Novel Mitogen-Activated Protein Kinase MpkC of \textit{Aspergillus fumigatus} Is Required for Utilization of Polyalcohol Sugars\textsuperscript{\textdagger}

Guadalupe Reyes, Angela Romans, C. Kim Nguyen, and Gregory S. May\textsuperscript{*}

Division of Pathology and Laboratory Medicine, University of Texas M. D. Anderson Cancer Center, Houston, Texas

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The genome of \textit{Aspergillus fumigatus} has four genes that encode mitogen-activated protein kinases (MAPKs), \textit{sakA}/\textit{hogA}, \textit{mpkA}, \textit{mpkB}, and \textit{mpkC}. The functions of the \textit{mpkB} and \textit{mpkC} MAPKs are unknown for \textit{A. fumigatus} and the closely related and genetically amenable species \textit{Aspergillus nidulans}. \textit{mpkC} deletion mutants of \textit{A. fumigatus} were made and their phenotypes characterized. The \textit{mpkC} deletion mutants were viable and had normal conidial germination and hyphal growth on minimal or complete media. This is in contrast to deletion mutants with deletions in the closely related MAPK gene \textit{sakA}/\textit{hogA} that we previously reported had a nitrogen source-dependent germination phenotype. Similarly, the growth of the \textit{mpkC} deletion mutants was wild type on high-osmolarity medium. Consistent with these two MAP kinase genes regulating different cellular responses, we determined that the \textit{mpkC} deletion mutants were unable to grow on minimal medium with sorbitol or mannitol as the sole carbon source. This result implicates \textit{MpkC} signaling in carbon source utilization.

Changes in mRNA levels for \textit{sakA} and \textit{mpkC} were measured in response to hypertonic stress, oxidative stress, and a shift from glucose to sorbitol to determine if there was overlap in the \textit{SakA} and \textit{MpkC} signaling pathways. These studies demonstrated that \textit{SakA}- and \textit{MpkC}-dependent patterns of change in mRNA levels are distinct and have minimal overlap in response to these environmental stresses.

\textit{Aspergillus fumigatus} is a filamentous fungus that is ubiquitous in the environment. It, like many other saprophytic fungi, contributes to the turnover of nitrogen and carbon in the environment through degradation of biological polymers such as cellulose and lignin. \textit{A. fumigatus} is also the cause of significant morbidity and mortality in patients with compromised immune systems (18). This fungus causes the life-threatening disease invasive pulmonary aspergillosis, a major clinical concern for patients with leukemia and lymphoma and bone marrow transplant patients. Despite the clinical importance of this fungus, it has not been the subject of intensive biological investigation. If novel therapies for invasive pulmonary aspergillosis are to be developed, a much better understanding of the basic biological processes of \textit{A. fumigatus} is required.

Mitogen-activated protein kinase pathways could decrease the virulence of the fungus in a host. A number of studies of plant and animal fungal pathogens have shown that mutants defective in MAPK signaling have reduced virulence (2, 5, 7, 10, 13, 16, 19, 26, 27). The genome of \textit{A. fumigatus} has four genes that code for MAPKs, \textit{sakA}/\textit{hogA}, \textit{mpkA}, \textit{mpkB}, and \textit{mpkC} (23). \textit{sakA} encodes the stress-activated kinase that responds to hypertonic stress, reactive oxygen species, and heat shock (28). This highly conserved MAPK’s functions are also well conserved among fungi (1, 11, 12, 14, 19, 26, 28). \textit{mpkA} is the ortholog of the \textit{SLT2} MAPK gene in \textit{Saccharomyces cerevisiae} that is responsible for monitoring cell wall integrity. Deletion of \textit{mpkA} in \textit{Aspergillus nidulans} produces a strain with delayed conidial germination and swelling at the hyphal tips. These phenotypes are largely remedied by growth on high-osmolarity complex media (3). Orthologs of this MAPK in plant-pathogenic fungi have been demonstrated to play an important role in pathogenesis (26). Similarly, the \textit{Candida albicans} ortholog \textit{MKC1} has been shown to play a role in virulence (6, 21, 22). \textit{mpkB} codes for a MAPK that is most similar to the MAPKs encoded by the \textit{S. cerevisiae} genes \textit{FUS3} and \textit{KSS1}. The \textit{Fus3p} and \textit{Kss1p} MAPKs function in the pheromone mating response pathway and the filamentation/invasive growth pathways, respectively. Homologues of these MAPK systems in other fungi have been shown to be important for pathogenesis in plant and animal models (7, 19, 26). Finally, \textit{mpkC} has no clearly orthologous gene in \textit{S. cerevisiae}, but the protein is most similar in sequence to \textit{Hog1p} and related stress-activated protein kinases.

These four MAPK genes also define the complete set for \textit{A. nidulans} and \textit{Aspergillus oryzae}, suggesting that they are all that is necessary to regulate filamentous-fungal-cell physiology (9, 20, 23). Nevertheless, the functions of the \textit{mpkA}, \textit{mpkB}, and \textit{mpkC} MAPK genes in \textit{A. fumigatus} remain unknown. Because of their central role in regulating fungal physiology and their role in virulence in other fungi, they should be investigated in \textit{A. fumigatus}. Thus, it is important that we determine how each of the MAPKs regulates fungal cell physiology in the human pathogen \textit{A. fumigatus}, as it will provide insight into the development of potentially novel antifungal drugs.

In this paper, we report the construction and phenotypic characterization of \textit{mpkC} deletion mutants. We determined that \textit{mpkC} is required for growth on media where sorbitol or...
mannitol is the sole carbon source. These results define a new and novel MAPK signaling pathway that regulates conidial germination in response to the carbon source in the medium.

**MATERIALS AND METHODS**

**Aspergillus fumigatus** growth media, strains, and transformation. *A. fumigatus* strain A293 was grown on minimal medium (MM) that was composed of 7 mM KCl, 2 mM MgSO₄, 20 mM ammonium tartrate, 12 mM KPO₄ (pH 6.8), 1% (wt/vol) glucose, vitamin mix (1 mg per each of p-aminobenzoic acid, choline HCl, niacin, pyrroline HCl, riboflavin, and thiamine HCl) and 2 ng/ml D-biotin), 0.2% (wt/vol) yeast extract, 0.2% (wt/vol) peptone, and 0.1% (wt/vol) tryptone (Difco Laboratories, Inc., Sparks, MD). To test growth on different carbon sources, glucose was omitted and the alternative carbon source was added to a final concentration of 100 mM. The alternative carbon sources that we tested were ethanol, galactose, glycerol, lactose, maltose, mannitol, raffinose, and sorbitol. To test growth on different nitrogen sources, ammonium tartrate was omitted and the alternative nitrogen source sodium nitrate, sodium nitrite, proline, or phenylalanine was substituted at a 20 mM final concentration. The solid media for the plates incorporated 1.5% (wt/vol) agar (Difco Laboratories, Inc., Sparks, MD). Transformation of the *A. fumigatus* mutants was performed as previously described (25). DNA was prepared from transformant strains (28), and deletion mutants were identified by Southern blot analysis. First-strand cDNA was primed for synthesis with oligo(dT)₁₂–₁₈ using SuperScript II reverse transcriptase under conditions described by the manufacturer (InVitrogen, Carlsbad, CA). This cDNA was used to prime quantitative RT-PCR (qRT-PCR) as described below.

**Construction of an mpkC deletion mutant.** The plasmid used to delete *mpkC* was made using the Gateway Multisite plasmid system (Invitrogen, Carlsbad, CA). Briefly, ∼1 kb of the 5’-end-flanking sequence beginning immediately upstream of the predicted start codon of *mpkC* was PCR amplified from A293 genomic DNA using oligonucleotides mpkC-5’-1 and mpkC-5’-2 (Table 1) and recombined into pDONR8-P1 by using a BP recombination reaction as described by the manufacturer. Similarly, approximately 1 kb of the 3’-end-flanking sequence beginning immediately after the predicted termination codon of *mpkC* was PCR amplified from A293 genomic DNA using oligonucleotides mpkC-3’-1 and mpkC-3’-2 and recombined into pDONRP2-R3 by using a BP recombination reaction. The *A. fumigatus* *pyrG* gene was PCR amplified from A293 genomic DNA with oligonucleotides *Ap*pyrG-1 and *Ap*pyrG-2 and recombined into pDONR221 in a BP recombination reaction. These three plasmids were then transformed into the *A. fumigatus* protoplasts using the LIE protocol. The *pyrG* gene flanked by the *mpkC* 5’- and 3’-end-flanking sequences. The *mpkC* deletion plasmid was transformed into protoplasts of A293.1, and trans-formants were selected for as previously described (25). DNA was prepared from transformant strains (28), and deletion mutants were identified by Southern blot analysis.

**RNA preparation.** Total RNA was prepared from freeze-dried mycelia using TRIzol reagent with modifications (Invitrogen Corp., Carlsbad, CA). Briefly, dried mycelia were ground to a fine powder using a 1-ml micropipette tip in a microcentrifuge tube and acid-washed glass beads (particle size, 425 to 600 μm) in a volume equal to that of the mycelia. An approximately 50-μl volume of the powder was rapidly vortexed in 1 ml of TRIzol reagent for 1 min. This mixture was allowed to incubate at room temperature for 5 min, 200 μl chloroform was added, the mixture was vortexed, and the phases were separated by centrifugation for 15 min at 14,000 × g at 4°C in a microcentrifuge. The TRIzol reagent phase was recovered, the chloroform extract was repeated, and the RNA was precipitated from the final TRIzol phase by the addition of 600 μl isopropanol. Poly(A)⁺ RNA was prepared from total RNA using a poly(A)-Quick kit (Qiagen, Valencia, CA). A primary library of approximately 1.15 × 10⁶ PFU was plated on 23 plates (150-mm diameter), and positive plaques were identified by hybridization, with

| Table 1. Oligonucleotides used in the preparation of plasmids |
|-----------------------|------------------|
| Oligonucleotide         | Sequence          |
| mpkC-5’-1              | GGGGACAACCTTGTATAGAAAGTGTCGCAGCACAGATTTGAGGA |
| mpkC-5’-2              | GGGGACTCTTTTATGACAACTGTGTCG |
| mpkC-3’-1              | GGGGACAGCTTCTTGTACAAAGTGTCG |
| mpkC-3’-2              | GGGGACACCTTGTATAGAAAGTGTCG |
| mpkC-5’-1              | GGGGACAACCTTGTATAGAAAGTGTCG |
| mpkC-5’-2              | GGGGACTCTTTTATGACAACTGTGTCG |
| mpkC-3’-1              | GGGGACAGCTTCTTGTACAAAGTGTCG |
| mpkC-3’-2              | GGGGACACCTTGTATAGAAAGTGTCG |

Data were then analyzed using the comparative cycle threshold method as
described by the manufacturer, and results are presented as change relative to the value at time zero. Each qRT-PCR was done in triplicate, and the mean value was plotted with the standard deviation.

Conidial germination on different carbon sources. Freshly prepared wild-type or mpkC deletion mutant conidia (4 × 10⁷) were placed into 100-mm petri dishes containing 20 ml of MM made with 100 mM glucose or 100 mM sorbitol as the sole carbon source. The plates had five sterile 18- by 18-mm cover glasses submerged in the medium, on which the conidia settled during germination. Plates were incubated without agitation at 37°C, and at 6 and 8 h, a cover glass was removed. The cover glass was inverted on a drop of 4% formaldehyde in phosphate-buffered saline (PBS) for 5 min and then rinsed in PBS and mounted on a slide in PBS-50% (vol/vol) glycerol. The absence or presence of a germ tube was then scored for at least 200 cells and the percent germination scored at each time point.

FIG. 1. Sequence and structure of the Aspergillus fumigatus MAPK gene mpkC. Protein-encoding DNA sequences are in uppercase and noncoding sequences in lowercase. The deduced amino acid sequence is below the DNA sequence in single-letter code. The DNA sequence coding for the upstream micro-open reading frame in the 5'-leader region of cDNA clones is in bold. The 5'-end nucleotides of cDNAs are in bold and double underlined, and the sites of 3'-polyadenylation are bold and underlined.

Nucleotide sequence accession numbers. The A. fumigatus mpkC cDNA sequence was deposited at the NCBI and assigned accession number DQ402475. The A. nidulans mpkC cDNA sequence was deposited at the NCBI and assigned accession number DQ402476.

RESULTS

Analysis of the mpkC gene and the predicted polypeptide. The structure of the mpkC gene and the properties of the predicted MpkC polypeptide were determined by the isolation and sequencing of mpkC cDNA clones. A total of eight positive cDNA clones were sequenced at their 5'-ends, and all
MAP KINASE REQUIREMENT IN CARBON SOURCE UTILIZATION

**TABLE 2.** The ΔmpkC mutant is defective for conidial germination on MM-sorbitol

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Growth (h)</th>
<th>Germination on glucose (%)</th>
<th>Germination on sorbitol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Af293</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>ΔmpkC mutant</td>
<td>6</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Af293</td>
<td>8</td>
<td>66</td>
<td>86</td>
</tr>
<tr>
<td>ΔmpkC mutant</td>
<td>8</td>
<td>56</td>
<td>3</td>
</tr>
</tbody>
</table>

**FIG. 2.** An mpkC deletion mutant is viable and is not growth inhibited on high-osmolarity medium. Growth of Af293, a sakA deletion mutant (ΔsakA1), and two mpkC deletion mutants (ΔmpkC1 and ΔmpkC2) on CM containing 1.4 M KCl, showing that the loss of MpkC function does not confer reduced growth on high-osmolarity medium.

RT-PCR amplified and cloned AnmpkC cDNAs and confirmed the alternative gene structure and predicted protein sequence (NCBI accession number DQ402476).

mpkC deletion mutant germinates poorly on and is unable to utilize polyalcohol sugars as sole carbon sources. A deletion mutant that replaced the mpkC coding region with the nutritionally selective gene pyrG was made. Two independent mpkC deletion mutants were isolated, and all phenotypic studies were conducted with both strains. In all these studies, the ΔmpkC1 and ΔmpkC2 mutants behaved identically. Our initial phenotypic studies, based on the assumption that SakA and MpkC signaling pathways may overlap, focused on growth conditions that we previously determined for the sakA deletion mutants and produced an identifiable phenotype. We tested for defects in growth at high osmolarity (1.4 M KCl) (Fig. 2), survival at high temperatures, and exposure to hydrogen peroxide. None of the conditions tested resulted in an observable phenotype for the mpkC deletion mutants. These studies demonstrate that SakA and MpkC MAPK signaling pathways are differentially utilized and have little or no overlap in nutritional sensing.

Failing to identify growth conditions under which the mpkC deletion mutant displays a phenotype based on our studies of sakA deletion mutants, we investigated the growth of the wild type, a sakA deletion mutant, and mpkC deletion mutants on a range of media in which we replaced glucose with different carbon sources and ammonium tartrate with different nitrogen sources (described in Materials and Methods). These growth studies determined that the mpkC deletion mutants were unable to grow on MM with sorbitol or mannitol as the sole carbon source (Fig. 3).

We measured conidial germination on MM-sorbitol compared to that on MM-glucose media for the mpkC deletion mutant and the wild type (Af293). We found that the deletion...
The mpkC deletion mutant showed only 2.5% germination on sorbitol compared to 86% germination for Af293. In contrast, both strains germinated well on MM-glucose, with each strain exhibiting about 60% germination on glucose at 8 h (Table 2). After 16 h in MM-sorbitol, the mpkC deletion mutant still had large numbers of conidia that did not germinate. We also demonstrated that mpkC is required for growth on sorbitol, by first germinating conidia overnight in MM-glucose and then transferring an agar plug of mycelia to a plate of MM-sorbitol, where the mycelium was unable to grow. These experiments demonstrate that MpkC function is required for efficient conidial germination on sorbitol as well as for hyphal growth.

mpkC mRNA increases in abundance in response to the carbon source in the medium and oxidative stress but not following osmotic stress. Since the mpkC deletion mutants were unable to grow on MM with sorbitol as the sole carbon source, we reasoned that a growth shift from glucose to sorbitol would activate the MpkC signaling pathway and might result in increased mpkC mRNA levels. We tested this by probing Northern blots of total RNA prepared at intervals following a shift from MM-glucose to MM-sorbitol from the wild type (Af293) and a mpkC deletion mutant (ΔmpkC1). We found that sakA mRNA levels appeared to transiently increase in response to the glucose-to-sorbitol shift, that actin mRNA levels remained unchanged, and that mpkC mRNA was virtually undetectable, producing a very weak signal in the wild-type 60-min sample and none at all, as would be expected, in the deletion mutant strain (Fig. 4a). We think that the low-level expression of mpkC combined with the multiple transcription start and stop sites identified from our analysis of cDNA sequences make detection by Northern blot analysis difficult. It has also been reported that AnmpkC mRNA was not detectable in A. nidulans (8). We therefore used RT-PCR and qRT-PCR to detect and quantify the mpkC mRNA and compared that amount to the levels of sakA and actin mRNA (Fig. 4b).

To further investigate whether mpkC may share functions with sakA, we examined changes in sakA and mpkC mRNA levels by qRT-PCR following hypertonic shock (1 M KCl) and oxidative stress (H2O2). We previously showed that sakA mRNA levels are increased following hypertonic stress (28). Since protein sequence analysis suggests that mpkC encodes a MAPK that is related to the SakA MAPK, it was possible that mpkC mRNA levels would increase following hypertonic or oxidative stress. Furthermore, the AnMpkC is reportedly activated by the same MAPK kinase, PbsB, in A. nidulans (8).
Using qRT-PCR, we found that mpkC mRNA levels were slightly reduced following a shift to growth medium with 1 M KCl (Fig. 5). Interestingly, mpkC mRNA levels displayed a rapid and sustained increase following exposure to H$_2$O$_2$. In contrast, sakA exhibited only a modest increase in response to oxidative stress. Thus, it appears that while MpkC and SkaA are similar in sequence, they do not respond transcriptionally in the same way to various environmental stresses.

**DISCUSSION**

We have deleted the mpkC gene of *A. fumigatus* and determined that it is not essential for fungal cell growth in culture. We did determine that MpkC is required for growth on the polyalcohol sugars sorbitol and mannitol, suggesting a role for MpkC in carbon source sensing. We have thus identified a novel MAPK signaling pathway in *A. fumigatus* that responds to specific carbon sources. It will be interesting to see if the mpkC deletion mutant of *A. nidulans* also fails to grow on these carbon sources (8). We also found that mpkC produces a number of different classes of transcripts and is expressed at very low levels. While the complexity of transcripts in *A. nidulans* has been mined that it is not essential for fungal cell growth in culture. We found that the stress-activated MAPK signal-ling pathway of specific carbon sources. The results from our study of MpkC in *A. fumigatus* illustrate this by demonstrating that this MAPK is required for germination and growth on sorbitol or mannitol as a sole carbon source. This extends our previous studies of SkaA, in which we identified a new role for this MAPK in regulating conidial germination in response to environmental nitrogen (28). Finally, these experiments demonstrate a role for MAPK function in the differential use of carbon sources in this fungus. To our knowledge, this is the first case of a fungal MAPK having such a function.

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**REFERENCES**


