**A MAP Kinase Necessary for Receptor-mediated Activation of Adenylyl Cyclase in *Dictyostelium***

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**Abstract.** Analysis of a developmental mutant in *Dictyostelium discoideum* which is unable to initiate morphogenesis has shown that a protein kinase of the MAP kinase/ERK family affects relay of the cAMP chemotactic signal and cell differentiation. Strains in which the locus encoding ERK2 is disrupted respond to a pulse of cAMP by synthesizing cGMP normally but show little synthesis of cAMP. Since mutant cells lacking ERK2 contain normal levels of both the cytosolic regulator of adenylyl cyclase (CRAC) and manganese-activatable adenylyl cyclase, it appears that this kinase is important for receptor-mediated activation of adenylyl cyclase.

*Dictyostelium discoideum* amoebae grow and divide as individual cells feeding upon bacteria or defined axenic media. Upon removal of the food source, a developmental process is initiated that culminates in the differentiation of cells into spore and stalk cells (12, 31, 45, 46). Starvation induces the expression of a number of proteins involved in the synthesis and sensing of cAMP. An adenylyl cyclase (53) synthesizes cAMP which is then secreted. Extracellular cAMP can bind to G protein-coupled receptors on the cell surface (25). The CAMP receptor CAR1 (64) and the G protein α subunit Gβγ (37) are necessary for initial responses to extracellular cAMP. They mediate chemotactic responses to cAMP and stimulate adenylyl cyclase, resulting in the synthesis and secretion of cAMP in response to the binding of extracellular cAMP to CAR1. The result is a positive feedback loop that generates waves of cAMP moving outward from aggregation centers, and cell movement towards aggregation centers. After the formation of a mound, an apical tip develops that elongates to a first finger that falls onto the substratum to form a migrating slug or pseudoplasmodium. Within the slug, ammonia, DIF, and cAMP are important signaling molecules that coordinate cell movement and cell-type differentiation (2, 9). Under appropriate conditions the slugs subsequently culminate to form fruiting bodies in which 80% of the cells differentiate into spores.

It has been demonstrated in a number of different systems that MAP kinases/ERKs are activated by extracellular stimuli (1, 3, 15, 56, 66). ERKs can be activated either by G protein-coupled receptors or tyrosine kinase receptors. For tyrosine kinase receptors, activation of ras is followed by activation of a kinase cascade involving raf, MEK, and then ERKs. For G protein-coupled receptors, α or βγ subunits can be important for activation, but the role of ras may vary with cell type (10, 16). A MEK kinase, distinct from raf, can activate MEK in responses activated by G proteins in mammalian cells (41). In *Saccharomyces cerevisiae*, the mating pheromone activates a kinase cascade involving a MEK kinase, MEK, and MAP kinase that leads to transcriptional activation of genes involved in mating and cell cycle arrest (15). Substrates for ERKs can include other kinases (including tyrosine kinase receptors), cytoskeletal elements, transcription factors, components of the cell cycle machinery, and other signal transduction enzymes (such as PLA2) (7, 30, 44, 49, 52, 58, 65). Such a wide range of substrates suggests that ERKs play a critical role in many signal transduction systems. In *Dictyostelium*, the MAP kinase ERK1 has been shown to be essential for growth and to play a role in multicellular development (19). Overexpression of ERK1 results in abnormal slug morphogenesis and fruiting body formation.

In this paper we identify an ERK in *Dictyostelium* which is important for receptor-mediated stimulation of adenylyl cyclase and subsequent cellular differentiation. Although ERKs have been shown to be stimulated in response to receptor activation in a variety of cell types, the data presented here present the first suggestion that they can play a role in the activation of adenylyl cyclase, and that ERKs are necessary for the normal developmental cycle of *Dictyostelium*.

**Materials and Methods**

**Generation of Gene Disruptions**

The plasmid DIV6 was constructed by inserting the PstI/SacI fragment from DIVI containing the pyr5-6 gene into pGEM5zf(+) REMI with DIV6 was
performed by digesting DIV6 with BamHI and electroporating into HL330 with 100 U of Sau3A enzyme per electroporation (38).

A second gene-disruption construct was made by inserting the full-length Thy1 gene (14) into the BglII site within an ERK2 cDNA clone. The DNA was purified and transformed into the Thy1 auxotrophic strain H110 (13, 22) and selected in standard HL5 medium in the absence of exogenous thymidine. Individual clones were isolated and screened for gene disruption by Southern blot. To complement the strain, the ERK2 cDNA was inserted into the expression vector Exp4(+) (47) downstream from the Actin 15 promoter. Mutants were transformed with the expression vector via electroporation and selecting for resistance to 10 μg/ml of G418.

**Sequencing**

Sequencing was performed using a Applied Biosystems sequencer and specific primers. The cDNA was isolated from a lambdaZAP library (59) generated from the RNA of cells starved for 12-16 h. The cDNA was sequenced in both directions, and the genomic flanking sequence was sequenced in one direction to confirm the cDNA sequence. Sequences were obtained and analyzed using the GCG analysis package (Genetics Computer Group, Madison, WI).

**Bacterial Expression of the ERK2 Protein**

A GST-ERK2 fusion protein in vector pGEX-KG (21) was made as described for the Dictyostelium ERK1 protein (19). Escherichia coli BL21 (DE3) was used for transformation with pGEX and pGEX-KG-ERK2. These transformants were cultured for 4-6 h at 30°C or overnight at 20°C after the addition of IPTG at 0.1 mM. Glutathione-S-transferase (GST) and GST-ERK2 fusion protein were prepared as previously described (62). Phosphorylation was performed for 10 min at 30°C in 20 μl of kinase mixture (50 μM Na3VO4, 10 mM MgCl2, 1 mM EGTA, 0.4 μg/ml leupeptin, 40 μM benzamidine, 0.4 μM microcystin LR, 25 mM β-glycerophosphate, pH 7.5, 100 μM ATP, 0.05 μCi/ml [32p]-3'-ATP, and 200 μM DTT) with or without myelin basic protein (MBP) at 0.5 mg/ml. GST or GST-ERK2 was used at 0.4 mg/ml. The reaction was stopped by adding 7 μl of 4X sample buffer for SDS-PAGE. The samples were fractionated on two 10% acrylamide gels. One gel was stained with Coomassie brilliant blue, while the other was used for electrotransfer of proteins to Immobilon-P membrane according to Harlow and Lane (24). The phosphorylated bands were then excised out, and hydrolyzed for 2 h at 110°C (33). After drying in vacuo, the hydrolysates were subjected to thin layer electrophoresis at pH 3.5 for 45 min (28). 1.5 mg protein was combined with a mixture of phosphoserine, phosphothreonine, and phosphotyrosine and used for the analysis. Phosphoamino acids were identified with ninhydrin.

**Measurement of Adenylyl Cyclase and Cytosolic Regulator of Adenylyl Cyclase**

Adenylyl cyclase activation was measured as described (53). Briefly, control and mutant cells were starved for 4 h in suspension with stimulation by 100 nM cAMP pulses. They were then lysed by passage through a 5-μm filter, and the total adenylyl cyclase activity of the lysate assayed using α-[32P]-ATP in the presence of 5 mM MnSO4. Cytosolic regulator of adenylyl cyclase (CRAC) activity was determined as the ability to reconstitute GTPγS-stimulation of adenylyl cyclase in lysates of synag 7 mutant cells. Synag 7 cells lack only endogenous CRAC activity and upon the addition of extracts from cells containing CRAC will activate adenylyl cyclase (43). Synag 7 cell lysates were prepared in the presence of cAMP and GTPγS, mixed with supernatants from control and mutant lysates, and then adenylyl cyclase activity measured in the absence of MnSO4.

**In Vivo Labeling of Cells**

Cells were labeled following a procedure developed by David Knecht (Univ. of Connecticut, Storrs, CT). Cells were grown in HL-5 and washed two times in phosphate buffer. They were resuspended in 1 mM CMFDA (C-2925; Molecular Probes) in phosphate buffer at 107/ml. The cells were gently shaken in this buffer for 15 min, then washed and resuspended in HL-5 for 1 h at 2 × 106/ml. They were then washed free of HL-5 and resuspended at 107/ml in phosphate buffer. Labeled cells were mixed with unlabeled cells in a 1:10 ratio. 100 ul was spread on a 1% agar plate containing 1 mM CaCl2, 1 mM MgCl2, in phosphate buffer and allowed to dry. The plate was then sealed and incubated in the dark at 23°C until analyzed. Labeling had no effect on the kinetics or morphology of development. The images were acquired using a BioRad MRC 600 confocal microscope using a 20 or 40x objective. A Z-series through the entire structure was acquired and then projected into a single plane for presentation.

**Figure 1.** Aggregation morphology of transformants growing on bacterial lawns. The edge of the colony is on the right hand side. For HS176, the positive control, cells to the left of the edge of the colony begin to starve due to the depletion of the bacterial food source. They form streams leading into aggregates and eventually fruiting bodies on the left side of the image. HS172 and HS173 are two transformants showing no aggregation.
Figure 2. Southern blot of recapitulation of IS240 insertions. Genomic DNA was cut with ClaI, blotted and probed with the recapitulation vector containing both the flanking sequences and selectable marker. HL330 (the parental strain) shows a single 3.8-kb band. The original mutant, AK240, and four aggregation-defective transformants show a shift to around 9-kb, indicating recapitulation of the insertion at the same site. HS176, the control transformant, shows the 3.8-kb band intact and the vector inserted into a high molecular weight band.

Results

Isolation of erkB Mutants

To identify genes encoding novel components essential for aggregation, we isolated a series of mutants that are defective in aggregation using restriction enzyme mediated integration (REMI) (38). This technique uses restriction enzymes to facilitate integration of a selectable vector into randomly distributed cognate restriction sites. Strain HL330 lacks the pyr5-6 gene (encoding the UMP synthase) and is therefore unable to grow in the absence of uracil (32). The plasmid DIV 6 (which contains the pyr5-6 gene) was linearized with BamHI-II and electroporated into HL330 cells together with the restriction enzyme Sau3A. Sau3A generates ends compatible with the ends produced by BamHI, and cuts frequently in the Dictyostelium genome. Transformants containing the DIV6 plasmid were selected in medium lacking uracil, and screened for strains that were defective in aggregation.

For strain AK240, colonies grown on bacterial lawns did not aggregate (Fig. 1). To rescue the DIV6 plasmid together with flanking sequences marking the insertion site (termed IS240), genomic DNA from AK240 was cut with ClaI (which does not cut in the DIV6 vector), religated, and used to transform E. coli. The resulting plasmid, p240ClaI, contains a 3.8-kb insert of flanking sequence, divided into 2.1 and 1.7-kb fragments by the insertion of DIV6. This plasmid was then used to recapitulate the insertion event in a new strain via homologous recombination, by linearizing the plasmid with ClaI and transforming in the absence of restriction enzymes. Roughly 90% of the transformants recovered by this procedure were defective in aggregation. Four independent aggregation-defective transformants, labeled HS172, HS173, HS174, and HS175, were chosen for further study along with a control strain, HS176, that aggregates normally (Fig. 1). A Southern blot of the transformants (Fig. 2), probed with the disruption vector p240ClaI, revealed that the 3.8-kb ClaI fragment in HL330 was shifted to around 9 kb in AK240 and the recapitulated insertions, HS172-175. The transformed control, HS176, had the 3.8-kb band intact, and the vector had inserted into a high molecular mass band. A vector-specific probe confirmed that the vector (which does not hybridize to HL330 genomic DNA), labeled the 9-kb band in HS172-175 and the high molecular mass band in HS176 (data not shown). Thus recapitulation of the insertion event resulted in recapitulation of the mutant phenotype, indicating that disruption of the encoded flanking sequences resulted in the mutant phenotype.

Structure of the erkB Gene

Sequencing the 2.1-kb flanking sequence of IS240 revealed an open reading frame. This fragment was used to identify a cDNA clone with sequence identical to the genornic open reading frame. A single intron interrupts the coding sequence in genomic DNA. When the 2.1-kb region was used to probe genomic Southern blots of HL330 cut with various restriction enzymes, only a single locus was recognized (Fig. 3 A). An arrayed set of YAC clones that represents the Dictyostelium MAP Kinase 407
tyrostelium genome (39) was also probed and a single locus was identified that was mapped to chromosome 4 (Fig. 3B). The sequence has been deposited in GenBank (accession number L33043).

The deduced amino acid sequence predicts a protein product of molecular weight 42,010 and pl 7.1. The sequence shows about 40% identity to MAP kinases/ERKs from diverse organisms (Fig. 4), and the encoded protein has been named ERK2 (the corresponding genetic locus is erkB). Similar homology is seen to another ERK gene from D. discoideum, ERK1. ERK1 shows a different pattern of expression and cells lacking ERK1 are not viable (19), indicating that the D. discoideum ERK1 and ERK2 proteins perform different functions. In addition, the D. discoideum ERK2 amino acid sequence shows ~40% identity to mammalian ERK1 and ERK2 while Dictyostelium ERK1 shows ~50% identity to those ERKs. The predicted ERK2 protein contains residues conserved in all kinases including the ATP-binding site in region I (Gly-X-Gly-X-X-Gly) and eight invariant residues present in protein kinases (23). In addition, there is the sequence TEY (starred), of which the threonine and tyrosine are phosphorylated when these protein kinases are activated (48, 54, 69).

**ERK2 Protein Kinase Activity**

To determine whether ERK2 has the biochemical properties...
Phosphorylates MBP predominately on threonine residues while the ERK2 cDNA showed that a 1.8-kb mRNA was present. In expression of erkB, phosphorylation is predominately on tyrosine and in amino acid analysis shows that the fusion protein phosphoamino acid analysis. Phosphorylated bands were excised from the GST-ERK2 fusion protein, but not purified GST protein. Phosphorylated bands present in the GST-ERK2 lanes are not always seen and may be breakdown products of GST-ERK2. After fractionation of phosphorylated proteins by SDS-PAGE, proteins were blotted onto a PVDF membrane and then exposed to x-ray film. Phosphorylated bands were excised from the membrane and subjected to acid hydrolysis, followed by thin layer chromatography. Of a MAP kinase, a GST/ERK2 fusion protein was expressed in E. coli, purified and assayed for the ability to autophosphorylate and to phosphorylate MBP, a known substrate for MAP kinases. Fig. 5A shows that affinity purified GST-ERK2 fusion protein, but not purified GST protein alone, phosphorylates MBP and also phosphorylates a band of the same size as the fusion protein (Fig. 5B). Phosphoamino acid analysis shows that the fusion protein phosphorylates MBP predominately on threonine residues while the autophosphorylation is predominately on tyrosine and serine residues (Fig. 5C). Autophosphorylation has been reported for other ERKs as well (11, 57, 60).

Expression of erkB

Probing Northern blots of RNA from wild-type cells with the ERK2 cDNA showed that a 1.8-kb mRNA was present at the start of development and increased two- to fivefold during early development (Fig. 6A). No cytoplasmic ERK2 mRNA accumulated in mutants in which the locus encoding ERK2 was disrupted, although a larger RNA of ~6 kb was seen in nuclear and whole cell preparations (Fig. 6B). The large nuclear RNA was recognized by a probe from the insertion vector, indicating that it arose by transcriptional read-through into the vector sequences. Since the vector inserted in the 3' untranslated region, such read-through could be expected.

We generated a separate gene disruption construct in which the ThyI (14) gene was inserted into the ERK2 cDNA clone at amino acid position 280. This was used to create a different disruption of the locus encoding ERK2 in the thymidine auxotroph JH110. The phenotype of the resulting strain was found to be similar to that of strains HS172-HS175 in that it fails to aggregate and shows reduced cAMP synthesis. Transformation of this strain with a multicopy vector carrying the gene encoding ERK2 fused to the actin 15 promoter region (26) resulted in strains that are once again able to aggregate and develop, confirming that ERK2 is essential for aggregation and subsequent development.

Phenotype of Cells Lacking ERK2

Aggregation in Dictyostelium cells relies upon the ability of cells to both sense gradients of extracellular cAMP (cAMP chemotaxis) and to generate these gradients via synthesis and secretion of cAMP (cAMP relay) (17, 36). In single-cell assays, the mutant cells respond chemotactically to gradients of cAMP in the nanomolar range (data not shown). Since the mutants are unable to aggregate on their own, it therefore seemed likely that the problem lay in cAMP production or relay. When we directly assayed cAMP synthesis following a pulse of exogenous cAMP, wild-type cells responded by a 10-fold increase in the amount of cAMP accumulated over a 5-min period while mutants lacking ERK2 showed a strongly reduced response (Fig. 7A).

The defect in signal relay could be due to alterations in the surface cAMP receptor or coupling of activated receptor to adenyl cyclase. However, we found that the mutants responded to a pulse of cAMP by synthesizing cGMP in a manner indistinguishable from that shown by wild-type cells (Fig. 7B). Since the cGMP response is mediated by the same receptor (CARI) and heterotrimeric G protein subunit (G2) as cAMP relay (50, 55), it appears that these components are fully functional in mutant cells lacking ERK2. Therefore, we looked to see if the genes encoding the cytosolic regulator of adenyl cyclase (CRAC [29, 43]) and adenyl cyclase (ACA) (53) were expressed in the mutant cells. When RNA taken at various times in development of wild-type and mutant cells was probed for CRAC and ACA mRNA, it was clear that both these genes are expressed normally in the mutant cells (data not shown). Moreover, when adenyl cyclase activity in cell extracts was determined in the presence of Mn²⁺, which bypasses...
Figure 7. Stimulated synthesis of cAMP (A) and cGMP (B). Cells starved for 6–8 h in suspension were stimulated with 10 μM 2'deoxy-CAMP and samples taken and assayed for cAMP (A) or cGMP (B) at the marked times as described. Data points marked with an asterisk are significantly different from control. Filled circles, HS176 (control); open squares, HS174 and HS175 (mutants, there was no difference between the two mutants). Data are the mean and standard error of the mean of five experiments for A and three for B.

Table I. Adenylyl Cyclase and CRAC Activities

| Strain    | Adenylyl cyclase (pmol/min/mg) | CRAC activity (pmol/min/mg) |
|-----------|-------------------------------|----------------------------|
| HS174 (mutant) | 18                           | 99                         |
| HS176 (control) | 20                           | 183                        |

Total adenylyl cyclase activity was measured using Mn2+-activated cell lysates. CRAC activity of cytosolic extracts from HS174 and HS176 was determined by addition of extracts to lysates of a CRAC mutant strain (synag 7), followed by measurement of adenylyl cyclase activity in the absence of Mn2+. In the absence of added cytosolic extracts the background CRAC activity was 12 pmol/min/mg.

Discussion

The data presented here demonstrate that the ERK2 protein is important for both activation of adenylyl cyclase and subsequent development. The initial integration site, IS240, although not within the coding sequence, appears to perturb expression of ERK2 by interfering with mRNA processing. The relatively large nuclear RNA present in these transformants could be produced by loss of the appropriate termination signals, leading to a mislocalization of the RNA, and loss of expression of the protein. The fact that disruption by a separate plasmid utilizing the Thyl selection marker at a site within the coding region results in the same phenotype indicates that the consequences of disruption of erkB are not allele-specific.

ERK2 could either be directly on the pathway activating adenylyl cyclase or indirectly regulate the coupling of adenylyl cyclase to the cAMP receptor. One form of indirect regulation of adenylyl cyclase by ERK2 could be through regulation of the expression of factors necessary for the activation of cyclase. Alternatively, one of the proteins directly involved in activation of cyclase may require phosphorylation by ERK2 in order to be competent for coupling the cAMP receptor to cyclase. For example, other signals such as PSF or CMF (8) might activate pathways necessary to allow coupling of the cAMP receptor to cyclase. The observa-
Figure 8. Localization of cells in mixtures of mutant and control transformants. 1:10 mixtures of HS174 to control cells were induced to undergo development on agar. Either mutant or 10% of control cells were labeled with CMFDA. Structures at each stage of development were analyzed with a confocal microscope and projections made of the entire structure, viewed from above. Left, mutant cells stained; right, control cells stained; top, aggregates. middle, slug stage; bottom, fruiting bodies.
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