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The *Dictyostelium* MAP kinase kinase DdMEK1 regulates chemotaxis and is essential for chemoattractant-mediated activation of guanylyl cyclase

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We have identified a MAP kinase kinase (DdMEK1) that is required for proper aggregation in *Dictyostelium*. Null mutations produce extremely small aggregate sizes, resulting in the formation of slugs and terminal fruiting bodies that are significantly smaller than those of wild-type cells. Time-lapse video microscopy and *in vitro* assays indicate that the cells are able to produce cAMP waves that move through the aggregation domains. However, these cells are unable to undergo chemotaxis properly during aggregation in response to the chemoattractant cAMP or activate guanylyl cyclase, a known regulator of chemotaxis in *Dictyostelium*. The activation of guanylyl cyclase in response to osmotic stress is, however, normal. Expression of putative constitutively active forms of DdMEK1 in a *ddmek1* null background is capable, at least partially, of complementing the small aggregate size defect and the ability to activate guanylyl cyclase. However, this does not result in constitutive activation of guanylyl cyclase, suggesting that DdMEK1 activity is necessary, but not sufficient, for cAMP activation of guanylyl cyclase. Analysis of a temperature-sensitive DdMEK1 mutant suggests that DdMEK1 activity is required throughout aggregation at the time of guanylyl cyclase activation, but is not essential for proper morphogenesis during the later multicellular stages. The activation of the MAP kinase ERK2, which is essential for chemoattractant activation of adenylyl cyclase, is not affected in *ddmek1* null strains, indicating that DdMEK1 does not regulate ERK2 and suggesting that at least two independent MAP kinase cascades control aggregation in *Dictyostelium*.

Keywords: chemotaxis/*Dictyostelium discoideum*/guanylyl cyclase/MAP kinase/MEK/signal transduction

Introduction

MAP kinase cascades are comprised of three evolutionarily conserved kinases, a MEK kinase (MAP kinase kinase kinase), a MEK (MAP kinase kinase) and a MAP kinase, that function sequentially (Davis, 1993; Errede and Levin, 1993; Herskowitz, 1995; Levin and Errede, 1995; Marshall, 1995; Bokemeyer *et al.*, 1996). These cascades are activated by various cell surface receptors, including receptor tyrosine kinases, receptors coupled to cytoplasmic tyrosine kinases, G protein-coupled receptors and histidine kinases that function as part of two-component systems. They control a diversity of intracellular responses that include: pathways leading to cell growth and cell type differentiation, mating in yeast, activation of adenylyl cyclase in *Dictyostelium* and cell viability after stresses such as osmotic or heat shock, low oxygen tension and UV radiation (Herskowitz, 1995; Levin and Errede, 1995; Marshall, 1995; Segall *et al.*, 1995; Bokemeyer *et al.*, 1996). In the yeast *Saccharomyces cerevisiae*, five independent MAP kinase cascades have been identified (Levin and Errede, 1995). Some of these cascades have overlapping components, but at least one component of each cascade is unique to a specific response pathway.

In *Dictyostelium*, formation of the multicellular organism results from the chemotactic aggregation of up to 10^5 cells. This process is controlled by a series of integrated signal transduction pathways that are activated by oscillatory pulses of extracellular cAMP interacting with G protein-coupled, serpentine receptors (cARs) (Devreotes, 1994; Firtel, 1995; Van Haastert, 1995; Chen *et al.*, 1996). As an aggregation domain forms, cAMP waves are initiated by a central core of cells, or oscillator, and move outward with a spiral or concentric pattern and a periodicity of ~6 min during the height of aggregation (Siegert and Weijer, 1991). Approximately 20 of these waves are required for the formation of a loose aggregate. cAMP binding to the receptors results in the activation of adenylyl and guanylyl cyclases. Activation of adenylyl cyclase results initially in a rise in intracellular cAMP, which is then released into the extracellular medium, relaying the signal from cell to cell.

The transient rise in intracellular cGMP in response to the activation of guanylyl cyclase has been linked directly to chemotactic movement, and the pathways that control chemotaxis in *Dictyostelium* are similar to those used in mammalian neutrophils (Devreotes and Zigmond, 1988). *Dictyostelium* mutations that are unable to activate guanylyl cyclase do not undergo chemotaxis (Kuwayama *et al.*, 1993, 1995; Liu *et al.*, 1993). A mutation in a cytosolic cGMP-specific phosphodiesterase (Ross and Newell, 1981; Newell, 1995), which results in a more extended cAMP-stimulated rise in cGMP, produces a protracted period of chemotactic movement. Both the adenylyl and guanylyl cyclase pathways are followed by a period of adaptation. Clearing of the extracellular cAMP signal by phosphodiesterase allows these pathways to become resensitized and the cells to respond to the subsequent wave of cAMP (Hall *et al.*, 1993). Activation of cell surface receptors also results in the induction of aggregation-stage gene expression (Firtel, 1995). Most of these genes require cAMP oscillations for expression and removal of the

cAMP signal or the addition of a continuous signal, which results in an adaptation of the receptors and repression of gene expression (Mann and Firtel, 1987, 1989; Wu *et al.*, 1995a). Other genes, such as the extracellular phosphodiesterase, are induced by either a pulsatile or continuous signal (Hall *et al.*, 1993; Wu *et al.*, 1995a).

Genetic and biochemical analyses have resulted in the identification of many of the components that regulate various aspects of the aggregation-stage signaling pathways (Devreotes, 1994; Firtel, 1995; Van Haastert, 1995; Chen *et al.*, 1996). The activation of both adenylyl and guanylyl cyclases requires the cAMP receptor-coupled G protein containing the Gα subunit Gα2, but the mechanisms by which the two pathways are activated are distinct. Data indicate that the Gα subunit is thought to activate guanylyl cyclase and phospholipase C, while the βγ subunits are directly involved in the activation of adenylyl cyclase (Okaichi *et al.*, 1992; Bominaar *et al.*, 1994; Valkema and Van Haastert, 1994; Wu *et al.*, 1995b; Chen *et al.*, 1996). In addition, other components, such as CRAC (cytosolic regulator of adenylyl cyclase), are essential for activation of adenylyl, but not guanylyl, cyclase (Insall *et al.*, 1994; Lilly and Devreotes, 1994, 1995). The MAP kinase ERK2 is also essential for activation of adenylyl cyclase (Segall *et al.*, 1995). *erk2* null cells have normal levels of adenylyl cyclase but do not exhibit receptor-mediated activation. ERK2 is activated rapidly in response to cAMP, but a significant component of this activation does not require receptor-coupled G proteins (Knetsch *et al.*, 1996; Maeda *et al.*, 1996). In addition to being required for aggregation, ERK2 is also essential for proper morphogenesis and pre-spore, but not pre-stalk, cell differentiation during the multicellular stages of development (Gaskins *et al.*, 1996). ERK2 activation and adaptation are negatively regulated by Ras and positively regulated by cAMP-dependent protein kinase (PKA), which is also required for other aspects of aggregation (Simon *et al.*, 1989; Firtel and Chapman, 1990; Schulkes and Schaap, 1995; Mann *et al.*, 1997). Analysis of these and other mutants has produced a picture of aggregation that is significantly more complex than might have been thought initially, containing multiple activation and feedback pathways that allow the organism to control the ability of the cells to undergo chemotaxis and produce an appropriate-sized multicellular organism (Chen *et al.*, 1996).

Two independently functioning MAP kinases have been identified in *Dictyostelium*, ERK1 (Gaskins *et al.*, 1994) and ERK2. Overexpression studies of wild-type and mutant ERK1 expressed in a variety of genetic backgrounds indicate that ERK1 plays an important role in aggregation and later multicellular stages (Gaskins *et al.*, 1994). Overexpression in wild-type cells produces multicellular aggregates with abnormal morphology which initiates at the slug stage. Overexpression of ERK1 in a protein tyrosine phosphatase 2 (PTP2) null background (*ptp2* null cells) results in large aggregation streams that break up into multiple, tiny aggregates that developmentally arrest (Gaskins *et al.*, 1994; Howard *et al.*, 1994). The activity of ERK1 is developmentally regulated, as indicated by measuring the activity of the immunoprecipitated ERK1 isolated from cells at various times during *Dictyostelium* development (Gaskins *et al.*, 1994). The inability to knock out the expression of ERK1 by either homologous recombination or antisense constructs has led to the suggestion that ERK1 function may be essential for growth.

Here we describe the identification and function of a *Dictyostelium* MAP kinase kinase designated DdMEK1 that is essential for proper aggregation. *ddmek1* null cells created by homologous recombination form very small aggregates. These cells show the normal wave patterning of cAMP signaling during the initial stages of aggregation, but the cells do not migrate towards the aggregation centers and are unable to migrate towards cAMP in standard chemotaxis assays. Instead, the aggregation domains break down and small groups of cells coalesce to form aggregates that are significantly smaller than those of wild-type cells. These aggregates continue to develop and show normal morphological differentiation, with the exception that the fruiting bodies are very small. These cells exhibit cAMP-stimulated adenylyl cyclase activity that is not significantly different from that in wild-type cells, but show a very severe defect in the cAMP-mediated activation of guanylyl cyclase. However, activation of guanylyl cyclase in response to osmotic stress is normal, suggesting that the intrinsic guanylyl cyclase activity is unaffected. Complementation of the *ddmek1* null strain with a putative Ddmek1 temperature-sensitive mutation results in normal-sized aggregates at the permissive temperature and very small aggregates at the non-permissive temperature. Temperature shift experiments indicate that DdMEK1 activity is required at the time of cAMP stimulation for guanylyl cyclase activation and continuously throughout aggregation. A mutation in which the conserved serine/threonine residues that are phosphorylated in response to MEKK stimulation are changed to alanine residues is unable to complement the null cells and functions as a dominant-negative mutation in wild-type cells, producing a phenotype like that of *ddmek1* null cells. *ddmek1* null or wild-type cells expressing DdMEK1 containing either glutamate or aspartate at the conserved positions of serine/threonine phosphorylation form almost normal sized aggregates, but many of these aggregates arrest at the mound stage. Furthermore, we show that the *ddmek1* null mutant does not affect the activation of the MAP kinase ERK2, which is also essential for aggregation and regulates the activation of adenylyl cyclase, suggesting that at least two independent MAP kinase cascades control aggregation in *Dictyostelium*. Our results identify a new and novel MAP kinase cascade that is essential for proper chemotaxis in *Dictyostelium* and appears to function by regulating receptor-mediated activation of guanylyl cyclase that is essential for aggregation.

Results

Cloning of DdMEK1

PCR was used to amplify expected conserved domains and components of MAP kinase pathways using *Dictyostelium* genomic DNA and cDNA libraries (see Materials and methods). One of the PCR products, evaluated after sequencing, was used to screen a λZap cDNA library. cDNA clones were isolated and sequenced. The derived amino acid sequence of these clones suggested that the gene encoded a MEK (MAP kinase kinase) and was

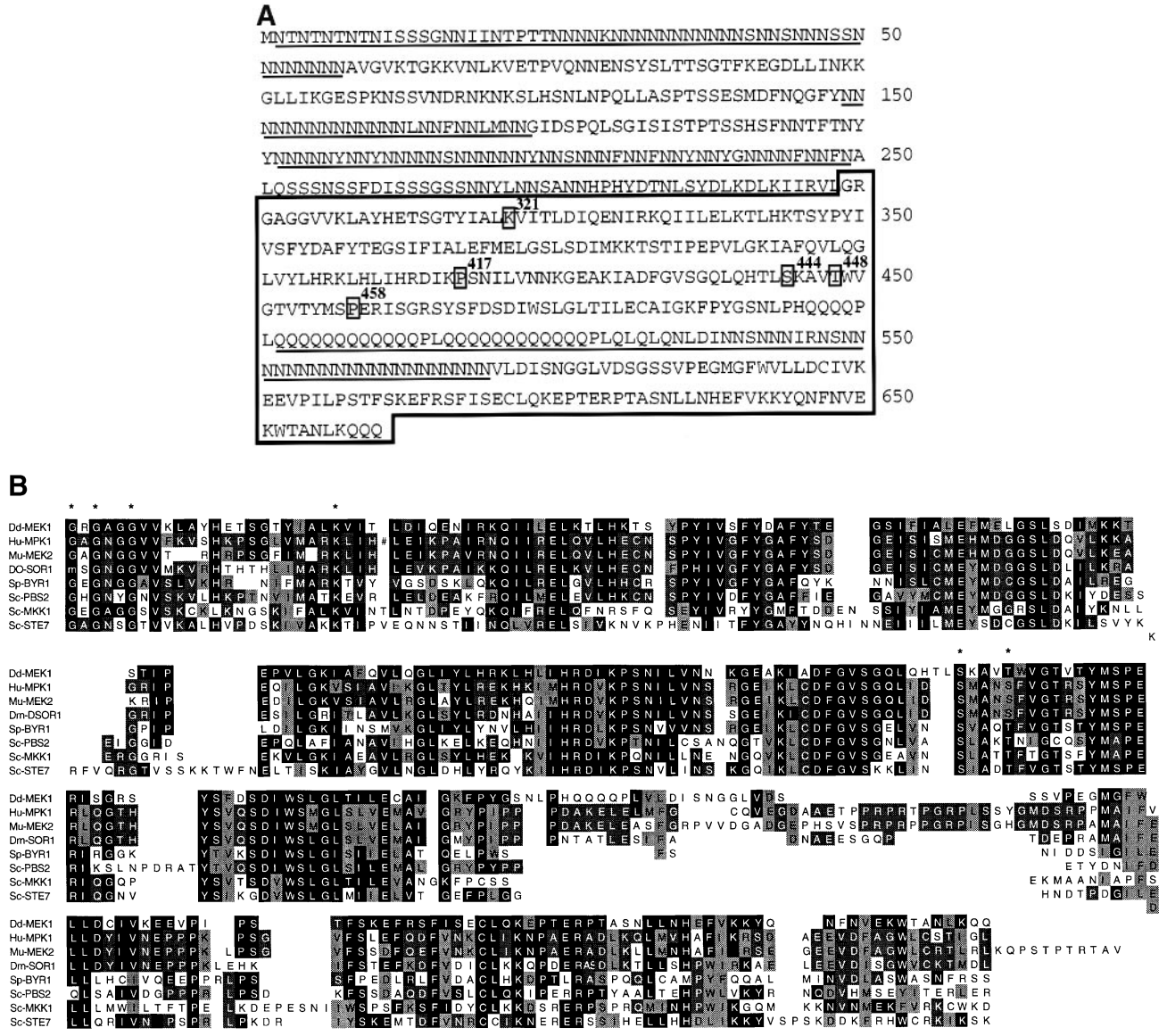


Fig. 1. Sequence of DdMEK1. (**A**) The amino acid sequence of DdMEK1 is shown. The boxed region depicts the kinase core. Underlined regions show homopolymer and homopolymer-rich runs. The boxed amino acids with numbering are positions of point mutations described in the text. (**B**) Amino acid sequence comparison of the kinase core domain of DdMEK1 with other MAP kinase kinases from a variety of eukaryotes. Hu-Mpk1, human MEK Mpk1, accession no. Q02750; Mu-MEK2, murine MEK MEK2, accession no. S68267; Dm-SOR1, *Drosophila* MEK SOR1, accession no. A45176; Sp-BYR1, *Schizosaccharomyces pombe* MEK BYR1, accession no. P10506; Sc-PBS2, *Saccharomyces cerevisiae* MEK PBS2, accession no. P08018; Sc-MKK1, *S.cerevisiae* MEK MKK1, accession no. P32490; *S.cerevisiae* MEK Ste7, accession no. P06784. Asterisks indicate conserved residues in the ATP-binding site and sites of MEK activating phosphorylation by MEKKs.

designated *DdMEK1*. The full open reading frame (ORF) and a comparison of the putative kinase domain with that of known MEKs are shown in Figure 1A and B, respectively. The amino acid sequence shows strong homology to known MEKs present in the GenBank database and includes the conserved serine/threonine residues that are phosphorylated by the upstream MEK kinase during activation in other MEKs. The highest level of homology is to the murine MEK2 (Brott *et al.*, 1993). Northern blot analysis indicates that *DdMEK1* is expressed at a low level in vegetative cells, with mRNA levels increasing during early development, peaking during late aggregation, and then decreasing during the later multicellular stages (Figure 2).

Phenotype of ddmek1 null cells

DdMEK1 was disrupted by homologous recombination by insertion of a blasticidin resistance cassette between codons 377 and 378 of the ORF. The disruption was confirmed by Southern blot analysis of randomly selected clones (data not shown; see Materials and methods). Northern blot analysis of a representative clone shows the loss of the full-length *DdMEK1* transcript and the presence of new, shorter transcripts (Figure 2). *ddmek1* null cells grow normally either in axenic medium or in association with bacteria. When these cells are plated on Na/K-buffered agar to allow multicellular development to ensue, the cells do not show normal aggregation streams but produce aggregates, slugs and fruiting bodies that are very

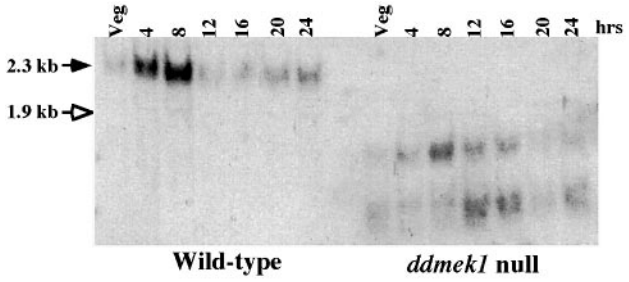


Fig. 2. Developmental kinetics of expression of *DdMEK1* in wild-type and *ddmek1* null cells. RNA was isolated from developing cells, at the times indicated, from wild-type and *ddmek1* null cells, size fractionated on a denaturing gel, blotted and hybridized with the *DdMEK1* probe. 'Veg' is RNA from log-phase vegetatively growing cells.

small compared with those of wild-type organisms. A good indication of the size differential is seen in Figure 3A, which shows photographs of wild-type and *ddmek1* null mounds and slugs at the same magnifications. As seen in the top panels, the mound sizes of the *ddmek1* null cells are significantly smaller than those of wild-type cells. As depicted in the wild-type control (lower panel), the one large slug is representative of the size of wild-type slugs when cells are plated at this density. In addition, there are a few smaller slugs composed of cells that did not aggregate into and become part of a normal, larger aggregate. As is evident, the sizes of all the *ddmek1* null strain aggregates and slugs are significantly smaller than the wild-type slugs when the strains are plated on the agar at the same density. Except for the size, the overall morphologies of the *ddmek1* null slugs, fruiting bodies and other multicellular stages are indistinguishable from those of wild-type cells (data not shown). [Note that in wild-type strains, normal morphology and spatial patterning is observed in aggregates that range in size over more than three orders of magnitude, with a maximum size of ~10^5 cells (Schaap, 1986).] This small-aggregate *ddmek1* null phenotype is fully complemented by expression of the full-length *DdMEK1* cDNA from the constitutively expressed *Actin 15* (*Act15*) promoter (Knecht *et al.*, 1986; data not shown). Overexpression of *DdMEK1* from the *Act15* promoter in wild-type cells does not produce an observable phenotype. Some mutants affecting aggregation can be rescued or partially rescued by first being pulsed with cAMP (Insall *et al.*, 1996), which maximizes the expression of the aggregation-stage cAMP receptor and other components of the signaling pathway. When both wild-type and *ddmek1* null cells are pulsed for 4.5 h and then plated for development, the aggregate size of the *ddmek1* null and wild-type strains are unchanged compared with unpulsed cells (Figure 3B), indicating that prior pulsing of *ddmek1* cells cannot rescue the null phenotype.

Northern blot analysis was performed to determine if the *ddmek1* null mutation affected the temporal or quantitative level of expression of cell type-specific genes. This analysis included the pre-stalk-specific gene *ecmA* (pre-stalk) (Williams *et al.*, 1987), the pre-spore-specific gene *SP60* (Mehdy *et al.*, 1983; Haberstroh and Firtel, 1990) and the sporulation-specific gene *SpiA* (Richardson *et al.*, 1994). No differences were observed (data not shown). Analysis of cell type proportioning and spatial localization of pre-stalk and pre-spore cells within the multicellular organisms using cell type-specific promoters expressing the *lac*Z reporter also showed no observable differences between *ddmek1* null cells and wild-type cells (data not shown).

Point mutations of DdMEK1 suggest that DdMEK1 encodes a MAP kinase kinase

MAP kinase kinases, or MEKs, are activated by phosphorylation at conserved serine/threonine residues by upstream MEK kinases (Mansour *et al.*, 1994; Pages *et al.*, 1994; Zheng and Guan, 1994). Mutations of these conserved serine/threonine residues to glutamates or aspartates can result in constitutively active forms of the enzyme, whereas mutations of these residues to alanines result in an enzyme that cannot be activated by phosphorylation and can yield mutations that are dominant-negative when overexpressed in an otherwise wild-type background (Mansour *et al.*, 1994; Pages *et al.*, 1994; Zheng and Guan, 1994; Huang *et al.*, 1995; Yashar *et al.*, 1995; Errede and Ge, 1996). Figures 1A and 4 show the amino acid sequence around these conserved residues and the various mutations that were made by site-directed mutagenesis. The different mutant DdMEK1 expression constructs were transformed into both wild-type and *ddmek1* null cells. Clonal isolates of stable transformants were obtained. As shown in Figure 5 and summarized in Table I, overexpression of DdMEK1 with the double glutamate or aspartate substitutions (DdMEK1^{S444E,T448E}; DdMEK1^{S444D,T448D}) in *ddmek1* null cells complemented the *ddmek1* null phenotype with respect to its aggregate size defect, although, on average, the aggregates were slightly smaller than those of *ddmek1* null cells complemented with the wild-type DdMEK1. *ddmek1* null cells expressing DdMEK1^{S444D,T448D} show defects in cAMP-mediated signaling pathways essential for aggregation (see below). While *ddmek1* null cells expressing DdMEK1^{S444E,T448E} form almost normal sized aggregates, many of these aggregates arrested at the mound stage (Figure 5). Approximately 30–50% of the cells continued through development and formed mature fruiting bodies. A similar phenotype was observed when the DdMEK1^{S444E,T448E} mutant protein was expressed in wild-type cells, indicating that the effect is dominant. The same phenotypes were observed for the overexpression of DdMEK1 with the aspartate (DdMEK1^{S444D,T448D}) mutations (data not shown). Expression of the double alanine mutant (DdMEK1^{S444A,T448A}) or a mutant in which the lysine residue required for ATP binding was converted to an alanine (DdMEK1^{K321A}) did not complement the *ddmek1* null cells and resulted in a dominant-negative phenotype when expressed in wild-type cells producing a *ddmek1* null phenotype (very small aggregate size; Figure 5; data for DdMEK1^{K321A} not shown). The phenotypes of these mutants are consistent with *Dictyostelium DdMEK1* encoding a MAP kinase kinase and these residues being essential for proper functioning of the protein. Table I summarizes the observed phenotypes of the different mutations.

The region N-terminal to the catalytic core has long homopolymers that are not uncommon in *Dictyostelium* proteins and are present, for example, in the catalytic subunit of cAMP-dependent protein kinase and the

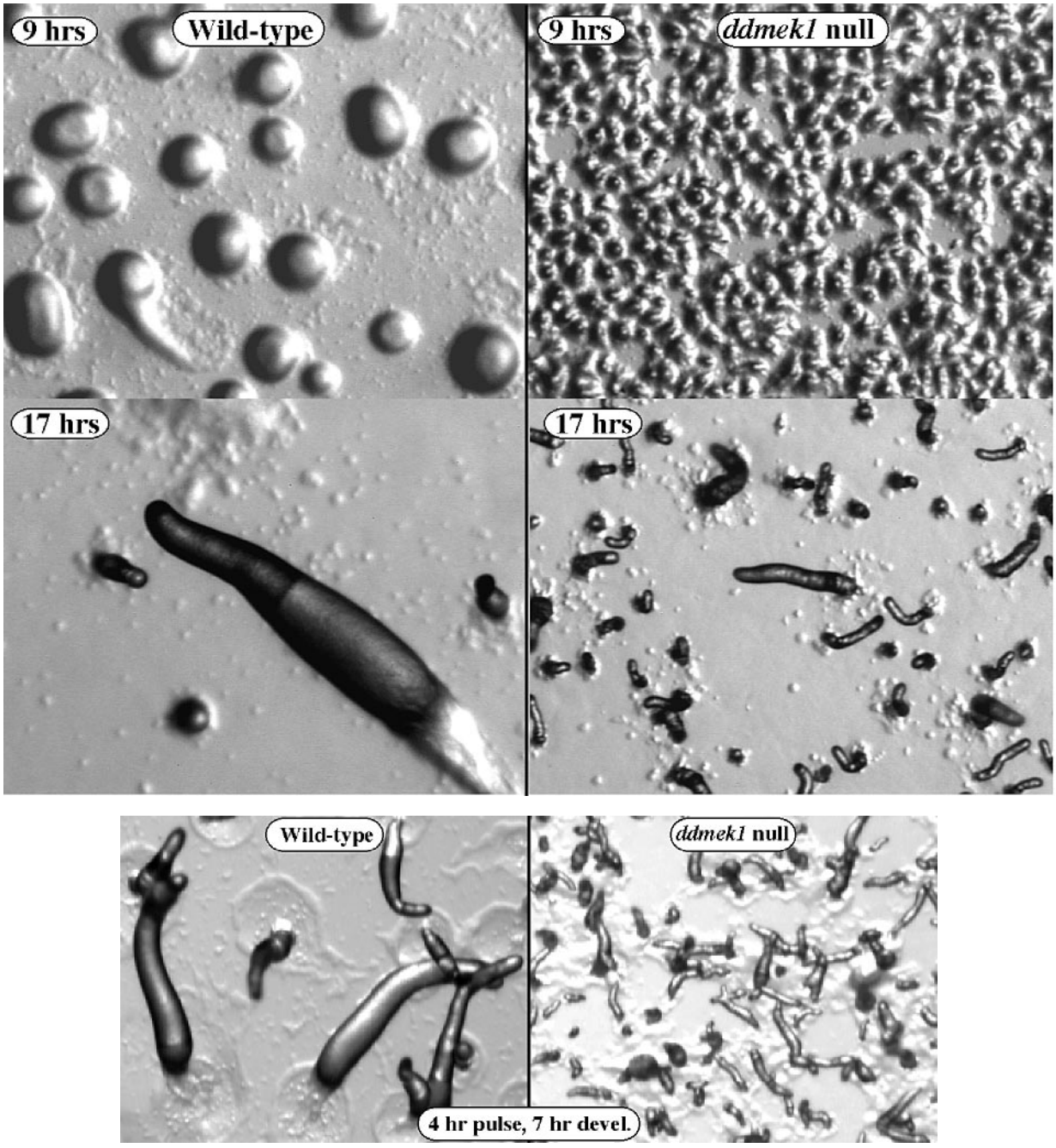


Fig. 3. Developmental morphology of wild-type and *ddmek1* null cells. (**A**) The figure shows the developmental morphology of wild-type and *ddmek1* null cells at 9 h (upper panels) and 17 h of development (the slug stage; bottom panels). The magnification of the two images in the top row and the two images in the bottom row is the same. (**B**) Log-phase wild-type and *ddmek1* null vegetative cells were washed and resuspended in 12 mM Na/KPO_4 (pH 6.2) at 5×10^6 cells/ml and pulsed with 30 nM cAMP at 6 min intervals for 4.5 h. Cells were then washed and plated for development on Na/KPO_4-containing agar at the same density. Photographs were taken 7 h after plating on agar at the time of initial slug formation. (Note: aggregation is significantly more rapid in cells that are pulsed with cAMP.) Both photographs are at the same magnification.

adenylyl cyclase ACA (Mann and Firtel, 1991; Pitt *et al.*, 1992). In addition, there is a domain near the N-terminus with weak homology to the MAP kinase-interacting domains that have been identified (Bardwell and Thorner, 1996; Bardwell *et al.*, 1996). It is possible that this region is involved in designating DdMEK1 specificity. A DdMEK1 construct carrying a deletion of the region upstream from the catalytic core cannot complement the *ddmek1* phenotype and inhibits proper aggregation and later morphogenesis in *ddmek1* null and wild-type strains (Figure 5, Table I).

ddmek1 null cells are defective in chemotaxis to cAMP

As indicated in the Introduction, aggregation is controlled by a series of integrated signal transduction pathways that are activated by extracellular cAMP interacting with G protein-coupled receptors. To obtain better insight into the inability of *ddmek1* null cells to undergo normal aggregation, the formation of multicellular aggregates in these cells was followed by time-lapse video microscopy using a phase contrast microscope. The videos were then compared with a similar analysis of wild-type cells. Figure

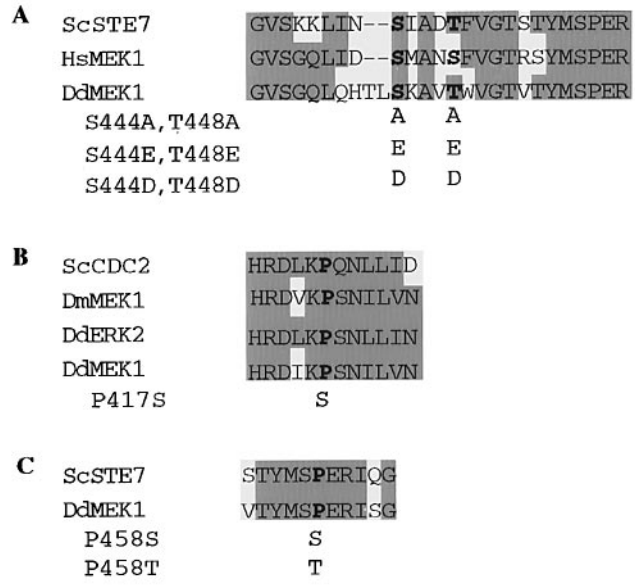


Fig. 4. Amino acid sequence substitutions in DdMEK1. (**A**) The sequence surrounding the putative activation/phosphorylation sites of DdMEK1, Ste7 and human MEK1. Point mutations made in DdMEK1 are indicated below. (**B**) The sequence surrounding sites of proline→serine mutation that results in temperature-sensitive mutants in yeast Cdc2, *Drosophila* DmMEK1 and *Dictyostelium* DdERK2. DdMEK1 is shown with the point mutation described in the text. (**C**) The sequence around another conserved domain in yeast Ste7 and *Dictyostelium* DdMEK1. Sites of proline→serine or threonine changes are indicated.

6A shows images from the time-lapse video recording of wild-type cells at different stages of the aggregation process taken at two different magnifications. Small, dark areas within the field of brighter rings of cells represent the initial formation of aggregation centers (see arrowheads). As the cAMP wave moves outward, responding cells elongate, producing lighter bands (Newell, 1995). As a cell adapts, the cell's shape becomes random and this area darkens. Moving waves can thus be seen as alternating light and dark bands moving through the field and as 'undulations' in the field of cells that can only be visualized by directly viewing the time-lapse video. At 3 h into development, the field appears uniform, although wave patterns can be seen in the video recordings. By 3.5–4 h, aggregation domains can be seen in the still images, which are very clear by 4 h 20 min (see arrowheads). In the 4 h 20 min and 4 h 40 min images, alternating light and dark domains can be identified. The time-lapse shows an oscillatory wave emitting through these various aggregation domains. Chemotaxis then initiates rapidly and, within 1 h, the cells are almost fully aggregated. In the mature mounds seen at 6 h, larger concentric rings due to cell movement and oscillations are visible. The oscillations can be observed in the video. By 7–8 h, the mounds start to spin rapidly as the tight aggregate forms and a tip starts to initiate.

Video imaging of *ddmek1* null cells shows a similar pattern to that of wild-type cells, with waves moving through the lawn of cells at the start of aggregation, although the bright central region of the aggregation domains is slightly smaller (Figure 6B, see arrowheads). However, videos of the wave patterns show that the waves extend outward to a size similar to that seen in wild-type cells (see arrowheads in Figure 6B, images at 3 h 50 min through 5 h 10 min). Moreover, the cells in the outer regions of these domains do not appear to be fully organized and the density of cell shape change that produces the light and dark bands is reduced. As can be observed, no concerted chemotaxis of the cells is observed. Instead of rapidly aggregating to form mounds, the domains start to dissipate and form microcenters or waves of centers (see image at 5 h 10 min). Eventually, the cells become microdomains that form the very small aggregates described above (Figure 3). By 6 h, the more organized domains also start to split into very small aggregates (at 5 h 30 min and later) until the entire center has split (see arrowheads in images at 6 h 10 min and 6 h 50 min). The change in light and dark wave patterns indicates that the cells undergo birefringent cell shape changes in response to cAMP waves; however, the cells do not move inward in response to the waves. The movement of waves through the lawn of cells suggests that the *ddmek1* null cells relay cAMP signals.

To examine directly the ability of *ddmek1* null cells to migrate towards cAMP, we used a standard small-population chemotaxis assay in which microdrops of cells are placed in close proximity to microdrops of different concentrations of cAMP (Konijn, 1970). Chemotaxis to cAMP at concentrations ranging from 0.01 to 100 μM was then examined by observing the movement of cells within the drop towards the drop of cAMP. Under the conditions tested, wild-type cells initiated chemotaxis towards 0.1, 1 and 10 μM cAMP under the assay conditions within 20 min (Figure 7A). At 70–80 min, the cells exhibited significant movement (Figure 7A and B), whereas *ddmek1* null cells showed no elongated cell shape changes that accompany chemotaxis at these times (Figure 7A and B). No chemotaxis of *ddmek1* null cells towards cAMP was observed at any cAMP concentration tested, even after several hours of assay (data not shown).

Requirement for DdMEK1 in aggregation-stage signal transduction pathways

To determine whether the levels and kinetics of activation of adenylyl and guanylyl cyclases, two signaling components essential for aggregation, are altered in *ddmek1* null cells, cAMP-mediated activation of these enzymes was measured. To maximize the levels of expression of all components involved in the cAMP-mediated signaling pathways, cells were pulsed with nanomolar concentrations of cAMP for 4–5 h before performing the biochemical assays (see legend to Figure 8; Devreotes *et al.*, 1987; Mann *et al.*, 1994). The time course of adenylyl cyclase activity in response to *in vivo* receptor stimulation was measured at time points after cAMP stimulation in lysed samples (Figure 8A). In addition, $MnSO_4$- and GTPγS-stimulated activities, which measure the intrinsic and maximal G protein-stimulated activity of the enzyme respectively, were quantified (Figure 8B; see Materials and methods). In *ddmek1* null cells, the receptor-mediated activation of adenylyl cyclase showed kinetics that were similar to those measured in wild-type cells, but the maximal level of activity was lower and the adaptation of the response was delayed (Figure 8A). However, both $MnSO_4$- and GTPγS-stimulated activities were slightly higher (Figure 8B). These results suggest that the overall

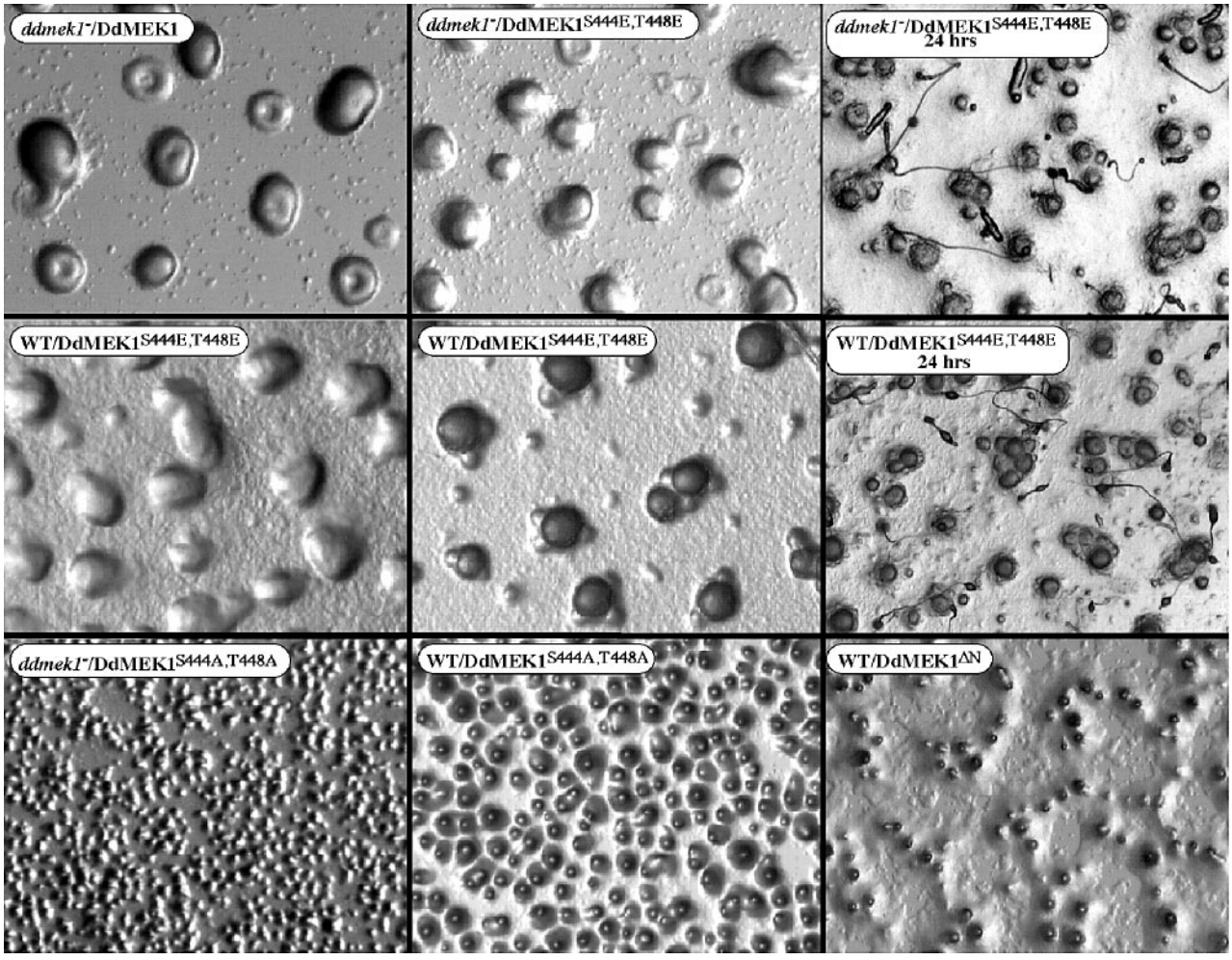


Fig. 5. Morphologies of DdMEK1 mutations expressed in wild-type and *ddmek1* null cells. Morphologies of *ddmek1* null and wild-type cells expressing wild-type and mutant DdMEK1 proteins. The strains and time in development are indicated. All images are at the same magnification, except *ddmek1* null and wild-type cells expressing DdMEK1^{S44E,T448E} at 24 h, which are at a lower magnification to show a larger field of view.

Table I. Summary of expression of mutant DdMEK1 on the small aggregate size phenotype

DdMEK1 mutant	Wild-type	*ddmek1* null
S444, T448→E, E	almost wild-type aggregate	almost wild-type aggregate
S444, T448→D, D	partial mound arrest	partial mound arrest
S444, T448→A, A	null phenotype (dominant-negative)	null phenotype
K321→A	null phenotype (dominant-negative)	null phenotype
P458→S	temperature sensitive (weak)	temperature sensitive (weak)
P458→T	temperature sensitive (strong) (dominant-negative)	temperature sensitive (strong)
P417→S	non-ts, wild-type	wild-type
DdMEK1ΔN	null phenotype (dominant-negative)	null phenotype

enzymatic activation of adenylyl cyclase in *ddmek1* null cells is not altered significantly with respect to cAMP signaling, although the pattern of cAMP-mediated activation is slightly aberrant. In *ddmek1* null cells expressing DdMEK1^{S444D,T448D}, the adenylyl cyclase activities were significantly reduced in all conditions tested.

In contrast to the comparison of adenylyl cyclase activation in *ddmek1* null and wild-type cells, cAMP-mediated activation of guanylyl cyclase was very greatly reduced in the null cells, with only a very minimal measurable increase (⩽5% of wild-type cells) in cAMP-stimulated cGMP levels (Figure 8C). Whereas basal levels of guanylyl cyclase are difficult to quantitate accurately, they are similar in *ddmek1* null and wild-type cells. This suggests that the guanylyl cyclase enzyme may be normal in these cells. To investigate this further, we took advantage of the fact that guanylyl cyclase is also activated in response to osmotic stress (Kuwayama *et al.*, 1996; Oyama, 1996). This activation is significantly slower than the response to cAMP signaling, but the level of total cGMP produced is higher and the activation is independent of receptors and heterotrimeric G proteins (H.Kuwayama and P.Van Haastert, personal communication). As shown in Figure 8D, *ddmek1* null cells had a level of guanylyl cyclase activation that was higher than that of wild-type cells, as measured by the production of cGMP. This result is consistent with the level of the guanylyl cyclase enzyme being normal in *ddmek1* null cells but not being able to be activated in response to cAMP chemoattractant signals. *ddmek1* null cells expressing the putative constitutively active form of DdMEK1 (DdMEK1^{S444D,T448D}) were also

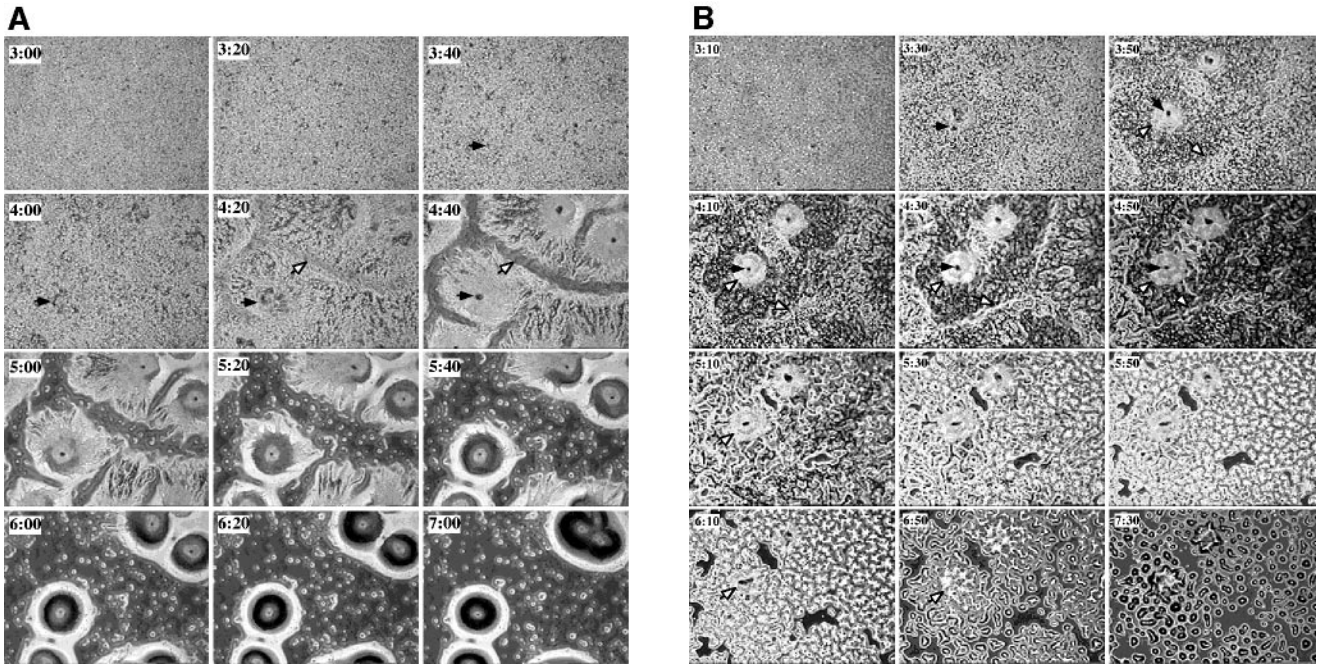


Fig. 6. Time-lapse video microscopy of wild-type and *ddmek1* null cells. The morphology of *Dictyostelium* cells was followed by time-lapse video phase microscopy taken with a 4× objective and recorded using an SVHS recorder. Zero time is the time of plating of the cells on agar. The time compression factor was 120. Shown are computer-grabbed images from the videotape at the approximate times in hours and minutes indicated. Images shown were captured from the video tape at ~20 min intervals of real time, except as indicated. (**A**) Wild-type cells. The solid arrowheads mark the center of the aggregation center, which is always dark, as these cells are always adapted. The open arrowheads mark the outer limits of the aggregation domain. (**B**) *ddmek1* null cells. In the images 3:30–5:10, the innermost open arrowheads mark the limits of the cells that show the strongest refractory changes. The arrowhead further out indicates the outer limits of the aggregation domain as determined by wave movement in the time-lapse video. In images 6:10 and 6:50, the open arrowheads mark the center of the aggregation domain that is splitting into multiple small aggregates.

assayed for cAMP stimulation of guanylyl cyclase activity. The basal level of guanylyl cyclase activity, as determined by the level of cGMP in unstimulated cells, is lower than that of wild-type or *ddmek1* null cells. Activity was stimulated by cAMP, but the kinetics of stimulation were slower and the maximum level of cGMP produced was lower, although the level of stimulation was ~3-fold compared with 6- to 8-fold in wild-type cells or *ddmek1* null cells complemented with wild-type DdMEK1 (Figure 8C).

Analysis of a DdMEK1 temperature-sensitive conditional mutant suggests that DdMEK1 function is required continuously during aggregation at the time of guanylyl cyclase activation

We attempted to create temperature-sensitive mutants of DdMEK1 by amino acid substitutions similar to mutations of conserved residues that are known to result in temperature-sensitive enzymes in other members of this broad class of structurally related kinases (Figure 4B and C; Carr *et al.*, 1989; Hsu and Perrimon, 1994; Gaskins *et al.*, 1996; M.Teague, R.Cade and B.Errede, personal communication). A DdMEK1 in which a conserved proline residue was mutated to serine or threonine (DdMEK1$^{P458S/T}$; Figure 4C) complemented *ddmek1* null cells forming wild-type-sized aggregates when cells were plated at the permissive temperature of 18°C (Figure 9; data for the serine mutation not shown). However, when the *ddmek1*$^{-}$/DdMEK1$^{P458S/T}$ cells were plated at 27°C, the aggregate size was indistinguishable from that of *ddmek1* null cells, indicating the DdMEK1$^{P458S/T}$ proteins were temperature sensitive. The threonine mutation had a stronger temperature-sensitive phenotype, exhibiting a phenotype at the restrictive temperature that was indistinguishable from that of the *ddmek1* null cells. At the restrictive temperature, the aggregate size of the serine mutation was slightly larger than that of the threonine mutant or the *ddmek1* null mutant (data not shown). P→S or T mutations at the equivalent site in the yeast MEK *Ste7* show the same differential effect of temperature sensitivity (M.Teague, R.Cade and B.Errede, personal communication). Mutation of Pro417 to serine (DdMEK1^{P417S}), a complementary mutation of which produces a temperature-sensitive mutant protein in the *Drosophila* MEK DmMEK1 (Hsu and Perrimon, 1994), yeast cdc2 (Carr *et al.*, 1989) and the *Dictyostelium* MAP kinase ERK2 (Gaskins *et al.*, 1996; Figure 4B), complemented the *ddmek1* null cells at both restrictive and permissive temperatures (data not shown). No temperature-sensitive phenotypes were observed when DdMEK1^{P417S} was expressed in either wild-type or *ddmek1* null cells. Table I summarizes the observed phenotypes of the different mutations.

We used the *ddmek1* null cells complemented with the DdMEK1^{P458T} conditional mutant in temperature shift experiments. Cells were allowed to aggregate for 6 h and then shifted to the 27°C non-permissive temperature. Under these conditions, forming aggregates split into multiple small aggregates similar in size to aggregates that form when cells are plated at the non-permissive temperature from the start of development. Cells that were maintained at the permissive temperature for 8 h formed

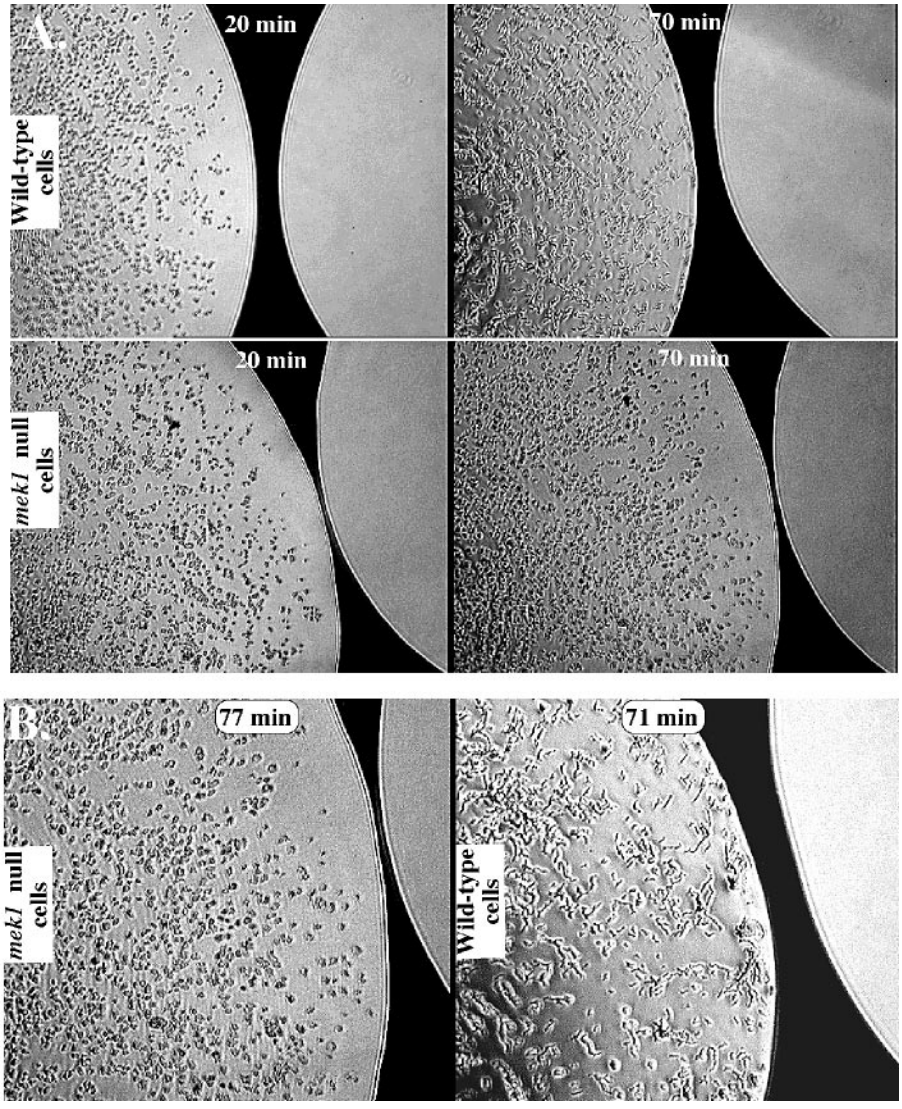
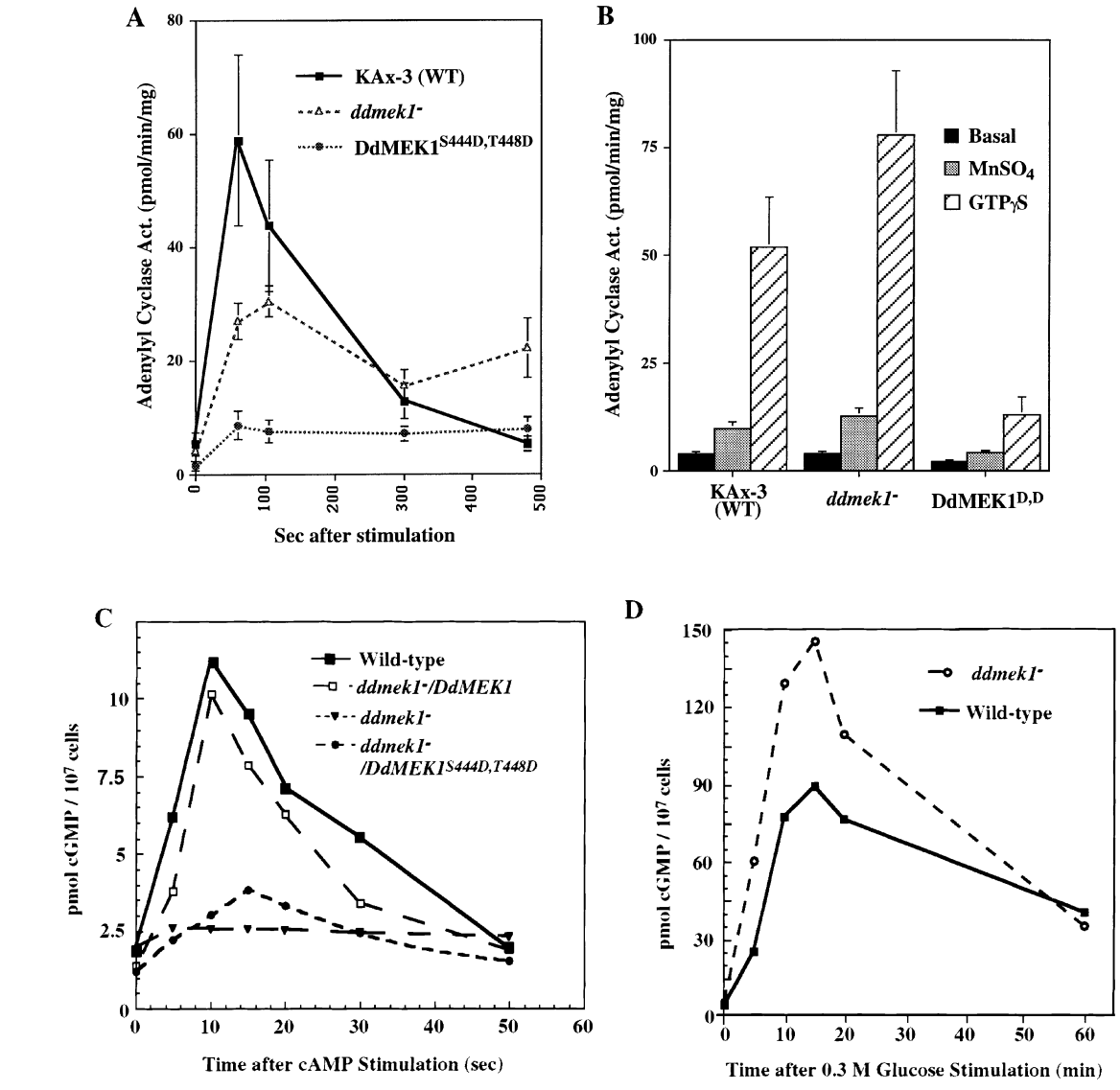


Fig. 7. Analysis of chemotaxis of *ddmek1* null and wild-type cells to cAMP. A small-population/drop assay was performed for chemotaxis (Konijn, 1970). Briefly, log-phase vegetative cells were washed, pulsed with cAMP (see legend to Figure 3; Materials and methods) and then placed on Na/KPO_4-containing agar plates in microdrops close to other microdrops containing different concentrations of cAMP. Multiple sets of microdrops were done for each concentration, and the assay was repeated on different days. The assay shown is for 1 μM cAMP. (**A**) Photographs taken at 20 and 70 min after placing the drops on the agar. Note the movement of wild-type cells but not *ddmek1* null cells towards the drop containing cAMP at the 70 min time point. (**B**) A similar assay done at a different time and images taken at a higher magnification. Note the elongation of wild-type cells. Some short streams of cells moving toward the drop containing the cAMP are observed. No chemotaxis towards the adjacent drop was observed if the drop did not contain cAMP.

wild-type-sized aggregates (Figure 9). Similar splitting of forming aggregates was observed in cells that were developed for 11 h before shifting the cells to the non-permissive temperature. Even though mounds had already started to form, significant splitting was observed (Figure 9). However, once a tip is formed, shifting of the cells from the permissive to non-permissive temperature did not alter the size of the aggregates (data not shown). When DdMEK1^{ts} (DdMEK1^{P458T}) is expressed in wild-type cells, development is normal at the permissive temperature. However, at the non-permissive temperature, small aggregates are formed, suggesting that DdMEK1^{ts} is dominant (Figure 9, Table I). When a temperature shift is performed at 6 h of development, small aggregates are formed (Figure 9), similar to our observations with DdMEK1^{ts} expressed in *ddmek1* null cells.

To determine if DdMEK1 was required at the time of cAMP-stimulated guanylyl cyclase activation or required for some earlier step that is permissive for guanylyl cyclase activation, we performed temperature shift experiments. *ddmek1* null cells expressing DdMEK1^{ts} and wild-type cells (control) were grown at the permissive temperature (18°C). The cells were harvested, washed, pulsed for 4.5 h at 18°C, and then split. The cells were then shaken at either the permissive or non-permissive temperature (27°C) for 30 min, washed and then activated and assayed at the respective temperatures. As observed in Figure 8E, *ddmek1* null cells expressing DdMEK1^{ts} showed a level of guanylyl cyclase activation (as measured by cGMP production) at the permissive temperature that was approximately two-thirds that of wild-type cells. For both wild-type and DdMEK1^{ts}-complemented null cells, cGMP levels peaked at 15 s compared with the 10 s observed for wild-type and wild-type DdMEK1-complemented *ddmek1* null cells



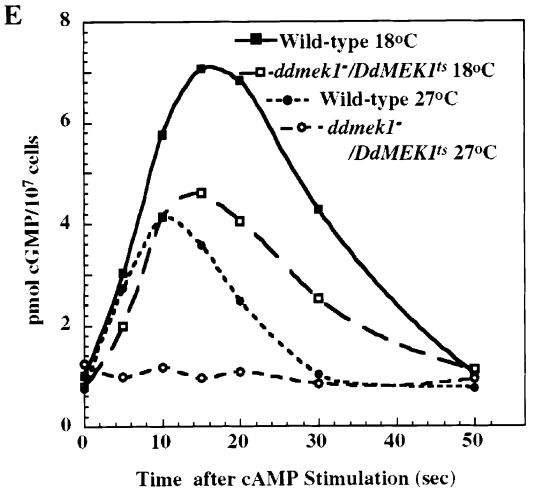


Fig. 8. Activation of adenylyl and guanylyl cyclases in wild-type and DdMEK1 mutants. (**A**) *In vivo* activation of adenylyl cyclase. Cells previously pulsed with 50 nM cAMP were stimulated with 10 μM cAMP and lysed, and enzyme activity was assayed for 1 min at room temperature. (**B**) *In vitro* activation of adenylyl cyclase. Cells were prepared as described for (A), lysed and assayed under basal conditions (no addition), or in the presence of 5 mM $MnSO_4$ or 40 μM GTPγS and 1 μM cAMP for 2 min at room temperature. See Materials and methods for details. The results presented are averages of 3–5 independent experiments. (**C**) cAMP-stimulated activation of guanylyl cyclase in cells pulsed with 30 nM cAMP for 4.5 h. cGMP levels were measured as described in Materials and methods in response to 1 μM cAMP. Guanylyl cyclase was measured at 22°C. Curves shown are from the same experiment and are representative of at least three independent experiments. (**D**) Osmotic shock activation of guanylyl cyclase in wild-type and *ddmek1* null cells. Cells that had been starved for 2 h were stimulated with 0.3 M glucose, which has been demonstrated to result in an extended activation of guanylyl cyclase (Kuwayama *et al.*, 1996; Oyama, 1996). Note that the time course on this curve is in minutes rather than seconds as in the other parts of the figure. Details and conditions were as described in (C). (**E**) Wild-type cells or *ddmek1* null cells expressing $DdMEK1^{ts}$ were grown and pulsed with 30 nM cAMP at 18°C as described in (C). The cells were then split during the last 30 min of shaking, and half were incubated at the non-permissive temperature (27°C) while the remainder were incubated at 18°C. The cells were then cAMP-stimulated at either 27 or 18°C. Details as described in (C).

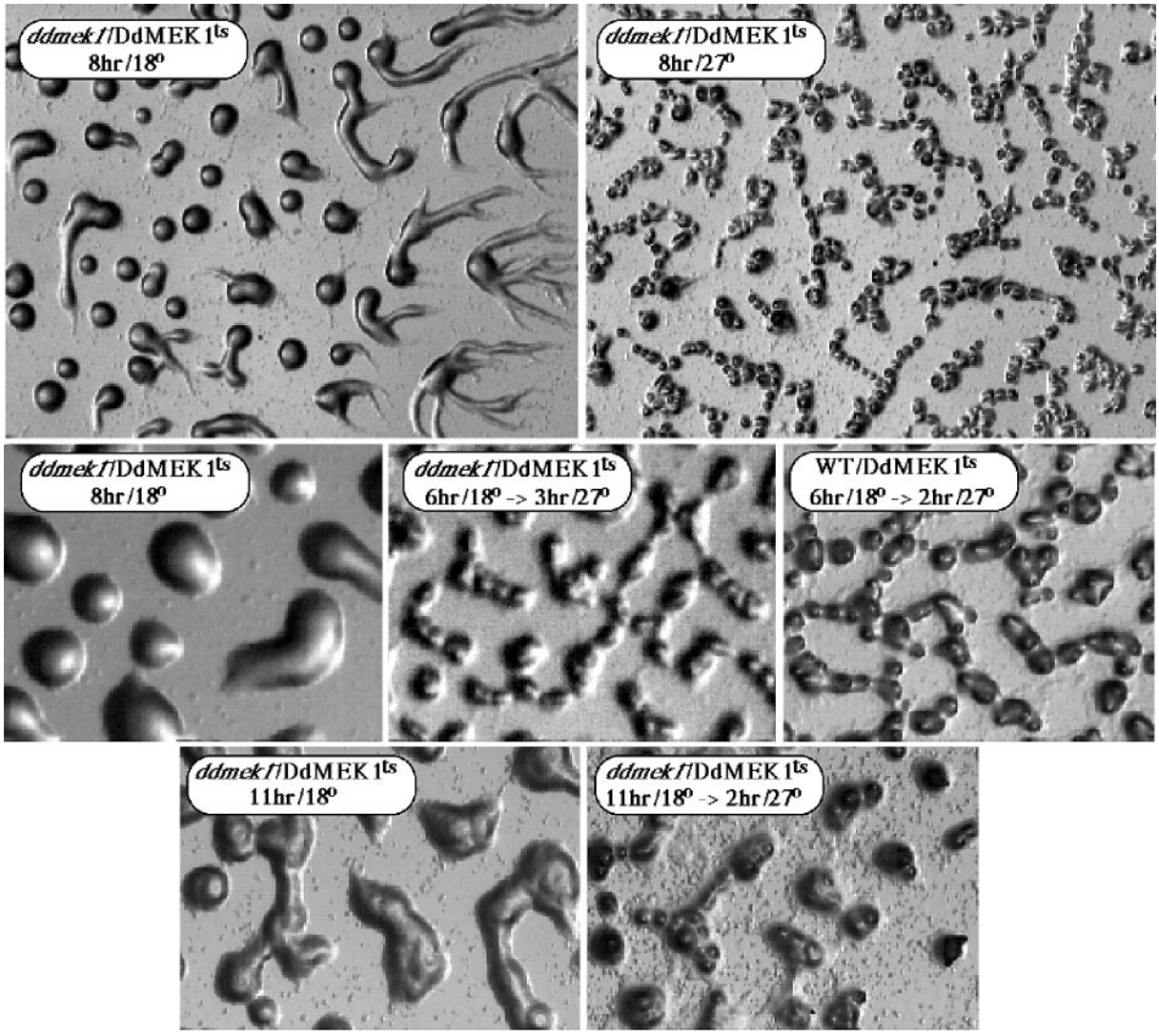


Fig. 9. Morphology of *ddmek1* null or wild-type cells expressing temperature-sensitive mutants of DdMEK1 (see text). In the upper panel, cells were developed for 8 h at either 18 or 27°C. Both photographs were taken at the same magnification. The middle and lower set of panels depict temperature shift experiments. The first panel in the middle shows DdMEK1 cells expressing DdMEK1^{ts} at 8 h of development at 18°C. The second panel shows the same strain, which was allowed to develop to 6 h at 18°C and shifted to 3 h at 27°C. The right-most panel shows wild-type cells expressing the temperature-sensitive DdMEK1 mutant that had been developed for 6 h at the permissive temperature and then shifted to 2 h at the non-permissive temperature. The lower panel shows cells developed for 11 h at 18°C (left panel) and then the same cells after having been shifted to 27°C for 2 h.

at 22°C (Figure 8C and E). At 27°C, wild-type cells had a level of guanylyl cyclase activity that was ~60% of that at 18°C, whereas *ddmek1* null cells expressing DdMEK1^{ts} showed no measurable activation. These results are consistent with DdMEK1 being required at the time of guanylyl cyclase activation by cAMP.

DdMEK1 is not required for cAMP activation of the MAP kinase ERK2

The activity of the *Dictyostelium* MAP kinase ERK2, which is required for the activation of adenylyl cyclase but not guanylyl cyclase, is stimulated ~40-fold in response to cAMP (Segall *et al.*, 1995; Maeda *et al.*, 1996). Although the phenotypes of the *ddmek1* and *erk2* null mutants suggest that DdMEK1 does not lie upstream from ERK2 (*erk2* null cells are aggregation deficient), we assayed cAMP-mediated activation of ERK2 by an 'in gel' kinase assay using myelin basic protein (MBP) as a substrate (Maeda *et al.*, 1996). There were no observable differences between the kinetics and level of ERK2 activation in *ddmek1* null cells and wild-type cells (data not shown).

Discussion

DdMEK1 encodes a MAP kinase kinase required for chemotaxis to cAMP

We have identified a MAP kinase kinase, DdMEK1, from *Dictyostelium* cells that is essential for proper aggregation and regulates a pathway not previously described for members of MAP kinase cascades. *Dictyostelium* DdMEK1 is highly related to known MEKs from a variety of organisms, and essential residues, including the sites of serine/threonine phosphorylation required for activation of MEKs, are conserved. Mutation of these residues to alanines or the lysine required for ATP binding to alanine

results in a protein that cannot complement the null mutation, consistent with these functionally conserved sites being required for activation and/or function of the protein, respectively. These mutant enzymes have dominant-negative effects when expressed in wild-type cells. Conversion of these sites to either Glu or Asp results in a protein that complements the aggregation-stage defect of *ddmek1* null cells. These data are consistent with *Dictyostelium* DdMEK1 encoding a MAP kinase kinase with properties that have been observed in MEKs from other organisms (Mansour *et al.*, 1994; Pages *et al.*, 1994; Zheng and Guan, 1994; Errede and Ge, 1996).

Dictyostelium DdMEK1 has a null phenotype that results in extremely small aggregates. cAMP wave patterns emitted through the field of cells are observed, and *in vitro* assays demonstrate that *ddmek1* null cells can activate adenylyl cyclase in response to cAMP signaling. As the wave patterns are visualizations of the changes in cell shape in response to cAMP stimulation, *ddmek1* null cells are capable of morphological changes in response to cAMP. However, in contrast to what is seen with wild-type cells, *ddmek1* null cells do not migrate inward towards an aggregation center in response to these cAMP waves, and only a very small aggregate is formed. The initial sizes of the aggregation domains are similar in wild-type and *ddmek1* null cells. In addition, these cells show no chemotaxis to cAMP in a microdrop assay. The cells demonstrated a minimal chemoattractant-mediated cell elongation in some assays.

cAMP-mediated stimulation of adenylyl cyclase in *ddmek1* null cells has a lower peak of activity and a delayed rate of adaptation. However, both intrinsic ($MnSO_4$) and total G protein-mediated activation of adenylyl cyclase remained unchanged compared with wild-type cells. We do not think that the modestly altered *in vivo* activation profile of adenylyl cyclase in *ddmek1* null cells is significant. Rather, we postulate that the formation of small aggregates is due to the inability of the cells to migrate inward towards the aggregation center in a concerted fashion. The aggregation domains cannot be or are not maintained, leading to a proliferation of smaller, weaker centers and eventually to the formation of very small aggregates. Whereas the cells cannot migrate normally to form aggregates, it is possible that stable associations are formed through cell–cell contacts maintained by cell adhesion molecules known to be induced by cAMP signals during aggregation (Gerisch, 1977; Gerisch *et al.*, 1977; Loomis, 1983; Noegel *et al.*, 1986). This may lead to the coalescing of small groups of cells into small aggregates.

DdMEK1 is required for cAMP-mediated activation of guanylyl cyclase

The major biochemical defect that can be observed is a significantly reduced ability of *ddmek1* null cells to activate guanylyl cyclase in response to cAMP signaling. It is thought that activation of guanylyl cyclase is essential for aggregation (Van Haastert and Devreotes, 1993; Newell, 1995). Basal levels of guanylyl cyclase activity, as determined by the basal levels of cGMP in unstimulated cells, appear normal, and osmotic shock-stimulated activation of guanylyl cyclase is normal in *ddmek1* null cells. Genetic analysis has suggested that there is a single guanylyl cyclase structural gene for basal, cAMP- and osmotic shock-stimulated guanylyl cyclase activity (Kuwayama *et al.*, 1993, 1995, 1996). Guanylyl cyclase-deficient mutants are unable to undergo chemotaxis. Our results indicate that DdMEK1 is essential only for the cAMP receptor-mediated guanylyl cyclase activation, indicating that the mechanisms by which guanylyl cyclase is activated in response to cAMP and osmotic shock are distinct.

ddmek1 null cells expressing putative constitutively active DdMEK1s ($DdMEK1^{S444E/D,T448E/D}$) show a normal aggregation response and produce aggregate sizes that are slightly smaller than those of wild-type cells. Guanylyl cyclase activity is not constitutive in these cells, but is activated in response to cAMP stimulation. While the absolute level of activation is significantly lower than that observed in wild-type cells, the increase in cGMP levels is several fold. The lower activity could be due to a lower activity of the $DdMEK1^{S444D,T448D}$ protein compared with that of wild-type activated (phosphorylated) DdMEK1, as has been observed with similarly mutated MEKs in other systems (Mansour *et al.*, 1994) or due to negative feedback loops that might be constitutive in cells expressing $DdMEK1^{S444D,T448D}$, as has been observed for the yeast MEK Ste7 (Errede and Ge, 1996). While these cells aggregate, the level of chemoattractant stimulation of adenylyl cyclase is also reduced in this strain, indicating that the putative constitutive forms of DdMEK1 may have effects on signaling pathways in addition to the activation of guanylyl cyclase. Alternatively, overexpression of active DdMEK1 might interfere with other MAP kinase cascades not normally regulated by DdMEK1, possibly due to mislocalization of the protein and/or cross-talk between pathways as has been observed in yeast (Yasar *et al.*, 1995). DdMEK1 may directly regulate pathways that may not be essential for aggregation under the conditions tested, and the dominant mutation unmasked these functions. The partial developmental arrest of these cells and of wild-type cells expressing $DdMEK1^{S444D,T448D}$ at the mound stage also suggests that DdMEK1 regulates additional pathways, possibly ones that are distinct from those that control aggregation.

Our analysis of $DdMEK1^{ts}$ sugggests that DdMEK1 may not be necessary for subsequent morphogenesis. The DdMEK1 temperature-sensitive mutation shows normal-sized aggregates at the permissive temperature and a null phenotype at the non-permissive temperature. Shifting of cells expressing $DdMEK1^{ts}$ during aggregation results in small aggregates; however, shifting of the cells after tight aggregate formation results in no observable morphological phenotype (H.Ma and R.A.Firtel, unpublished observations). It is possible that the absence of phenotypes later in development is due to guanylyl cyclase not being required during these stages. Alternatively, DdMEK1 may be essential for the potentiation of chemotaxis and the activation of guanylyl cyclase only during aggregation and not in the later stages. For example, we cannot discount the possibility that the mechanism of receptor-mediated guanylyl cyclase activation does not require DdMEK1 at this stage or that the temperature shifts might be less effective at affecting accumulated DdMEK1 at these later times. We also note that the chemotaxis-deficient phenotype observed for *ddmek1* null cells is quite different from that observed for other chemotaxis-defective mutants, such as those directly affecting guanylyl

cyclase activity and a downstream cGMP-binding protein (Kuwayama *et al.*, 1993, 1995). While *ddmek1* null cells form small aggregates, the other mutants do not form aggregates of any size. The very small aggregates that are formed in *ddmek1* null cells show normal morphogenesis, indicating that DdMEK1 is not essential for these processes; however, *ddmek1* null cells show a very low cAMP-mediated activation of guanylyl cyclase, and it may be possible that this is sufficient for morphogenesis. We also note that *ddmek1* null cells are capable of some cell shape changes in response to wave patterns, as determined by the time-lapse video microscopy, but are unable to move significant distances. It is possible that, in small aggregates, the cells are capable of moving sufficient distances to allow morphogenesis. The fact that *Dictyostelium* spatial patterning is independent of aggregate size may allow the *ddmek1* null cells to undergo morphogenesis if the organism is sufficiently small. Alternatively, morphogenesis during the later stages involves guanylyl cyclase-independent pathways, and these pathways are distinct from those regulating chemotaxis. The temperature-sensitive mutant also has a dominant phenotype during aggregation but not during later development. We would therefore expect that not only is the temperature-sensitive mutant inactive at the non-permissive temperature, but it also interacts with, and may sequester, either an upstream or downstream component of this pathway. We have also observed this with expression of a temperature-sensitive mutant of the MAP kinase ERK2 (Gaskins *et al.*, 1996; L.Aubry and R.A.Firtel, unpublished observations).

Multiple MAP kinase cascades regulate aggregation

We have identified two distinct putative MAP kinase cascades, one of which is required for the proper activation of adenylyl cyclase, while the other, as described in this study, is required for proper activation of guanylyl cyclase in response to chemoattractant signaling (Figure 10). The lack of overlapping phenotypes of *erk2* and *ddmek1* null cells and the normal cAMP-mediated activation of ERK2 in *ddmek1* null cells strongly suggest that ERK2 and DdMEK1 are components of distinct MAP kinase cascades. Whether these pathways contain common upstream elements (e.g. MEKK) is not known. The fact that we have a temperature-sensitive phenotype in the cAMP-mediated stimulation of guanylyl cyclase 30 min after cells carrying the DdMEK1^{ts} were shifted to the non-permissive temperature suggests that DdMEK1 directly regulates cytoplasmic responses and does not control guanylyl cyclase activation via a transcriptional activation pathway. The fact that DdMEK1 is maximally expressed during the aggregation stage of development is consistent with its role in mediating chemotaxis. We note that DdMEK1 is expressed later in development and that receptor activation of guanylyl cyclase occurs at these stages, although at a lower level than during aggregation (Schaap and Wang, 1985). We cannot exclude the possibility that DdMEK1 is involved in mediating responses in addition to the activation of guanylyl cyclase. Moreover, while our data are consistent with a cytoplasmic or membrane role for DdMEK1 in controlling this process, it is not excluded that DdMEK1 functions in a MAP kinase cascade that also involves nuclear signaling and transcriptional regulation. Determination of whether DdMEK1 activity is stimulated in response to cAMP will be important in elucidating its function.

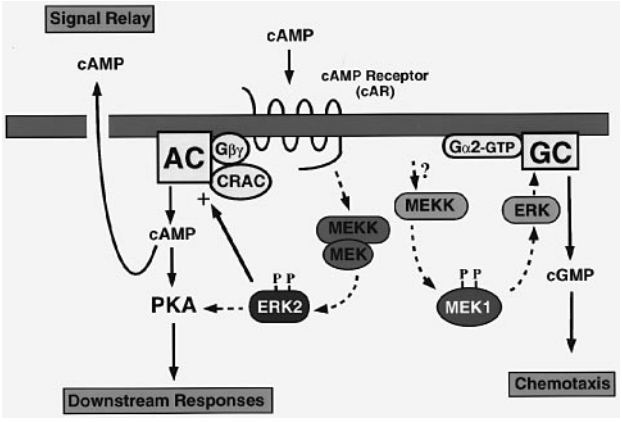


Fig. 10. Cartoon of the MAP kinase cascades involved in aggregation. The cartoon shows the involvement of two MAP kinase cascades, one containing the MAP kinase ERK2, which has been shown to be required for the activation of adenylyl cyclase (Segall *et al.*, 1995; Maeda *et al.*, 1996), and DdMEK1, which we show is essential for cAMP-mediated activation of guanylyl cyclase. As described in the text, adenylyl cyclase activation requires the Gβγ subunit and CRAC, a PH-domain-containing soluble protein that translocates to the plasma membrane in response to cAMP signaling (Lilly and Devreotes, 1995; Chen *et al.*, 1996). Guanylyl cyclase, as indicated in the text, is left to be activated by the Gα2 protein in the GTP-bound state (Kumagai *et al.*, 1989, 1991; Okaichi *et al.*, 1992; Valkema and Van Haastert, 1994). The model depicts an upstream MEKK and a downstream ERK, although these have not been identified. The mechanisms by which such a putative upstream MEKK and thus DdMEK1 are activated is not known. A dotted arrow with a question mark is shown to indicate this point. Production of cGMP is required for mediating chemotaxis (see Introduction). Adenylyl cyclase produces cAMP, which is involved in signal relay and the activation of PKA (Firtel, 1995; Chen *et al.*, 1996; Aubry *et al.*, 1997). See text for additional references and discussion.

The activation of DdMEK1 is necessary, but not sufficient, for guanylyl cyclase activation. We know that the Gα subunit Gα2 is required for cAMP-stimulated activation of guanylyl cyclase (Snaar-Jagalska *et al.*, 1988; Kumagai *et al.*, 1989, 1991). We do not know whether Gα2 directly participates in the activation of DdMEK1, guanylyl cyclase or possibly both pathways. The pathway leading to the activation of DdMEK1 is not known, although we presume it includes an upstream MEKK and a downstream MAP kinase (Figure 10). Activation of adenylyl cyclase is thought to be mediated by the Gβγ subunit and requires other components including CRAC, a PH-domain-containing protein, the activation of ERK2 and the activity of the putative Ras exchange factor Aimless (Chen *et al.*, 1996). Neither CRAC nor Aimless is required for activation of guanylyl cyclase, which is thought to require the GTP-bound state of the Gα2 subunit (Okaichi *et al.*, 1992; Van Haastert and Devreotes, 1993; Insall *et al.*, 1994, 1996; Valkema and Van Haastert, 1994). Adenylyl and guanylyl cyclases regulate distinct downstream signaling pathways. There must be specific physiological reasons why two at least partially non-overlapping MAP kinase pathways evolved to regulate adenylyl and guanylyl cyclases. These include a possible requirement to activate adenylyl and guanylyl cyclases independently in response to different chemoattractant signals or to other stimuli at other times in development.

Guanylyl cyclase can be activated by two independent pathways, one through chemoattractant receptors and one through an osmotic stress pathway. Only the receptor-mediated pathway requires DdMEK1. Interestingly, we have shown that DdMEK1 is hyperphosphorylated transiently in response to osmotic stress (as determined by a mobility shift on SDS–PAGE that is sensitive to PP2A treatment; H.Ma, M.Gamper and R.A.Firtel, unpublished observations) but not in response to cAMP even when assayed very rapidly (5 s) after cAMP stimulation. The phosphorylation responsible for the mobility shift is not on the activating Ser/Thr residues, as the DdMEK1^{S444A,T448A} mutant protein, which cannot be activated, is hyperphosphorylated. Moreover, this phosphorylation occurs in a *ddmek1* null background. It is possible that this phosphorylation, which is not the result of phosphorylation on the activating Ser/Thr residues, may be involved in differentially regulating guanylyl cyclase in response to the different signaling pathways.

Materials and methods

Cell culture, transformation and development

Dictyostelium cells were grown and transformed using standard techniques (Nellen *et al.*, 1987). Clonal isolates of non-drug-resistant strains were selected by plating clonally on SM medium containing agar in association with *Klebsiella aerogenes* as a food source (Firtel and Chapman, 1990). DdMEK1 knockout vectors were transformed into KAx-3 wild-type cells and selected for blasticidin resistance as described (Brott *et al.*, 1993). Clonal isolates for strains carrying DdMEK1 expression vectors or *lac*Z reporter constructs were transformed using G418 as a selectable marker (Nellen *et al.*, 1987). Clonal isolates were selected on DM plates in association with *Escherichia coli* B/r carrying neomycin resistance (Hughes *et al.*, 1992).

Morphology was examined by plating cells at different densities on non-nutrient agar plates containing 12 mM Na/KPO_4 (pH 6.2) containing agar.

For time-lapse video microscopy, cells were plated as a monolayer on a very thin layer of Na/KPO_4-containing agar (2.5 ml of agar was added to a 60 mm Petri dish). Cells were visualized on inverted plates using a Nikon Optiphot-2 phase contrast microscope with 4× or 10× objectives, as indicated in the figure legends. Time-lapse videos were recorded on a Mitsubishi SVHS time-lapse video recorder (S-H5600) with a compression ratio of 120 (2 h tape recorded over 240 h). Images from the videotape were grabbed using NIH Image 1.59 and a Scion image board.

Molecular biology

PCR cloning of DdMEK1 was achieved using the following degenerate primers made against conserved domain members of the MAP kinase family: primer 1 is the sense strand coding for the conserved sequence PYIVG/Q/DFYGA (5′ CC[A/T]TA[T/C]AT[T/C/A]GT[T/A/C][A/C/G][A/G]NTT[T/C]TA[T/C]GG 3′); primer 2 is the antisense strand coding for the sequence CDFGVSGQ (5′ TG[A/T/G]CC[A/T/G]GA[A/T/G]AC[A/T/G]CC[G/A]AA[G/A]TC [A/G]CA 3′). Amplification was done at 72°C. A λZap library made from RNA taken between 8 h (late aggregation) and ~16 h (slug stage) was screened for isolation of full-length DdMEK1 cDNA clones containing the full-length ORF (Schnitzler *et al.*, 1994).

Site-directed mutagenesis was done using the Transformer Site-Directed Mutagenesis kit from Clontech. All constructs containing site-directed mutants were sequenced to confirm the nucleotide substitutions and the absence of other mutations.

RNA and DNA blot hybridizations were done using standard techniques (Nellen *et al.*, 1987).

The plasmid for homologous recombination was constructed by insertion of the 1.3 kb fragment encoding blasticidin resistance (Bsr) (Sutoh, 1993) into the *Eco*RV site located 1135 bp downstream from the translation initiation codon of *DdMEK1* cDNA in pBluescript SK(–). The plasmid was linearized by digestion with *Nde*I and *Kpn*I (274 bp 5′ and 679 bp 3′ from the *Eco*RV site, respectively) and transformed into KAx-3 cells by electroporation (Nellen, 1987) followed by selection with blasticidin (5 μg/ml). Single clones were isolated by growing transformants on a lawn of *K.aerogenes*, and genomic DNA was collected and subjected to Southern blot analysis.

All DdMEK1 constructs were expressed in *Dictyostelium* as stable G418-resistant transformants downstream from the *Act15* promoter as previously described (Dynes *et al.*, 1994).

Assays of adenylyl and guanylyl cyclases and chemotaxis

Adenylyl cyclase assays were performed as previously described (Devreotes *et al.*, 1987). Briefly, the cells were pulsed with 50 nM cAMP for 5 h, washed and resuspended at 8×10^7 cells/ml in 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 pH 6.2 and 2 mM $MgSO_4$. For *in vivo* stimulation, the cells were stimulated with 10 μM cAMP, lysed at specific time points and assayed for 1 min at room temperature. For *in vitro* stimulation, the cells were lysed and assayed under basal conditions (no addition), or in the presence of 5 mM $MnSO_4$ or 40 μM GTPγS and 1 μM cAMP for 2 min at room temperature. For both *in vivo* and *in vitro* procedures, the assays were performed with 100 μM ATP spiked with 5 μCi [α-^{32}P]ATP. The [^{32}P]cAMP produced was purified by sequential Dowex and alumina chromatography (Salomon, 1979).

Guanylyl cyclase was assayed using the cGMP assay kit from Amersham. Assay conditions were as described previously (Van Haastert and Van der Heijden, 1983; Kumagai *et al.*, 1991). Specific conditions are described in the legend to Figure 8C–E. Assays were done at 22°C unless otherwise specified.

Chemotaxis was measured using the small-population assay as described by Konijn (1970). Briefly, small microdrops of cAMP (concentrations tested: 0 and from 10^{-8} M to 10^{-4} M) and either wild-type or *ddmek1* null cells that had been pulsed for 4 h with 30 nM cAMP and then washed were placed in close proximity on fresh Na/KPO_4-containing agar plates. The shape and position of the cells were visualized periodically by phase contrast microscopy and the images recorded.

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