erk2 null cells can recover from a 5-hr shift to the nonpermissive temperature to form normal or almost normal fruiting bodies, depending on the time of the temperature shift. However, when a higher permissive temperature (22°C) was used, the shift to 27°C for 5 hr resulted in more severe phenotypes. Our results suggest that ERK2 may play a continual role in mediating morphogenesis throughout the multicellular stages. The known plasticity of Dictyostelium development may explain why cells shifted from and to the lower temperature (19°C) show normal development.

Components of a MAP kinase cascade functioning in two independent regulatory pathways have been seen previously in Saccharomyces cerevisiae (Roberts and Fink 1994). In this case, the MEK, MEK kinase, and an upstream kinase (Ste20) are required for both mating and pseudohyphal growth, although the MAP kinases involved in the two pathways appear to be distinct. In Dictyostelium, we do not know whether the upstream regulators of the aggregation-stage pathways and those that regulate ERK2 activity in later development are the same. We have shown that cAMP will stimulate ERK2 activation in cells isolated from migrating slugs [Maeda, M., and R.A. Firtel, unpubl.], however, it is possible that the pathway regulating ERK2 activity for the prespore pathway is distinct. We know that ERK2 activity is rapidly stimulated and the activity rapidly adapts upon cAMP stimulation during the aggregation stages [M. Maeda, L. Aubry, R. Insall, C. Gaskins, P. Devreotes, and R. Firtel, in prep.]. Prespore gene expression, however, is stimulated by a continuous cAMP flux, a condition that causes only a transient stimulation of the ERK2 during early development. This suggests that distinct pathways may control ERK2 activation in response to cAMP and possibly other stimuli that lead to cell-type differentiation and morphogenesis. Future studies should identify upstream components and determine whether ERK2 functions to directly modify prespore- and/or prestalk-specific transcription factors or functions farther upstream in the regulatory network.

Materials and methods

Strains

erk2 null cells were obtained by retransforming the recovered...
plasmid from the initial REMI mutation [Kuspa and Loomis 1992] into DH1, a uracil auxotroph, selecting for uracil prototrophs, and screening for aggregation-deficient clones. The disruption of the ERK2 locus was determined by Southern blot analysis. This disrupts the ERK2 open reading frame (ORF) near the carboxyl terminus, and the strain does not produce ERK2 mRNA or ERK2 protein [data not shown]. This erk2 null strain has properties identical to the original REMI mutation [Kuspa and Loomis 1992; Segall et al. 1995]. Both this strain and the original erk2 null strain were used for the experiments. In addition, we created another erk2 null strain in which the gene was disrupted at the BglII site in the catalytic domain. These cells also have the same properties. Strain KAx-3, the parental of DH1, or DH1 cells transformed with a Pyr5-6 uracil vector were used for wild-type cells.

Molecular biology, vectors, and cell culture
All experiments were performed using standard molecular biological and cell culture techniques for Dictyostelium and have been described previously [Dynes et al. 1994].

The temperature-sensitive mutant was made by site-directed mutagenesis with an oligonucleotide containing the proline to serine amino acid substitution using single-stranded template DNA and the Bio-Rad Muta-Gene phagemid kit. The resultant plasmids were sequenced to identify one carrying the substitutive of pATANB43, a Ddpl-based extrachromosomal plasmid [Dynes et al. 1989]. When transformed into erk2 null cells, the erk2 null phenotypes are complemented and cells develop normally. The ERK2-null mutation was expressed in the same vector by replacing the wild-type ERK2 gene with the ERK2-null gene.

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References


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Karim, M. 1995. The regulation of AP-1 activity by mitogen-
Molecular genetic analysis of two Ga protein subunits in
Karin, M. 1995. The regulation of AP-1 activity by mitogen-
Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integra-
Loomis, W. F., [ed.] 1982. Development of Dictyostelium dis-
Mann, S.K. and R.A. Firtel. 1989. Two-phase regulatory path-
1928.
early gene expression in Dictyostelium discoideum Media-
7: 458–469.
threonine protein kinase is essential for development in Dictyos-
Marshall, C.J. 1985. Specificity of receptor tyrosine kinase sig-
naling: Transient versus sustained extracellular signal-regu-
AMP jointly regulate cell-type-specific gene expression in
plete sequence and transcript regulation of a cell adhesion
protein from aggregating Dictyostelium cells. EMBO J.
5: 1473–1476.
Pereira da Silva, L.H., M. Darmon, P. Brachet, C. Klein, and P.
Barrand. 1975. Induction of cell differentiation by the che-
motactic signal in Dictyostelium discoideum. FEBS Proc.
Pitt, G.S., R. Brandt, K.C. Lin, P.N. Devreotes, and P. Schaap.
1993. Extracellular cAMP is sufficient to restore develop-
mental gene expression and morphogenesis in Dictyos-
telium cells lacking the aggregation adenylyl cyclase (ACA).
Genes & Dev. 7: 2172–2180.
Reymond, C.D., R.H. Gomer, M.C. Mehdy, and R.A. Firtel.
1984. Developmental regulation of a Dictyostelium gene en-
coding a protein homologous to mammalian ras protein.
kinase cascade in Saccharomyces cerevisiae mediate two de-
velopmental programs in the same cell type: Mating and
invasive growth. Genes & Dev. 8: 2974–2985.
Schaap, P. 1991. Intercellular interactions during Dictyostelium
development. In Microbial cell-cell interactions [ed. M. Dworkin], pp. 147–178. American Society for Microbiology,
Washington, D.C.
Schnitzler, G., W. Fischer, and R. Firtel. 1994. Cloning and char-
acterization of the G-box binding factor, an essential com-
ponent of the developmental switch between early and late
1995. Serpentine cAMP receptors may act through a G-protein-
independent pathway to induce post-aggregative develop-

Segall, J., A. Kuspa, G. Shaulsky, M. Ecke, M. Maeda, C.
Gaskins, R. Firtel, and W. Loomis. 1995. A MAP kinase nec-
essary for receptor-mediated activation of adenylyl cyclase
Extracellular cAMP can restore development in Dictyostel-
ium cells lacking one, but not two subtypes of early cAMP
receptors [cARs]: Evidence for involvement of cAR1 in ag-
pathways in Dictyostelium. In Advances in second messen-
ger and phosphoprotein research [ed. P. Greengard and G.A.
Williams, J., N. Hopper, A. Early, D. Traynor, A. Harwood, T.
Abe, M. Simon, and M. Veron. 1993a. Interacting signalling
pathways regulating prestalk cell differentiation and move-
ment during the morphogenesis of Dictyostelium. Develop-
ment [Suppl.]: 1–7.
Williams, J.G., A. Ceccarelli, S. McRobbie, H. Mahhubani, R.R.
Kay, A. Early, M. Berks, and K.A. Jermyn. 1987. Direct in-
duction of Dictyostelium prestalk gene expression by D1F
provides evidence that D1F is a morphogen. Cell 49: 185–
192.
Williams, J.G., K.T. Duffy, D.P. Lane, S.J. McRobbie, A.J. Har-
the prestalk-prespore pattern in Dictyostelium develop-
Williams, J.G., A.J. Harwood, N.A. Hopper, M.N. Simon, S.
Bouzid, and M. Veron. 1993b. Regulation of Dictyostelium
morphogenesis by CAMP-dependent protein kinase. Philos.

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The Dictyostelium MAP kinase ERK2 regulates multiple, independent developmental pathways.

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*Genes Dev.* 1996, 10:
Access the most recent version at doi:10.1101/gad.10.1.118

### References
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