Dictyostelium Stress-activated Protein Kinase α , a Novel Stress-activated Mitogen-activated Protein Kinase Kinase Kinase-like Kinase, Is Important for the Proper Regulation of the Cytoskeleton

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Mitogen-activated protein kinase cascades regulate various cellular functions, including growth, cell differentiation, development, and stress responses. We have identified a new *Dictyostelium* kinase (stress-activated protein kinase [SAPK] α), which is related to members of the mixed lineage kinase class of mitogen-activated protein kinase kinases. SAPK α is activated by osmotic stress, heat shock, and detachment from the substratum and by a membrane-permeable cGMP analog, a known regulator of stress responses in *Dictyostelium*. SAPK α is important for cellular resistance to stresses, because SAPK α null cells exhibit reduced viability in response to osmotic stress. We found that SAPK α mutants affect cellular processes requiring proper regulation of the actin cytoskeleton, including cell motility, morphogenesis, cytokinesis, and cell adhesion. Overexpression of SAPK α results in highly elevated basal and chemoattractant-stimulated F-actin levels and strong aggregation and developmental defects, including a failure to polarize and chemotax, and abnormal morphogenesis. These phenotypes require a kinase-active SAPK α . SAPK α null cells exhibit reduced chemoattractant-stimulated F-actin levels, cytokinesis, developmental and adhesion defects, and a motility defect that is less severe than that exhibited by SAPK α -overexpressing cells. SAPK α colocalizes with F-actin in F-actin-enriched structures, including membrane ruffles and pseudopodia during chemotaxis. Although SAPK α is required for these F-actin-mediated processes, it is not detectably activated in response to chemoattractant stimulation.

INTRODUCTION

The actin cytoskeleton plays a vital role in numerous cellular processes, such as cell locomotion, phagocytosis, cytokinesis, cell adhesion, cell shape changes, and stress responses. During these processes, the actin cytoskeleton is dynamically changed, a process that involves F-actin polymerization and depolymerization and the reorganization of existing filament networks. Mutations of signaling molecules or structural elements involved in these changes affect cellular processes to various extents.

Eukaryotic cells are constantly exposed to stress conditions. To survive, cells adopt various mechanisms to adapt to environmental changes. Mitogen-activated protein kinase (MAPK) pathways regulate cellular responses to external stress in various organisms, including yeast, plants, and mammals (Canman and Kastan, 1996; Jonak *et al.*, 1996; Gustin *et al.*, 1998; Hirt, 2000; Kyriakis and Avruch, 2001; O'Rourke *et al.*, 2002). In *Saccharomyces cerevisiae*, the well-characterized HOG1 MAPK pathway is activated by osmotic stress and leads to gene expression and glycerol production, which is critical for yeast cells to withstand osmotic stress (Gustin *et al.*, 1998; O'Rourke *et al.*, 2002). In mammalian cells, the c-Jun NH₂-terminal kinase/stress-activated protein

kinase (JNK/SAPK1) pathway and the p38/SAPK2 MAP kinase pathway are both activated by diverse stimuli including UV, osmotic, and thermal stresses (Canman and Kastan, 1996; Chang and Karin, 2001; Kyriakis and Avruch, 2001). The activation of the JNK/SAPK1 pathway results in the phosphorylation and activation of transcription factors, which in turn regulate gene expression to resist stress conditions. The p38/SAPK2 MAPK pathway, in addition to its role in regulating gene expression by phosphorylation of transcription factors, has been linked to F-actin reorganization, possibly through the actin polymerization modifier HSP27 (Landry and Huot, 1995, 1999). In addition to their roles in stress responses, the JNK/SAPK1 and p38/SAPK2 pathways mediate other cellular responses. Both pathways are activated by cytokines such as tumor necrosis factor and interleukin-1 and are thereby implicated in cell differentiation and apoptosis (Ichijo, 1999; Rincon *et al.*, 2000). However, these two pathways also exhibit specificity toward different stimuli and participate in different cellular processes. For example, in endothelial cells, the p38/SAPK2 pathway, but not the JNK/SAPK1 pathway, is activated by vascular endothelial growth factor and mediates actin reorganization and cell migration (Rousseau *et al.*, 1997).

The activation of MAPK pathways is mediated by a family of protein kinases, MAP kinase kinases (MAPKKs) or MEK kinases (MEKKs). Identified mammalian MEK kinases include the Raf family, the MEK kinase (MEKK) family, and the mixed lineage kinase (MLK) family. The Raf family kinases selectively activate the extracellular signal-

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regulated kinase (ERK) pathway, which regulates growth, cell differentiation, and developmental processes (Nishida and Gotoh, 1993; Treisman, 1996). Members of the MEKK family activate both the ERK and JNK/SAPK pathways (Schlesinger *et al.*, 1998). The recently identified MLK family of MEKKs possesses a kinase domain exhibiting characteristics of both Ser/Thr and Tyr protein kinases (Gallo and Johnson, 2002). Several members of this kinase family activate JNK/SAPK stress signaling pathways. Recently, MLK family kinases (MLTK/ZAK) were reported to activate the JNK/SAPK and p38 pathways (Gotoh *et al.*, 2001; Gallo and Johnson, 2002). MLTKs are activated by osmotic shock, and overexpression of MLTK α , but not MLTK β , induces the disruption of actin stress fibers and dramatic changes in cell morphology mediated through the p38 MAP kinase pathway (Gotoh *et al.*, 2001). *Dictyostelium* grows as single-celled amoebae, which, upon starvation, aggregate to form a multicellular organism (Aubry and Firtel, 1999; Chen *et al.*, 1996). Both aggregation and subsequent morphogenesis involve chemotaxis to one or more secreted factors. Dissection of the chemotaxis pathways to cAMP, which mediates aggregation, has led to important new insights into this evolutionarily highly conserved process. Chemotaxis, or directional movement toward a small molecule ligand, involves the spatially regulated polymerization of F-actin at the leading edge resulting in the forward protrusion of a pseudopod. This is followed by a myosin II/F-actin-mediated contraction of the posterior of the cell or uropod (Parent and Devreotes, 1999; Firtel and Chung, 2000; Chung *et al.*, 2001; Iijima *et al.*, 2002). In response to hyperosmotic stress, *Dictyostelium* cells withstand the extensive cell shrinkage caused by osmolarity change, largely by remodeling of the cortical cytoskeleton (Insall, 1996; Kuwayama *et al.*, 1996; Zischka *et al.*, 1999). This remodeling includes the rearrangement of F-actin and myosin II fibers at the cell cortex to form the core of a rigid network resembling a shell-like structure, thereby providing the physical strength to resist the osmotic shock (Insall, 1996). Other known cytoskeletal changes activated by osmotic shock in *Dictyostelium* include tyrosine phosphorylation of actin, threonine phosphorylation of myosin II, and an increase in the levels of RMLC (regulatory myosin light chain) and cofilin and a decrease in DdLIM in the cortex (Howard *et al.*, 1993; Jungbluth *et al.*, in 1994, 1995; Aizawa *et al.*, 1999; Gamper *et al.*, 1999; Zischka *et al.*, 1999). In addition, the F-actin cross-linking protein α -actinin and the 120-kDa gelation factor are important for protection of *Dictyostelium* cells from osmotic stress and support the pivotal role the cytoskeleton plays in the osmotic response (Rivero *et al.*, 1996). Osmotic shock results in an ~ 1.6 -fold increase in F-actin content in *Dictyostelium*, as it does in neutrophils ($\sim$ twofold) (Hallows *et al.*, 1996; Zischka *et al.*, 1999; Rizoli *et al.*, 2000). In contrast to the signaling pathways controlling F-actin reorganization in response to chemoattractant stimulation, little is known about the signaling events mediating cytoskeleton reorganization in response to stress. The response of *Dictyostelium* cells to osmotic stress is mediated, at least in part, by cGMP and cAMP (Kuwayama *et al.*, 1996; Oyama, 1996; Kuwayama and Van Haastert, 1998; Ott *et al.*, 2000; Rizoli *et al.*, 2000), which are also important for controlling *Dictyostelium* chemotaxis and development. Hyperosmotic treatment of cells induces both cAMP and cGMP production, with a peak at 2 and 10 min, respectively (Oyama, 1996; Ott *et al.*, 2000). Biochemical and cell biological data indicate that cAMP accumulation in response to osmotic shock depends on DokA, which controls the intracellular cAMP phosphodiesterase RegA (Ott *et al.*,

2000). Although there are two guanylyl cyclases in *Dictyostelium*, cGMP accumulation is primarily due to the activation of the soluble guanylyl cyclase both during osmotic shock and chemoattractant stimulation (Roelofs and Van Haastert, 2002). Whereas the immediate downstream target of cAMP during osmotic shock is unknown, cGMP production is required for myosin II heavy chain phosphorylation, resulting in the disassembly of myosin II filaments, a prerequisite for their reassembly at the cell cortex (Egelhoff *et al.*, 1993; Kuwayama *et al.*, 1996).

Dictyostelium has three partially characterized MAP kinase pathways. The pathway containing the MAPK ERK2 regulates the activity and stability of the intracellular cAMP-specific phosphodiesterase RegA, whose proper regulation is essential for aggregation, morphogenesis, patterning, and cell-type differentiation (Shaulsky *et al.*, 1998; Thomason *et al.*, 1999; Mohanty *et al.*, 2001). The pathway containing MEK1 and ERK1 is essential for proper chemotaxis and aggregation (Ma *et al.*, 1997; Sobko *et al.*, 2002). One MEKK, MEKK α , has been characterized (Chung *et al.*, 1998). The phenotypes of *mekk α* null and overexpression strains suggest that MEKK α is required for proper morphogenesis and cell-type patterning. MEKK α does not couple downstream with either the ERK1 or ERK2 pathways.

Here, we describe a putative *Dictyostelium* MEKK-like kinase, SAPK α , which we demonstrate is involved in stress responses and is also required for other cellular processes. *Dictyostelium* SAPK α is activated by hyperosmotic and heat shock. SAPK α (*spkA*) null cells are more sensitive to hyperosmotic stress but also exhibit an altered F-actin cytoskeleton, resulting in cytokinesis and chemotaxis defects. *spkA* null cells aggregate, but form very small fruiting bodies when cells are plated at lower densities. In addition, *spkA* null cells seem to have reduced adhesion to the substratum and SAPK α is transiently activated when cells are detached from the substratum. Overexpression of SAPK α results in an elevated level of F-actin in resting cells. These cells do not polarize and move poorly toward the chemoattractant cAMP. Our results identify a novel MEKK-like kinase that is important for stress responses and other cellular processes, possibly through a direct or indirect involvement in remodeling the actin cytoskeleton.

MATERIALS AND METHODS

Materials

The anti-FLAG monoclonal antibody (mAb), fluorescein isothiocyanate (FITC)-phalloidin, tetramethylrhodamine B isothiocyanate (TRITC)-phalloidin, 8-bromo-cGMP (8-br-cGMP), latrunculin B, sodium orthovanadate, β -glycerophosphate, aprotinin, and leupeptin were purchased from Sigma-Aldrich (St. Louis, MO). Myelin basic protein (MBP) was purchased from Roche Diagnostics (Indianapolis, IN). [32 P] γ -ATP was from ICN Pharmaceuticals (Costa Mesa, CA). Protein G plus agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrocellulose filters (13 mm, 0.025 μ m, catalog no. VSWP01300) used for the adhesion assay were from Millipore (Bedford, MA).

Cell Culture and Development of *Dictyostelium* Cells

pdeD $^{-}$ cells in the Ax2 background were a gift from Marcel Meima and Pauline Schaap (School of Life Sciences, University of Dundee, Dundee, Scotland). Wild-type *Dictyostelium* strains KAx3 and Thy minus JH10 were used to generate various mutant strains as indicated. Cells were grown in nutrient medium (Watts and Ashworth, 1970) at 23°C either in shaking culture or on a Petri dish. For the developmental study, cells were washed twice in 12 mM Na/K phosphate buffer and resuspended at 7×10^7 cells/ml and plated at various densities on agar plates.

Generation of *spkA* Null Cells and Cells Expressing Wild-Type and Mutant SAPK α

The *spkA* null mutant was obtained by a gene replacement technique based on that of Manstein *et al.* (1989). Because there are two copies of SPKA genes, *spkA* null cells were generated in JH10 cells by using two targeting constructs containing a Thy cassette and a Bsr cassette, respectively.

To make wild-type SAPK α expression constructs, SAPK α was tagged at the C terminus with either the FLAG epitope or GFP. FLAG-tagged SAPK α was produced by polymerase chain reaction (PCR) of the coding region of SAPK α with primers 5'-GTTTTTACTAGTAAAAAAT GTCATCAACTCAACAA-CAACAACATC-3' and 5'-GTTTTCTCGAGTTATTGTC ATCGTCATCTT-TATAATCTTCTTGATTCTTTGGAAGTGG-3'. The PCR fragment was digested with *SpeI* and *XhoI* and cloned directly into the Exp4 (+) expression vector. For making SAPK α -GFP or N-terminal SAPK α -GFP constructs, GFP and SAPK α or N-terminal SAPK α were amplified by PCR and cloned simultaneously into an Exp4(+) vector through a three-piece ligation approach. For making the SAPK α K378A mutation, site-directed mutagenesis was done by a two-step PCR approach by using overlapping primers containing the desired mutation (Li and Wilkinson, 1997). The overlapping primers used were 5'-GAAGTTGCAATTGCAAGTATTAATCAATGACTG-3' and 5'-GATTTTAATCTGCAATTGCAACTTCTTTACCTC-3'. The DNA fragments containing the mutation were used to replace the corresponding fragments in the wild-type SAPK α constructs. All constructs were sequenced and transformed JH10 or KAx-3 or *spkA* null cells.

All transformations were done as described previously (Nellen *et al.*, 1987). Transformants were selected with 10 or 20 μ g/ml G418 and clones were isolated by plating cells on DM agar (12 mM Na/K phosphate buffer pH 6.1, 1% Bacto peptone, 0.2% D-glucose, 1.5% agar) containing 40 μ g/ml G418. In the case of making *spkA* null cells, cells were selected either with 7.5 μ g/ml blasticidin for the Bsr cassette or HL5 media without thymidine supplement for the Thy cassette.

Chemotaxis Assay

Aggregation-competent cells were made by pulsing cells with cAMP as described previously (Meili *et al.*, 1999), except that cells were pulsed at 6-min intervals. Pulsed cells of 4×10^5 cells/ml were plated onto a small dish with a hole covered by a 0.17-mm glass coverslip at 40 μ l/dish and allowed to adhere for 15 min. A micropipette filled with 150 μ M cAMP was positioned close to cells by using a micromanipulator (Eppendorf Patchman), and the response of cells was recorded at 6-s intervals by using a time-lapse video recorder and NIH Image software. Cell movement was analyzed using the DIAS program (Wessels *et al.*, 1998).

Immunofluorescence Microscopy

Cells expressing green fluorescent protein (GFP) fusion or FLAG-tagged SAPK α were fixed with 3.7% formaldehyde in Na/K phosphate buffer for 5 min, washed with Na/K phosphate buffer three times, and permeabilized with 0.1% Triton X-100 for 1 min. Cells were washed and incubated with phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20 for 1 h. For cells expressing FLAG-tagged SAPK α , cells were first incubated with anti-FLAG (1:200) for 1 h. Cells were stained with FITC-conjugated anti-mouse IgG (1:200) and TRITC-labeled phalloidin for 1 h, washed, and mounted for observation with a 60 $\times$ oil immersion lens on a Nikon Microphot-FX microscope. For cells expressing GFP fusion SAPK α proteins, cells were directly stained with TRITC-labeled phalloidin for 1 h, washed, and mounted for observation. For nuclei staining, after fixation and permeabilization, cells were mounted with mounting medium containing 0.0001% Hoechst dye.

In Vivo Actin Polymerization Assay

Cellular F-actin was quantified using a method originally developed by Howard and Oresajo (1985), except that TRITC-phalloidin was used. Cells were pulsed with 30 nM cAMP at 6-min intervals for 5 h and were then collected by centrifugation and resuspended in Na/K phosphate buffer to 1×10^7 cells/ml and shaken at 200 rpm with 2.5 mM caffeine for 20 min. Cells were spun down, resuspended again with phosphate buffer containing 2.5 mM caffeine, and stimulated with 100 μ M cAMP. Aliquots of cells (100 μ l) were taken at 0, 5, 10, 15, and 30 s and mixed with 1 ml of actin buffer (20 mM KH₂PO₄/10 mM PIPES pH 6.8/5 mM EGTA/2 mM MgCl₂) containing 3.7% formaldehyde, 0.1% Triton X-100, and 0.4 μ M TRITC-phalloidin. Cells were fixed and stained for 1 h and spun down at $10,000 \times g$ for 5 min in the microcentrifuge. The pellet was extracted with 1 ml of 100% methanol and fluorescence was measured (525 excitation/565 emission).

Kinase Assay

For cAMP stimulation, kinase assays were performed as described previously (Meili *et al.*, 1999). For responses to various stress conditions, cells were washed twice with 12 mM Na/K phosphate buffer, pH 6.1, and resuspended at 3×10^7 cells/ml in the same buffer. Cells were then shaken at 150 rpm for

1 h and treated by various stressors as indicated. For kinase assay after cell detachment, cells were first attached to plates for 30 min, starved for 1 h, and quickly resuspended in Na/K phosphate buffer by pipetting. In each case, an aliquot of 500 μ l of cells was taken at the indicated time point and lysed immediately by mixing with an equal volume of 2 $\times$ lysis buffer containing 50 mM Tris, pH 7.6, 200 mM NaCl, 20 mM NaF, 2 mM vanadate, 50 mM β -glycerophosphate, 6 mM sodium pyrophosphate, 2 mM EDTA, 2 mM EGTA, 4 μ g/ml leupeptin, 4 μ g/ml aprotinin, 2% NP-40, 20% glycerol, 2 mM dithiothreitol. The lysate was precleared by centrifugation. One microliter of anti-FLAG antibody (4 μ g/ μ l) was added and incubated with agitation in a cold room for 1 h. The formed immune complexes were collected with 40 μ l of a 1:1 slurry of protein G beads by incubation under agitation for 1 h at 4°C. The beads were washed twice with lysis buffer and twice with kinase buffer (25 mM MOPS, pH 7.4, at room temperature, 25 mM glycerophosphate, 20 mM magnesium chloride, 1 mM dithiothreitol). The kinase activity was measured using MBP as a substrate in a reaction in 60 μ l of kinase buffer containing 5 μ Ci of [³²P] γ -ATP, 5 μ M cold ATP, and 5 μ g of MBP. The reactions were stopped by adding 20 μ l of 4 $\times$ SDS buffer and boiling for 5 min. Samples were resolved by SDS-PAGE (12.5%), blotted onto polyvinylidene difluoride membrane (Millipore), and exposed to film.

Cell Viability Assay

Measurement of cell survival was performed as follows. Briefly, cells were resuspended at 3×10^7 cells/ml in Na/K phosphate buffer and stimulated with 400 mM glucose. Cells were diluted 1000 times at each time point and 200 cells were mixed with *Escherichia coli* B/r and plated on 1/3 SM plates. The number of surviving cells was counted several days later. The cell viability after osmotic stress is presented as percentage of cells that survived after treatment compared with the survival rate of untreated cells.

Cell Adhesion Assay

The cell-substrate adhesion assay was performed with cells attached to nitrocellulose filters. Cells were washed twice with Na/K phosphate buffer and resuspended at 2×10^7 cells/ml. The amount of 4×10^5 cells in 200 μ l were plated onto 13-mm circular nitrocellulose filters (Millipore). After 30 min, unattached cells were removed by dipping filters into Na/K phosphate buffer. Filters were transferred to microcentrifuge tubes filled with 1 ml of Na/K phosphate buffer with the side of the filter containing the cells facing away from the wall of the tube. All of the tubes were placed simultaneously onto an Eppendorf 5432 mixer and vortexed for 10 s. Cells left in the buffer were counted and regarded as detached. Filters were transferred to fresh tubes each containing 1 ml buffer with 10 mM EDTA. Cells were completely detached from filters by vortexing for 1 min and were counted. These cells were regarded as attached. Cell adhesion is presented as percentage of cells attached compared with those detached. This experiment was repeated three times, each time with three filters for each strain.

RESULTS

Identification of the Novel Protein Kinase SAPK α

spkA, was identified by PCR with degenerate primers based on the conserved sequences within the kinase domain of MAP family members (Ma *et al.*, 1997). The full-length cDNA was subsequently obtained by screening a λ ZAP cDNA library (see MATERIALS AND METHODS). The full-length cDNA contains an open reading frame encoding a protein of 638 amino acids and is named SAPK α . SMART analysis predicts five ankyrin motifs at the N terminus followed by a SAM domain (sterile α -motif) and a dual-specificity kinase domain at the C terminus of the protein (Figure 1A). SAM domains are involved in protein-protein interactions and dimer formation of several signaling molecules and transcription regulators (Ponting, 1995). A BLAST search against current databases failed to identify any orthologs in other organisms. However, the kinase domain showed the highest homology to MEKK family members in mammals and plants (Figure 1B). Among these are EDR1 (36% identity, 51% similar), which negatively regulates defense responses in plants (Frye *et al.*, 2001); mammalian MLTK α (also known as ZAK, 34% identity, 54% similar), an MEKK that can activate the ERK, JNK/SAPK, p38, and ERK5 pathways (Gotoh *et al.*, 2001); CTR1 (36% identity, 51% similar), a RAF-like kinase and a negative regulator of ethylene response in plants (Kieber *et al.*, 1993); and MAP3K10

Figure 1. Sequence analysis of *Dictyostelium* SAPKα. (A) Deduced amino acid sequence of SAPKα from the 2075-base pair cDNA clone. The boxed region at the N terminus is the ankyrin repeat domain. The kinase domain at the C terminus is underlined. The SAM domain in the middle is also underlined but with dashed lines. (B) Alignment of the kinase domain of SAPKα with that of other kinases including tomato CTR1 (AF110519), barley EDR1 (AF305912), human MAP3K (NM_002446), and mouse MLTKα (AB049731). (C) Schematic diagram of the domain structures of SAPKα and other kinases with close homology in the kinase domain. Ank, ankyrin repeat motifs. (D) Northern blot showing the developmental time course of SAPKα expression. Total RNA of 8 μg per sample was resolved on a 1.0% denaturing agarose gel, blotted and probed as described previously (Datta and Firtel, 1987). The 0-h time point is for vegetative cells.

(also known as MST/MLK2, 34% identity, 50% similar), a member of the MLK family which directly phosphorylates and activates SEK1, an activator of JNK/SAPK (Hirai *et al.*, 1997). MLTKα also has a SAM domain; however, unlike SAPKα, the MLTKα SAM domain is C-terminal to the kinase domain (Figure 1C).

To examine the timing of SAPKα gene expression during the *Dictyostelium* developmental cycle, we performed RNA blot analysis. As depicted in Figure 1D, there are two SAPKα transcripts. The smaller transcript is high in vegetative cells and during early development and rapidly decreases after 12 h, the time of tipped aggregate formation. A larger transcript occurs at 8 h of development, remains high through the 12-h time point, and then decreases, like the smaller transcript. Neither transcript is detected in the *spkA* null strain (our unpublished data). In strains in which one of the two copies of the *SpkA* gene was disrupted, both transcripts are still observed. These data indicate that both transcripts derive from the same gene. As is the case for other genes in *Dictyostelium* expressing two transcripts, we expect this is due to two promoters rather than differential splicing within the open reading frame (Faure *et al.*, 1990; Mann and Firtel, 1991; Saxe *et al.*, 1991; Mann *et al.*, 1992; Rogers *et al.*, 1997).

spkA Null Cells Are Defective in Cytokinesis

To investigate the function of SAPKα, we generated *spkA* null strains by gene disruption via homologous recombination as described in the MATERIALS AND METHODS and confirmed by Southern and Northern blot analyses (our unpublished data). Our analyses indicate that there are two identical copies of *SpkA* in the genome, probably due to a duplication of a region of the genome. We therefore used two sequential gene disruptions with two selectable markers to obtain *spkA* null strains (see MATERIALS AND METHODS; our unpublished data). We identified three independent *spkA* null strains (both copies disrupted). All exhibited the same growth and developmental defects (our unpublished data). One was used for all of the experiments described.

spkA null cells were very large when grown on Petri dishes, suggesting the cells have a cytokinesis defect (Figure 2A). These giant cells often rounded up and detached from plates. *spkA* null cells also show a very distinct cell morphology. Cells are often round or irregular with essentially no filopodia. Nuclear staining with 4,6-diamidino-2-phenylindole indicated that these giant *spkA* null cells could have as

Figure 2. Cytokinesis defect of *spkA* null cells. (A) Images of *spkA* null cells growing either on plates or in shaking culture. Cells were fixed with 3.7% formaldehyde on coverslips and stained with Hoechst dye. *spkA* null cells grow on plates as large cells with multiple nuclei, but they divide much better in shaking culture. Control JH10 cells and *spkA* null cells expressing exogenous SAPKα divide normally. Bar, 30 μm. (B) Quantitative analysis of the cytokinesis defect of *spkA* null cells. Cells either grown on plates or 24 h after being transferred from plates to shaking culture were allowed to adhere on coverslips, fixed, and stained with Hoechst dye. The nuclei from as many as 300 cells were counted for each strain. (C) Myosin localization does not seem to be affected in *spkA* null cells. JH10 and *spkA* null cells expressing myosin-GFP were plated on coverslips and cell divisions were recorded using the GFP channel. For JH10 cells, frames were taken every 30 s. For *spkA* null cells, frames were taken at 1-min intervals due to increased sensitivity of cells to UV light. Bar, 10 μm. (D) Time-lapse recording of division of JH10 and *spkA* null cells. Although JH10 cells divide normally, *spkA* null cells often fail to divide due to incomplete separation and subsequent fusion of the two daughter cells. Bar, 10 μm. (E) Image showing a large dividing *spkA* null cell stained for actin, myosin, and nuclei. Bar, 30 μm. (F) Time-lapse recording of division of a large *spkA* null cell. One large *spkA* null cell eventually divided into 4 daughter cells, as indicated by arrows. Bar, 50 μm.

many as 80 nuclei/cell. Approximately 92% of the cells of the parental strain JH10 have one or two nuclei compared with ~41% for *spkA* null cells (Figure 2B). Furthermore, ~30% of *spkA* null cells have at least seven nuclei per cell. We examined whether *spkA* null cells are multinucleate when grown in suspension (shaking) culture. Twenty-four hours after transferring the cells from a Petri dish to shaking culture, *spkA* null cells with one or two nuclei/cell increased to ~69% compared with ~78% for JH10 cells. The decrease in the number of multinucleate cells when grown in suspension culture is the opposite of observations for mutants in the myosin II pathway (e.g., myosin II, PAKa, RLC null strains; Fukui *et al.*, 1990; Zang *et al.*, 1997; Chaudoir *et al.*, 1999; Chung and Firtel, 1999). These strains produce large, multinucleate cells in suspension but are often mono- or dinucleate when grown on Petri plates, as the cells are able to pull themselves apart by traction-mediated cytofission (Fukui *et al.*, 1990). Interestingly, the F-actin binding protein coronin is also required for proper cytokinesis. Like *spkA* null cells, *coronin* null cells exhibit a stronger cytokinesis defect when grown on Petri dishes than when grown in suspension culture (de Hostos *et al.*, 1993). Expression of exogenous SAPK α under the Actin 15 promoter in *spkA* null cells restored normal cytokinesis (Figure 2, A and B).

To better study cytokinesis, we examined GFP-myosin II localization during cytokinesis. As shown in Figure 2C, the localization of GFP-myosin II was similar in *spkA* null cell and the parental strain JH10. These results are consistent with the *spkA* null cytokinesis defect not being associated with a defect in the myosin II pathway.

We performed a more detailed time-lapse video analysis of cells undergoing cytokinesis to further understand the *spkA* null cells' cytokinesis defects (Figure 2D). Because of *Dictyostelium* cells' sensitivity to the light required for fluorescence imaging in our system, these more detailed studies were not practical with GFP fusion proteins as markers. We therefore examined cells by differential interference contrast imaging. In *spkA* null cells, dividing cells form a cleavage furrow normally and the two daughter cells migrate away from each other. However, in many cases, the two daughter cells fail to separate and are connected by a thin "bridge" and fuse as one cell eventually. We also noticed that, whereas JH10 cells remain fully attached to the surface of the coverslip during division, the dividing *spkA* null cells do not remain well attached. As a result, the two *spkA* null daughter cells are sometimes unable to effectively pull themselves apart when trying to separate (our unpublished data). This observation might explain why *spkA* null cells divide better in shaking culture. We expect that the shear forces in shaking culture might break the bridge between the two cells, allowing the daughter cells to separate.

The profile of the number of nuclei per cell (some cells have a small number of nuclei; Figure 2B) indicated that cells are periodically able to undergo cytokinesis. We followed the division of larger cells labeled with GFP-myosin II. Figure 2E shows one representative large *spkA* null cell in the process of dividing into smaller multinucleate daughter cells. In this cell type, myosin II localized to the place where daughter cells were going to separate and nuclei were distributed along the periphery of the cell and eventually separated into a few daughter cells. Figure 2F shows a time-lapse recording of the division of one representative large *spkA* null cell. From these observations, we conclude that the cytokinesis defect of *spkA* null cells is probably not due to a defect in the myosin II pathway. The high frequency of reversion of cytokinesis during the final separation of

daughter cells suggests that SAPK α function might be required, directly or indirectly, in the severing of the midbody. The cytokinesis defect could be due to an altered actin cytoskeleton and/or a strong adhesion defect (see result below). We do not know whether SAPK α is directly involved in cytokinesis or the cytokinesis defect is due to a pleiotrophic defect in a yet-to-be-defined pathway affecting the cytoskeleton.

spkA Null and SAPK α^{OE} Cells Exhibit Developmental Defects

Figure 3 illustrates the development of *spkA* null cells, cells overexpressing SAPK α (SAPK α^{OE} cells), and parental JH10 cells. When plated at a low density, *spkA* null cells formed much smaller mounds and fruiting bodies compared with the parental strain JH10 cells, which formed nearly normal-sized mounds when plated at the same density (our unpublished data). However, the developmental timing was not affected and both *spkA* null cells and JH10 cells were able to produce viable spores. To create cells overexpressing SAPK α^{OE} , wild-type cells were transformed with an expression vector expressing SAPK α with a C-terminal FLAG tag. Clones were screened for SAPK α expression by Western blot analysis and clones with three different expression levels of SAPK α were examined for developmental phenotypes. Cells expressing the highest level of SAPK α did not aggregate within 24 h (our unpublished data). Cells expressing a medium level of SAPK α exhibited very delayed aggregation and formed large, amorphous aggregates (Figure 3). Small, abnormal fruiting body-like structures emerged from the mounds. No detergent-resistant spores were formed at 24 h of development. Cells exhibiting the lowest level of overexpression formed mounds with normal developmental timing, but the aggregates were very small. Some of these mounds eventually developed into very small fruiting bodies, although many remained at the mound stage through 24 h of development. Expression of a putative kinase dead SAPK α (mutation of the putative Lys in the ATP binding site to Ala, SAPK α^{K378A}) in wild-type cells produced developmental phenotypes that were similar to but less severe than those of *spkA* null cells (our unpublished data).

Overexpression of SAPK α in *spkA* null cells produced phenotypes similar to those of overexpression of SAPK α in wild-type cells, although not as severe. Cells aggregated with normal timing and formed larger mounds. Many of these broke up into smaller aggregates, some of which produced multiple abnormal-looking structures that were similar to those produced by overexpression of SAPK α in wild-type cells. We view the phenotypes as being due predominantly to overexpression of SAPK α , but we cannot exclude the possibility that some of the defects result from misexpression of SAPK α from the Actin 15 promoter.

spkA Null and SAPK α^{OE} Cells Exhibit Motility Defects

The aggregation and morphogenesis defects suggest that *spkA* null and SAPK α^{OE} cells may exhibit motility and/or chemotaxis defects. Chemotaxis was examined by assaying the ability of aggregation-competent cells to migrate toward a micropipette emitting the chemoattractant cAMP. Figure 4A shows that *spkA* null cells are less polarized compared with JH10 cells and move more slowly. *spkA* null cells exhibit directionality similar to that of the parental strain. SAPK α^{OE} cells exhibit very strong chemotaxis defects. These cells are very poorly polarized and lack a defined pseudopod. In addition, the cells undergo considerably more directional change than JH10 cells, and the speed of movement is

Figure 3. Developmental phenotypes of *spkA* null cells and cells expressing wild-type SAPKα. (A) Cells grown in axenic medium were washed twice with Na/K phosphate buffer and plated on nonnutrient agar plates. Photographs were taken at various developmental stages at the same magnification. For *spkA* null cells, developmental phenotypes at both lower and higher density are shown. (B) Western blot showing levels of expression of SAPKα in three individual clones: 1, highest expression level (developmental data not shown); 2, medium level (shown as SAPKα^{OE} in A); and 3, lowest level (shown as SAPKα^{OE} lower in A).

reduced significantly. Expression of SAPKα in *spkA* null cells resulted in an intermediate phenotype (Figure 4A). Overexpression of a putative dominant-negative mutant of SAPKα in a wild-type background resulted in phenotypes

similar to those of *spkA* null cells. To eliminate the possibility that the chemotaxis defects observed were due to the cells not being properly developed and thus unable to generally respond to chemoattractant signals, we performed a Northern blot analysis to check the expression of chemoattractant receptor cAR1 at different time points after pulsing the cells with cAMP, which is known to induce cAR1 expression. As shown in Figure 4B, cAR1 mRNA levels were very similar in the four cell types examined: parent strain (JH10), *spkA* null cells, SAPKα^{OE} cells, and SAPKα/*spkA* null cells.

To examine whether these chemotaxis phenotypes may be due to general defects in cell motility, we examined random cell movement of these strains. For each strain, 15–20 cells were randomly chosen and the speed of movement was measured using DIAS software (Wessels *et al.*, 1998). JH10 cells moved faster than *spkA* null cells (5.81 ± 0.49 versus 4.52 ± 0.51 μm/min, respectively). For the SAPKα overexpression cells, we measured clones expressing different levels of SAPKα (Figure 3B). Cells expressing the highest level of SAPKα showed a significantly reduced speed of random movement (3.55 ± 0.57 μm/min), whereas cells with the lowest level of expression exhibited a speed (5.10 ± 0.62 μm/min) similar to that of the parental strain. These data indicate that SAPKα affects the speed of random motility. Whether the chemotaxis defects are the result of general defects in motility or whether SAPKα also directly affects chemotaxis is not known.

SAPKα Is Involved in Modulating the Actin Cytoskeleton

Because *spkA* null cells exhibit altered aggregation and morphogenesis, we examined the possibility that the SAPKα mutants might affect the actin cytoskeleton. To test this, we examined the distribution of F-actin by using FITC-phalloidin staining of F-actin in the SAPKα mutant strains under various physiological conditions (Figure 5, A and B). In wild-type cells, F-actin localized predominantly to the cortical areas at the sites of cell protrusions. SAPKα^{OE} cells seem to have an increase in the level of F-actin staining and an increase in the number of visible membrane ruffles. *spkA* null cells exhibited actin staining strikingly distinct from that of wild-type cells. As shown in Figure 5A, we observed three types of staining patterns. In large multinucleated cells, F-actin is localized in flattened lamellipodia (indicated by white arrow with a black center) and eupodia (indicated by white arrow), cortical structures proposed to be responsible for cell-substratum anchorage during amoeboid movement, and occur transiently in multiple units at the base of certain pseudopodia (Fukui and Inoue, 1997). Although these structures are present in a large proportion of *spkA* null cells, they are only seen occasionally in wild-type cells. Some *spkA* null cells exhibit a broad ring-like actin staining in the cortex region, which is not normally seen in wild-type cells (Figure 5A, 2). A small fraction of *spkA* null cells (<10%) look similar to wild-type cells (Figure 5A, 3). In SAPKα reconstituted *spkA* null cells, the normal, wild-type-cell distribution of F-actin is restored (Figure 5A).

In cells starved for 6 h or pulsed for 6 h with 30 nM cAMP every 6 min to make cells maximally express components of the signaling pathway required for chemotaxis to cAMP (Insall *et al.*, 1994), SAPKα^{OE} cells do not polarize and F-actin localizes to either multiple crown-like structures or ruffles (Figure 5B). Starved *spkA* null cells do not polarize, and we observed actin staining along the cell cortex. Pulsed *spkA* null cells elongate, but F-actin localizes along the entire cell cortex in small protrusions along the edge of the cells. These observations suggest that SAPKα may be important

A

Figure 4. Chemotaxis analysis of *spkA* null cells and cells expressing wild-type SAPK α . (A) Chemotaxis assay. Cells were washed and pulsed every 6 min for 5 h with 30 nM cAMP (see MATERIALS AND METHODS). Cells were plated on a Petri dish specifically made for observing cells by using an inverted microscope and placed close to a micropipette filled with 150 μ M cAMP. Cell movements were recorded with NIH Image software and analyzed using the DIAS program. Comparison of cell shape, direction change, and speed of the movement of different cells is shown. A representative cell from each type of cells is shown. The recordings are made at 10 frames/min and 15–20 randomly chosen cells were analyzed for each strain. The image was obtained by superimposing nine frames at 1-min intervals between frames. (B) cAR1 Northern blot. Cells were pulsed and 2×10^7 cells were sampled at the indicated time points. Total RNA was prepared and 6 μ g was resolved on a 1% formaldehyde denaturing agarose gel, transferred to nylon membrane, and hybridized to a cAR1 probe.

B

for a normal actin cytoskeleton. To further study F-actin levels in SAPK α mutant strains, we examined F-actin levels in pulsed cells before and after cAMP stimulation. Figure 5C shows that SAPK α ^{OE} cells have a significantly elevated basal level of F-actin compared with the parental strain as determined by our assay for F-actin levels (see MATERIALS AND METHODS). On chemoattractant stimulation, SAPK α ^{OE} exhibited an $\sim$ 30% increase, compared with the approximate doubling of F-actin levels in wild-type cells. The level of F-actin after stimulation in SAPK α ^{OE} cells was still higher than that in stimulated wild-type cells. In *spkA*

null cells, the basal level of F-actin was similar to that of wild-type cells, but there was only an $\sim$ 60% increase after stimulation by cAMP. Because our results suggest that SAPK α is involved in modulating the actin cytoskeleton, we tested whether SAPK α colocalizes with F-actin. Cells expressing either SAPK α -GFP or SAPK α -N-GFP were fixed and stained for actin with TRITC-phalloidin (Figure 6A). The result showed that both full-length SAPK α and the N terminus of SAPK α were enriched in cell surface actin-rich structures, inferring the N-terminal domain mediates the targeting of SAPK α . We also examined the localization of

Figure 5. Abnormal actin organization in *spkA* null cells and *SAPK α* ^{OE} cells. Cells in the vegetative growth state (A) or after pulsing (B) were plated on cover slips and fixed and stained with phalloidin as described in MATERIALS AND METHODS. For vegetative *spkA* null cells, representative cells from three different populations were shown (1–3). Population 1 (1) are mainly large,

SAPK α -FLAG in chemotaxing cells. *SAPK α* -FLAG seems to localize in the leading edge of chemotaxing cells. However, as *SAPK α* ^{OE} cells polarized poorly and many cells did not show directional movement up the chemoattractant gradient, we only examined cells that exhibited some polarization. In these cells, *SAPK α* localized to cell surface protrusions during cell movement (Figure 6B, top). To confirm this result, we examined *SAPK α* localization during chemotaxis in *spkA* null cells expressing *SAPK α* -FLAG. These cells chemotax significantly better than *SAPK α* ^{OE} cells and we still observed localization of *SAPK α* -FLAG in the leading edge of the cells (Figure 6B, bottom). We used the F-actin-binding proteins ABP120 and coronin fused with GFP to examine F-actin dynamics in *spkA* null cell chemotaxis (Figure 6C). In JH10 cells, both a GFP fusion of the F-actin-binding domain of ABP120 (ABP-GFP) and coronin-GFP localized to a well-defined single pseudopod at the leading edge of chemotaxing cells, as described previously (Gerisch *et al.*, 1995; Pang *et al.*, 1998). In *spkA* null cells, ABP-GFP and coronin-GFP had a significantly broader leading edge and we observed labeling at sites along the lateral edge and posterior of cells. These combined results suggest that *SAPK α* is involved in cortical F-actin organization.

SAPK α Is Activated by Stress and Required for Full Osmoprotection

In response to cAMP stimulation during chemotaxis or to osmotic shock, the actin cytoskeleton undergoes a major reorganization. We therefore examined whether *SAPK α* kinase activity increases in response to these stimuli (see MATERIALS AND METHODS). As depicted in Figure 7A, despite the strong developmental and motility defects exhibited by *SAPK α* ^{OE} cells and *spkA* null cells, *SAPK α* kinase was not detectably activated by cAMP stimulation in wild-type cells. In contrast, hyperosmotically shocking cells by treatment with 0.4 M sorbitol resulted in a very significant activation of *SAPK α* kinase activity in 5 min, as observed by the phosphorylation of the heterologous substrate MBP. There is a subsequent gradual decline in kinase activity. The kinetics of increase in kinase activity are similar to those of other responses stimulated by osmotic shock in *Dictyostelium* and much slower than responses stimulated by chemoattractants, which normally peak between 5 and 40 s, depending on the response (Kuwayama *et al.*, 1996; Oyama, 1996). The putative kinase dead *SAPK α* , *SAPK α* ^{K378A}, did not have any kinase activity. In addition to the phosphorylation of MBP, we observed the phosphorylation of a band that comigrates with tagged *SAPK α* . We suggest that this is autophosphorylated *SAPK α* rather than being due to another protein that is phosphorylated by and coimmunoprecipitates and comigrates with *SAPK α* . This labeled band is not observed when kinase dead *SAPK α* is assayed, indicating this band is probably not due to another kinase. In addition, we observed a third phosphorylated band with a mobility corresponding to ~40 kDa, which is not detected in

multinucleate cells which were also stained for nuclei. Population 2 (2) are cells with one or two nuclei and show ring-like staining. Population 3 (3) are cells with one or two nuclei and have a staining pattern more like that of parental JH10 cells. White arrows indicate flattened lamellipodia of different sizes typically seen in these cells. Multiple eupodia were also present in these cells (indicated by white arrows with black centers). Bar, 10 μ m. (C) Actin polymerization assay. F-actin levels before and after cAMP stimulation were measured and normalized against the amount of protein.

Figure 6. Localization of SAPK α . (A) Wild-type cells expressing full-length SAPK α -GFP or N-terminal SAPK α -GFP were pulsed with cAMP, fixed, and stained with TRITC-phalloidin as described in MATERIALS AND METHODS. Phase contrast and fluorescence images indicate that SAPK α was enriched in multiple actin-rich structures on the cell surface. Bar, 5 μ m. (B) Localization of SAPK α in chemotaxing cells. Wild-type cells (top) or *spkA* null cells (bottom) expressing full-length SAPK α -FLAG were pulsed with cAMP and cells were plated onto cover slips and allowed to aggregate. Cells were then fixed and stained with TRITC-phalloidin and anti-FLAG mAb as described in MATERIALS AND METHODS. White arrows indicate the directions of movement. Bar, 10 μ m. (C) Chemotaxis of *spkA* null cells was examined using ABP-GFP and coronin-GFP as markers. In JH10 cells, both ABP-GFP and coronin-GFP localized to a well-defined leading edge pseudopodium. *spkA* null cells, however, had a broader leading edge and both markers localized to the lateral cortex (as indicated by arrows).

assays by using kinase dead SAPK α . We suggest that this protein coimmunoprecipitates with and is phosphorylated by SAPK α .

To further test whether SAPK α is involved in generally responding to cellular stress, we examined the effects of other stress conditions on SAPK α kinase activity (Figure 7B). Other hyperosmotic stresses, NaCl and glycerol, produced a similar activation of SAPK α . Heat shock at 30°C for 5 min strongly activated the kinase. However, cold shock at 4°C or treatment of cells with NaN₃ or H₂O₂ did not increase kinase activity.

Although *spkA* null cells seemed to round up faster than JH10 cells (our unpublished data), SAPK α was not activated by UV light treatment. We think that the faster rounding-up of *spkA* null cells may be due to an adhesion defect as discussed below (see Figure 9C). Our results suggest that SAPK α kinase activity is selectively regulated by certain stress responses and may play a role in these responses.

It was shown previously that guanylyl cyclase is activated within a few minutes after osmotic shock, resulting in large increases in cGMP (Kuwayama *et al.*, 1996; Oyama, 1996;

Figure 7. Activation of SAPK α by various stimuli. (A) Activation of SAPK α by osmotic shock but not by cAMP. For cAMP stimulation, aggregation-competent cells made by pulsing (see MATERIALS AND METHODS) were stimulated with 100 μ M cAMP. For osmotic shock stimulation, vegetative cells were washed with Na/K phosphate buffer, starved for 1 h, and treated with 0.4 M sorbitol. Aliquots were taken at the indicated time points. Cells were lysed, SAPK α or its mutant was immunoprecipitated with anti-FLAG mAb, and kinase activity was measured as described in MATERIALS AND METHODS. The kinase dead SAPK α K378A was used as a negative control. (B) Activation of SAPK α in other stress conditions. To confirm the osmotic response of SAPK α , 0.4 M NaCl, and 0.4 M glycerol were also tested. For heat shock treatment, cells were incubated at 30°C. For cold shock, cells were incubated at 4°C. For UV treatment, cells were placed in a dish on a shaker and exposed to 300-nm UV light. Cells were also treated with 3 mM H₂O₂ and 1 mM NaN₃. The top band shows the autophosphorylated SAPK α . The bottom band shows phosphorylated MBP. The middle band might represent a protein coimmunoprecipitated and phosphorylated by SAPK α . To examine the immunoprecipitated SAPK α protein level, a Western blot was carried out on the same blot used for kinase assay (bottom).

Roelofs and Van Haastert, 2002). Total intracellular and extracellular cGMP levels start to rise significantly after 2 min, reach a peak at $\sim$ 10 min, and slowly decrease. Intracellular cGMP levels peak at 3–5 min. To test whether SAPK α can be activated by cGMP, we assayed SAPK α kinase activity after addition of the membrane-permeable cGMP (8-Br-cGMP). Figure 8A indicates that 8-Br-cGMP addition results in a rapid increase in SAPK α activity, although the level is lower than that observed after sorbitol treatment. The fact that SAPK α was activated by several stress treatments suggests that SAPK α might be required for cells to survive osmotic stress. Figure 8B shows that *spkA* null cells exhibit a decreased resistance to osmotic stress. This increased sensitivity was complemented by expression of SAPK α in these cells.

Because cGMP can activate SAPK α kinase activity, we reinvestigated chemoattractant activation of SAPK α in cells lacking the cGMP phosphodiesterase (*pdeD* null cells) primarily responsible for degrading cGMP in response to cAMP stimulation (Meima *et al.*, 2002). In this phosphodiesterase null mutation, cAMP-stimulated cGMP levels are highly elevated. As shown in Figure 8B, cAMP does result in a low-level activation of SAPK α kinase activity in PDE null but not wild-type cells with kinetics of activation similar to those of the kinetics of cGMP accumulation. The results suggest that chemoattractants may stimulate SAPK α kinase activity in wild-type cells but the level of activation is too low to be measured using our assay.

SAPK α Is Activated by Detachment and Might Be Involved in Cell Adhesion

Our experiments assayed sensitivity to stress in cells grown in suspension culture (Figure 7). We also performed experiments to test the effect of UV irradiation on SAPK α activity by using attached cells, because *spkA* null cells grown on Petri plates seemed to round up more rapidly than wild-type cells after treatment with UV light (our unpublished

data). Unexpectedly, when cells grown on plates were assayed for SAPK α kinase activity, we observed elevated SAPK α activity in unstimulated cells, immediately after they were detached from the substratum (comparable with unstimulated cells grown in suspension; our unpublished data). These data suggested that the detachment of cells from the plate resulted in SAPK α kinase activation. To test this possibility, we assayed SAPK α kinase activity at different times after detachment from Petri plates and in suspension in buffer. The result showed that SAPK α was activated upon cell detachment, followed by its inactivation within 5 min (Figure 9B). We think this activation occurred after cell detachment because, in a control experiment in which cells were lysed directly on a plate before detachment, the kinase activity was significantly lower (Figure 9B, lane 1). This proposed activation by detachment probably takes place through a different mechanism than by osmotic shock, because latrunculin B treatment of cells inhibits the detachment-mediated activation of SAPK α but has no effect on the osmotic shock stimulated activation (Figures 8A and 9B). Because latrunculin B specifically disrupts the F-actin cytoskeleton, it is possible that cell detachment-mediated activation is transmitted through the actin cytoskeleton.

spkA null cells are more easily detached from Petri dishes than wild-type cells and can be readily detached by gentle pipetting. Figure 9B quantifies the attachment and demonstrates that *spkA* null cells exhibit a greatly reduced adhesion to the substratum compared with wild-type, SAPK α^{OE} , and SAPK α /*spkA* null cells (see MATERIALS AND METHODS).

DISCUSSION

We have identified a putative novel member of the MEKK class of kinases that are most closely related to the MLK subfamily. The N terminus of SAPK α , which is required for SAPK α subcellular localization, contains five ankyrin repeats and a SAM domain, which we expect mediate protein–

Figure 8. SAPK α provides protection against osmotic stress. (A) SAPK α activation by 8-Br-cGMP. A kinase assay was performed with the addition of 20 mM 8-Br-cGMP, a cell membrane-permeable cGMP. As a positive control, cells were treated with 0.4 M sorbitol. To examine the effects of latrunculin B, cells were treated with 1 μ M latrunculin B (L.B.) for 10 min before osmotic stress stimulation. (B) SAPK α activation by cAMP in *pdeD* null cells. Cells were pulsed and stimulated with 150 μ M cAMP for 5 h and SAPK α kinase assays were performed as usual. (C) Cell survival after osmotic stress. Cells were shaken in Na/K phosphate buffer in the presence or absence of 0.4 M sorbitol for the times indicated, diluted with buffer, and plated onto SM agar plates with *Klebsiella aerogenes* bacteria. Cell viability was determined as the percentage of colonies compared with wild-type controls.

protein interactions. Analysis of the phenotypes of *spkA* null and SAPK α overexpressing cells suggests that disruption of normal SAPK α function affects various cellular processes, presumably due to the altered actin cytoskeleton observed in these cells. Both strains exhibit defects in cell motility, morphogenesis, and aggregation. *spkA* null cells also have a reduced adhesion to the substratum and seem to have increased number of eupodia, a cytoskeletal structure that has been linked in *Dictyostelium* to the substratum adhesion. Overexpression of SAPK α leads to very elevated F-actin levels in unstimulated cells that increase further upon stimulation. However, the ability of *spkA* null cells to increase F-actin levels in response to chemoattractant stimulation, albeit to only 50% of the level observed in stimulated wild-type cells, suggests SAPK α is not essential for this process. Furthermore, SAPK α is associated with the cytoskeletal cortex in response to chemoattractant stimulation. Our results indicate that both *spkA* null and SAPK α overexpressing cells exhibit general motility defects, and SAPK α is not activated by cAMP in a wild-type background. It is therefore possible that the effects of SAPK α mutants or overexpressor strain on

chemotaxis are indirect. However, we cannot completely rule out a possible involvement of SAPK α in chemotaxis, because we observe a low-level stimulation of SAPK α kinase activity in cells lacking one of the cGMP PDEs, demonstrating that the chemoattractant pathway is able to activate this response. If SAPK α does play a role in chemotaxis, we expect that SAPK α is activated in wild-type cells in response to chemoattractant stimulation, but the activity may be highly localized within the cell or highly transient and thus may not be detectable in our assays.

Interestingly, SAPK α kinase is rapidly activated in response to several stress stimuli, including heat and osmotic shock. We found that SAPK α activity can be stimulated by 8-Br-cGMP, a membrane-permeant cGMP. cGMP levels rapidly increase in response to osmotic stress to a point that is significantly higher than their activation in response to chemoattractant stimulation, and guanylyl cyclase activation has been linked to stress survival. The activation of SAPK α by several stress stimuli and 8-Br-cGMP suggests that SAPK α is a major downstream effector pathway controlling the cell's response to certain stress stimuli. Consistent with

Figure 9. SAPK α affects cell adhesion. (A) Activation of SAPK α by detachment. Cells were plated onto culture plates and allowed to attach for 30 min. Cells were then quickly resuspended by pipetting and transferred to shaking culture. At the time point indicated, cells were lysed in an equal volume of 2 $\times$ lysis buffer and SAPK α kinase activity was determined accordingly. For the 0-min time point, cells were lysed immediately after detachment from the plate. For the -5-min time point, cells were lysed 5 min before the experiment directly on the plate by adding 1 $\times$ lysis buffer. Kinase activation by detachment was also measured after treatment of cells with 1 μ M latrunculin B. (B) Cell-substratum adhesion assay on *spkA* null and SAPK α ^{OE} cells. Cell adhesion assays were performed using nitrocellulose filters as described in MATERIALS AND METHODS. Adhesion was measured by the ratio of attached compared with unattached cells. Experiments were performed at least three times. Bar, 10 μ m.

this result, *spkA* null cells exhibit a reduced ability to survive osmotic shock treatment. At present, we do not know which pathway lies between cGMP and SAPK α activation, although recently two cGMP binding proteins that are putative downstream effectors of cGMP have been identified and may lie upstream of SAPK α .

In addition, SAPK α is stimulated by detaching cells adhered to a substratum. This stimulation does not occur when cells are pretreated with latrunculin B, suggesting that a cytoskeleton-mediated pathway may sense the disruption that leads to SAPK α stimulation. This may parallel responses of mammalian cells, which also stimulate stress-related pathways upon detachment from the substratum. In mammalian cells, cell detachment activates the p38 MAP kinase, which is required for detachment-induced apoptosis (Rosen *et al.*, 2002). There is evidence suggesting that MAP kinase pathways may participate in modulating focal adhesion and/or cell adhesion (Schneider *et al.*, 1998; Takino *et al.*, 2002). Alternatively, as detachment by pipetting (the method used in this article) applies mechanical force to cells,

it is possible that shear stress may also trigger signaling pathways leading to SAPK α activation. In mammalian cells, shear stress activates MAP kinases and may participate in wound healing and other cellular responses (Kippenberger *et al.*, 2000a,b; Langille, 2001).

The level of cGMP produced in response to chemoattractant stimulation is significantly lower than that produced in stress pathways. It is possible that this lower level of cGMP production may be insufficient to induce an observable SAPK α activity if cGMP is the sole mechanism of SAPK α activation. However, it is possible that low levels of SAPK α activity may be activated by basal or chemoattractant-stimulated levels of cGMP, and these levels of kinase activity may suffice to mediate cellular responses under these physiological conditions but may be insufficient to mediate an observed increase in biochemical activity that is measurable in our assays.

In conclusion, we have identified a novel MEK kinase-like kinase that is a downstream effector of several stress-induced pathways. Results suggest that SAPK α might be in-

volved in a number of actin-based cellular processes. The presence of an MEKK-like domain suggests that SAPK α may lie at the top of a MAP kinase cascade, although downstream components have not been identified. The N-terminal domain mediates subcellular localization, presumably through protein-protein interactions. Although SAPK α does not have a direct ortholog in mammalian cells, there are significant parallels between SAPK α function and the function of members of the MLK subfamily of MEK kinases and the role of MAP kinases in stress and detachment-mediated responses. For example, MLK family members mediate the activation of p38 as well as JNK MAPK pathways by multiple stimuli, including cellular stresses (Kyriakis and Avruch, 2001). One of these, MLTK β , induces actin cytoskeleton reorganization through the p38 MAPK pathway and is activated by osmotic stress (Gotoh *et al.*, 2001). Furthermore, the p38 MAPK pathway directly phosphorylates the actin polymerization modifier HSP27 and regulates actin reorganization (Landry and Huot, 1995, 1999). This finding suggests that the *Dictyostelium* SAPK α -regulated pathway may have direct parallels in other systems.

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