Signaling by the Pathogenicity-Related MAP Kinase of *Cochliobolus heterostrophus* Correlates With Its Local Accumulation Rather Than Phosphorylation

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Submitted 23 April 2009. Accepted 5 May 2009.

Phosphorylated mitogen-activated protein kinases (MAPK) transmit signals by activation of their targets. The extent of signal transduction could depend on MAPK phosphorylation level, concentration, and subcellular localization. The pathogenicity MAPK Chk1 of the fungal corn pathogen *Cochliobolus heterostrophus* is required for central developmental functions, including appressoria formation, conidiation, melanization, virulence, and female fertility. We followed *CHK1* transcript level, protein localization, quantity, phosphorylation, and expression of downstream genes during conidial germination on a surface inductive for appressoria formation and in suspension. The Chk1-GFP protein representing a translational fusion of Chk1 and GFP (green fluorescent protein) was very abundant in ungerminated conidia, accumulated in maturing appressoria and appressorial nuclei, but was uniformly distributed in suspension-grown hyphae. Expression of Chk1-dependent genes was upregulated in appressoria-forming hyphae but not in suspension. Despite Chk1 activation, there was no change in its phosphorylation and total protein quantity. Of all conditions tested, a temperature shift caused a decrease whereas hyperosmotic stress caused an increase in Chk1 phosphorylation. Activation of Chk1 during appressoria formation is apparently manifested by its local accumulation but not by significant changes in phosphorylation.

Mitogen-activated protein kinases (MAPK) are ubiquitous and evolutionarily conserved enzymes involved in transduction of environmental signals to activate an appropriate cellular response. The budding yeast *Saccharomyces cerevisiae* has five MAPK, encoded by *FUS3*, *KSS1*, *HOG1*, *SLT2*, and *SMK1*. Slr2 homologs in filamentous fungi (cell integrity MAPK) function to maintain the cell wall and are essential for the development of functional appressoria in appressoria-forming fungi and for virulence (Hou et al. 2002; Kojima et al. 2002; Mehrabi et al. 2006; Mey et al. 2002b; Xu et al. 1998). Fus3/Kss1 homologs (pathogenicity MAPK) are involved in appressoria formation and pathogenicity in general but have diverse functions in hyphal growth, sexual and asexual repro-

duction, and conidial germination (Di Pietro et al. 2001; Lev et al. 1999; Mendoza-Mendoza et al. 2007; Mey et al. 2002a; Moriwaki et al. 2007; Mukherjee et al. 2003; Pandey et al. 2004; Ruiz-Roldan et al. 2001; Takano et al. 2000; Xu and Hamer 1996; Zheng et al. 2000). Hog1 homologs (stress-activated MAPK) are needed to cope with high-salt environments or oxidative stress (Jin et al. 2005; Kawasaki et al. 2002).

The involvement of pathogenicity MAPK in major functions necessary for fungal survival implies their activation at least at the crucial moments of developmental transitions. The classical ERK (extracellular signal-related kinase) activation path includes their phosphorylation on the key threonine and tyrosine residues by upstream MEK (MAPK/ERK kinase) and translocation into the nucleus to phosphorylate nuclear targets, such as transcription factors. ERK activity is terminated by dephosphorylation by specific phosphatases (Turjanski et al. 2007). Indeed, yeast Fus3 constitutively shuttles between the cytoplasm and nuclei of vegetative cells, but activation of the mating pathway by pheromone stimulates Fus3 phosphorylation and its nuclear localization. Pheromone also induces upregulation of *FUS3* transcription and leads to an increase in the total amount of Fus3 in stimulated cells (Blackwell et al. 2003; Choi et al. 1999; van Drogen et al. 2001). In contrast to Fus3, Kss1 is constitutively nuclear (Ma et al. 1995). Activation of this class of MAPK has been studied in some filamentous fungi. The *Magnaporthe grisea* Fus3/Kss1 homolog Pmk1 was shown to interact with upstream MAPK kinase Mst7 during appressoria formation (Zhao and Xu 2007). Furthermore, Pmk1 accumulated in maturing appressoria, as compared with vegetative hyphae, and was found in nuclei in a certain percentage of appressoria (Bruno et al. 2004). Phosphorylation of Pmk1 was detected during appressoria formation but not in vegetative mycelium (Zhao et al. 2005). In *Neurospora crassa*, increased phosphorylation of Mak2 was associated with conidial germination and formation of an interconnected hyphal network (Pandey et al. 2004). In *Sclerotinia sclerotiorum*, phosphorylation of Smk1 was induced during sclerotigenesis (Chen et al. 2004). In *Fusarium graminearum*, both the pathogenicity (Gpmk1) and the cell integrity (Mgv1) MAPK were increasingly phosphorylated in response to the *Medicago* spp. defensin MsDef1 (Ramamoorthy et al. 2007).

Transcriptional upregulation of Fus3/Kss1 homologs has been reported in several studies. Expression of *Stagonospora nodorum* MAK2 was upregulated in vitro upon nitrogen and carbon starvation and during sporulation in planta (Solomon et al. 2005). In *Ustilago maydis*, transcription of *KPP6* was upregulated by the bW/bE heterodimer and by pheromone stimulation (Brachmann et al. 2003). Transcription of *Scle-*

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The gene *MAS3* has been deposited under accession EU159530.

*The e-Xtra logo stands for “electronic extra” and indicates that a supplemental figure as well as supplemental methods information are published online.

rotinia sclerotiorum *SMK1* was dramatically induced by acidification of the growth medium and during production of sclerotia initials (Chen et al. 2004).

Cochliobolus heterostrophus, the cause of Southern corn leaf blight, is a filamentous necrotrophic ascomycete. In the presence of moisture, large melanized spores of this fungus adhere to the host leaves and germinate to form small appressoria. Appressoria-forming hyphae often intermingle and form more reinforced structures that appear as hyphal knots on the leaf surface. Penetration into the plant tissue occurs at the points of attachment of appressoria and hyphal knots. *C. heterostrophus* is also able to infect the host plant by penetration through stomata or other natural openings without forming appressoria (Horwitz et al. 1999). Successful infection results in formation of eyespot-like lesions on the maize leaves. We have previously characterized the *C. heterostrophus* pathogenicity MAPK gene *CHK1* (Lev et al. 1999). *Chk1*-deletion mutants are female sterile, lack conidia, do not form appressoria on an inductive surface, have reduced pigmentation, and are im-

paired in their ability to infect maize plants. Functional Chk1 is required for expression of a variety of genes, including the melanin biosynthesis genes scytalone dehydratase *SCD1* and tetra- and trihydroxynaphthalene reductases *BRN1* and *BRN2*, respectively, and cell wall-degrading enzymes including endoglucanase *EG6* and cellobiohydrolase *CBH7* (Eliahu et al. 2007; Lev and Horwitz 2003). It is still unclear whether Chk1 regulates the expression of melanin biosynthesis and cellulose genes directly through phosphorylation of transcriptional regulators. Chk1 may be necessary for differentiation of hyphae into appressoria and conidia that, once formed, activate the expression of additional, specialized sets of genes.

Recently, we have isolated a *C. heterostrophus* homolog of yeast *SLT2* designated *MPS1* (Igbaria et al. 2008). *mps1* deletion mutants resemble *chk1* mutants in their appearance. They have reduced conidiation, pigmentation, and virulence. In contrast to *chk1*, *mps1* deletion strains form normal-looking appressoria on artificial surfaces (Igbaria et al. 2008). In accordance with the similarity of *chk1* and *mps1* mutant phenotypes, Mps1 and Chk1

Fig. 1. Strategy for construction of *CHK1^{WT}-GFP*-, *CHK1^{AEF}-GFP*-, and *CHK1^{K49R}-GFP*-expressing fungal lines and their verification. **A**, Scheme of consecutive transformations ensuring introduction of AEF and K49R mutations. P_{trp} = constitutive promoter, *NAT* = nourseothricin acetyltransferase encoding gene, *HYG* = hygromycin phosphotransferase encoding gene, and *GFP* = green fluorescent protein encoding gene. Black arrows indicate positions of primers used for mutagenesis, vector construction, and verification of transformants. **B**, Transformants were verified by polymerase chain reaction (PCR) on genomic DNA as a template, using the following primer pairs: 1, *NheI*-s and *HindIII*-a; 2, *NAT*-a and *DCover*; 3, *HYG*-a and *DCover*; 4, *GFP* and 3s. **C**, Expression of wild-type and mutant variants of *CHK1-GFP* in transformant strains was confirmed by reverse transcriptase-PCR with a *GFP* and 3s primer pair. Expression of actin-encoding gene was used as a positive control.

share a subset of common target genes, including those involved in melanin biosynthesis (Eliahu et al. 2007).

In this study we assessed Chk1 activity under varying growth conditions and during conidial germination on a hydrophobic surface and in suspension. Our results suggest that Chk1 is constitutively phosphorylated, and its activation during appressoria formation is achieved by accumulation in appressoria and appressorial nuclei.

RESULTS

Construction of fungal lines expressing intact and mutated CHK1 fused to green fluorescent protein (GFP).

To follow Chk1 expression and localization, we constructed *CHK1-GFP*-expressing lines in two steps. We first deleted the entire *CHK1* coding region by homologous recombination, replacing it with the nourseothricin resistance cassette. Second, we reintroduced the *CHK1* coding region fused to *GFP* into the original *CHK1* chromosomal location, exchanging nourseothricin resistance for resistance to hygromycin (Fig. 1A). We also introduced *CHK1* bearing substitution mutations in the TEY amino acid sequence. The TEY sequence is conserved in all MAPK of the class to which Chk1 belongs and is phosphorylated by upstream MAPK kinase. The original Chk1 TEY motif was changed to AEF. Another mutation we introduced was a substitution of lysine 49 to arginine (K49R), this mutation is expected to cause loss of Chk1 kinase activity, as was described for its close homolog in *M. grisea*, Pmk1 (Bruno et al. 2004). The original *CHK1* promoter was retained to control the expression of the *CHK1-GFP* fusion genes. We used the strategy of *CHK1* removal and reintroduction into the same locus, since homologous recombination in *C. heterostrophus* is usually much more efficient than ectopic integration (unpublished observation).

Nourseothricin-resistant transformants showed all the characteristics of *chk1* deletion mutants, as described by Lev and associates (1999). Reintroduction of the *CHK1^{WT}-GFP* fusion gene restored the wild-type phenotype, including normal pigmentation, conidiation, and appressoria formation. The integration of *CHK1^{WT}*, as confirmed by polymerase chain reaction (PCR) on genomic DNA, was into the original *CHK1* location (Fig. 1A and B). Integration of the *CHK1^{AEF}*, also to the original location, did not restore the wild-type phenotype, and the transformant was indistinguishable from the *chk1* deletion mutant. The *CHK1^{K49R}-GFP*-expressing transformant possessed the same phenotype as *Chk1^{AEF}* and *Δchk1* lines. PCR verification of the *Chk1^{K49R}* transformant revealed ectopic integration of the construct into the genome (Fig. 1B). We confirmed the expression of the *CHK1-GFP* fusion gene in all transformants by reverse transcriptase (RT)-PCR (Fig. 1C). GFP fluorescence was observed in the transformants, and the background autofluorescence of WT was very low compared with the GFP signal (Supplementary Fig. S1).

Chk1 phosphorylation in *Chk1^{WT}*, *Chk1^{AEF}*, and *Chk1^{K49R}* transformant lines.

The presence of the Chk1-GFP protein and its phosphorylation in transformant colonies grown in stationary culture was tested by Western blot, using anti-P42/44 and anti-phospho-P42/44 antibodies (Fig. 2A). Anti-P42/P44 antibodies recognize MAPK proteins homologous to mammalian ERK1/ERK2, whereas anti-phospho-P42/44 antibodies recognize only individually or dually phosphorylated ERK1/ERK2 homologs. As expected, the addition of GFP caused an approximately 30-kDa shift in Chk1 migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). *Chk1^{AEF}-GFP* protein was readily detected by anti-P42/44 antibody but not by

anti-phospho-P42/44 antibody, thus confirming that substitution of TEY by AEF completely abolishes Chk1 phosphorylation (Fig. 2A), as was demonstrated for its orthologs in *M. grisea* (Pmk1) and yeast (Kss1) (Bardwell et al. 1998b; Bruno et al. 2004). Furthermore, this result provides strong evidence for specificity of the antibody, since a polypeptide of the expected size is detected by anti-P42/P44, but not anti-phospho-P42/P44. Unlike *Chk1^{AEF}*, *Chk1^{K49R}* was highly phosphorylated as compared with wild type and the complemented strain *Chk1^{WT}*. A possible explanation for this result is that, in the absence of the catalytic activity of *Chk1^{K49R}*, a negative feedback mechanism that normally would ensure the proper level of Chk1 phosphorylation was not activated.

In yeast, unphosphorylated Kss1 represses Ste12, and the presence of nonphosphorylatable or catalytically inactive forms of Kss1 is sufficient for this repression, whereas Kss1 deletion causes Ste12 derepression (Bardwell et al. 1998a and b). If unphosphorylated Chk1 has a repressive role, its target genes are expected to be more repressed in *Chk1^{K49R}* and *Chk1^{AEF}* than in *Δchk1* mutants; in other words, the *Chk1^{K49R}* and *Chk1^{AEF}* phenotypes would be expected to be more severe than that of *Δchk1*. This does not seem to be the case, since all three mutants have a similar appearance. Furthermore, the Chk1-dependent gene *BRN1* is underexpressed in all three mutants (Fig. 2B). The phosphorylation of Chk1 and its catalytic activity are thus crucial for its action, and the presence of unphosphorylated or inactive Chk1 is apparently not enough for fulfillment of any of its cellular functions.

Chk1-GFP accumulates in conidia and appressoria.

Bright fluorescence was observed in cross-sections of fungal colonies expressing *CHK1^{WT}-GFP*, both in agar-submerged

Fig. 2. Phosphorylation of Chk1 and expression of downstream gene *BRN1* in wild type and mutant strains. All fungal strains were grown for 3 days in complete medium stationary culture under continuous white illumination. **A**, Western blots were hybridized with anti-phospho-P42/44 and anti-P42/44 antibodies. Positions of Chk1 (41 kDa), Mps1 (47 kDa), and Chk1-GFP (68 kDa) are indicated. Total protein was visualized using Ponceau stain. Data are typical of three independent experiments. **B**, Expression of Chk1-dependent melanin biosynthesis gene *BRN1* was quantified in the RNA isolated from the samples shown in A, using real time polymerase chain reaction.

and aerial parts and, especially, in conidia (Fig. 3A). Background autofluorescence in wild type was at the limit of detection under these conditions. When germinating in shake culture, conidia and germ tubes were uniformly fluorescent (Fig. 3B). In appressoria formed by germ tubes on an inductive surface, the Chk1-GFP fluorescence was considerably higher than in conidia and surrounding hyphae (4 h postinoculation [hpi]) (Fig. 3C and D). We noticed that accumulation of Chk1-GFP occurred only in existing appressoria but not in hyphal tips prior to formation of appressoria. Some appressoria lacked the increased fluorescence. These may be newly formed appressoria, suggesting that Chk1 accumulation occurs only during appressoria maturation.

Appressoria-forming hyphae from one or several conidia often form 'knots' representing infective structures more complex than a single appressorium. To create these structures, adjacent hyphae seem to be attracted to each other, possibly by the mechanism responsible for hyphal fusion in *N. crassa* (Pandey et al. 2004). The observation that *chk1* mutants are

impaired in their ability to form knots as well as in ability to form appressoria supports this hypothesis, since *N. crassa* hyphal fusion is MAPK-dependent. It is difficult to establish if hyphal fusion occurs inside mycelial knots, but these structures function as alternative entry points into plant tissue, possibly serving as more reinforced structures than individual appressoria. Increase in fluorescence due to accumulation of Chk1-GFP was observed in hyphal knots, usually together with the adjoining hyphal segment (Fig. 3E and H).

The distribution of fluorescence in Chk1^{AEF} and Chk1^{K49R} mutants growing on a glass surface from small mycelial inocula was uniform throughout each individual hypha and varied only between different hyphae (Fig. 3F and G). No appressoria were formed by these mutants and no local fluorescence increase was observed. The Chk1^{WT} strain formed numerous appressoria when inoculated from mycelia, and Chk1-GFP accumulated inside these structures in the same pattern as in appressoria formed by conidial germ tubes (not shown).

Fig. 3. Localization of Chk1^{WT}-GFP in fungal hyphae by fluorescence imaging of transgenic lines. **A**, Colony cross-section. Note increased accumulation of Chk1-GFP in conidia. **B**, Conidia germinating in shake culture. **C** and **D**, Chk1-GFP accumulates in appressoria and adjacent hyphal segments. **E**, Hyphal knots formed on glass or **H**, infected leaf surface. **F**, Chk1^{K49R} and **G**, Chk1^{AEF} mutant strains growing on the glass surface. **a** indicates appressorium, **c**, conidium and **k**, hyphal knot.

We noticed that sometimes a small restricted area of more intense fluorescence appeared inside the appressoria and hyphal knots (Figs. 3C and E and 4). Nuclear staining confirmed that these brighter areas were, indeed, nuclei (Fig. 4). Since cytosolic fluorescence in appressoria was very high, it was difficult to establish the percentage of appressoria with increased nuclear accumulation of Chk1-GFP.

Activity of Chk1-GFP during conidial germination.

Several lines of evidence indicate that Chk1 is active during appressoria formation: i) *Achk1* mutants do not produce appressoria and ii) Chk1 protein accumulates in appressoria and their nuclei. Phenotypes of Chk1^{AEF} and Chk1^{K49R} mutants confirm that phosphorylation of Chk1 and its catalytic activity are required for appressoria formation. We examined Chk1 activation during conidia germination on an inductive surface as compared with germination in suspension. Chk1 activation was estimated on multiple levels, i.e., transcript and protein abundance, degree of phosphorylation, protein localization, and expression of genes *BRN1*, *SCD1*, *MAS3*, and *EG6* (Eliahu et al. 2007; Lev and Horwitz 2003). Although the expression of melanin biosynthesis and cellulase genes does not necessarily directly report Chk1 activity, it serves as an indicator of the developmental changes caused by Chk1 activation.

Microscopic examination of *CHK1-GFP*-expressing spores revealed very bright fluorescence, which decreased slightly during the first hour of incubation on a glass surface (Fig. 5C). Emerging germ tubes were also brightly fluorescent. After 2 h, the fluorescence of conidia and germ tubes further decreased and the first brightly fluorescent appressoria appeared. Between 2 and 4 h of incubation, the mycelium extended considerably and an increasing number of highly fluorescent appressoria and hyphal knots appeared (Fig. 5C). Expression of *BRN1*, *SCD1*, *MAS3*, and *EG6* was significantly induced between 4 and 8 h, after formation of numerous appressoria. Expression of melanin biosynthesis genes was very high in ungerminated conidia (data not shown), and in some experiments, this high expression was detected also at an early germination stage (2 h; Fig. 5B, lower left panel). Surprisingly, no detectable increase in Chk1 protein quantity or phosphorylation was found at any time during incubation on the inductive surface or in suspension (Fig. 5A). The transcript level of *CHK1* in samples from the inductive surface increased slightly over the time-course shown in the upper panel of Figure 5B; however, this did not occur in all experiments (data not shown) and, thus, is within the experimental variability. This is consistent with the constant level of Chk1 protein detected by anti-P42/44 (Fig. 5A, middle panel).

In suspension, high hydrophobicity of conidia caused them to adhere to each other and form clumps shortly after inoculation. Despite the high density of hyphae in these clumps, germ tubes continued to elongate. No appressoria were formed under these conditions. The fluorescence of conidia remained high during incubation up to 3.5 h, unlike the conidia germinating on a hard surface (Fig. 5C). The germ tubes were weakly but uniformly fluorescent. Again, Chk1 protein and phosphorylation levels in suspension-grown mycelium were the same as in germ tubes growing on an inductive surface (Fig. 5A).

In *M. grisea* two cell surface proteins, *GAS1/MAS3* and *GAS2/MAS1*, are specifically expressed in appressoria of the wild-type strain but not in *pmk1* deletion mutants (Xue et al. 2002). Interestingly, in the nematode-trapping fungus *Monacrosporium haptotylum*, a homolog of *MAS3* is expressed in the knob structures involved in trapping, suggesting a general role of this family of proteins in structures that have evolved to contact or penetrate the host (Ahren et al. 2005). We identified

a homolog of *MAS3* in the *C. heterostrophus* genome (Supplementary Methods) and tested its expression as a potential marker for appressoria formation. *C. heterostrophus MAS3* was strongly induced in surface-growing hyphae (starting from 4 hpi) but was almost unchanged in mycelium growing in suspension (Fig. 5B). Induction of *MAS3* further confirms the presence of a surface signal perceived by wild type growing on an inductive surface but not in suspension that is not reflected in a change of Chk1 phosphorylation status.

External and internal conditions affecting Chk1 phosphorylation.

We tested the phosphorylation of Chk1 under different environmental and physiological conditions to determine which factors might influence it. Out of all conditions tested, transfer of the fungal mycelium from 25 to 38°C caused a rapid decrease in Chk1 phosphorylation (Fig. 6A). On the other hand, addition of 1 M sorbitol triggered a rapid increase in Chk1 phosphorylation (Fig. 6B; Igbaria et al. 2008). These findings indicate that Chk1 phosphorylation is tightly controlled, and the fungus is able to respond swiftly to environmental changes with the adjustment of Chk1 phosphorylation to an appropriate level. Interestingly, the phosphorylation of Mps1 changed in the opposite manner from Chk1, i.e., it increased following transfer to 38°C and decreased after addition of sorbitol (Fig. 6A and B; Igbaria et al. 2008).

A minor increase in Chk1 phosphorylation was observed in germinating conidia (4 hpi) as compared with vegetative hyphae, but it was associated with germination itself rather than with surface sensing, since the same increase in phosphorylation was detected in conidia germinating in suspension (Fig. 6C). Phosphorylation of Chk1 was nearly the same in mycelium growing in shake culture, on agar plates, or in aerial hyphae (Fig. 6C). Yet, the differences in expression of *BRN1* were dramatic. It was highly expressed in colonies growing on agar plates and, especially, in samples of aerial hyphae with conidia and was very low in germinating conidia and mycelium grown in shake culture (Fig. 6D). The peak *BRN1* expression was found in conidia (data not shown). This finding is consistent with the distribution of Chk1-GFP fluorescence in

Fig. 4. Chk1-GFP accumulates in nuclei of appressoria. Left panels show green fluorescent protein fluorescence; middle panels, DAPI (4',6'-diamidino-2-phenylindole) nuclear staining; and right panels, bright-field illumination.

the colony cross-section, as shown in Figure 3A, in which it was low in hyphae and very high in conidia.

Interestingly, in the samples with high *BRN1* expression, phosphorylation of Mps1 was also upregulated, though very

moderately. As we established previously, *BRN1* expression depends on two MAPK, Chk1 and Mps1 (Eliahu et al. 2007). Joint signaling by both MAPK might contribute to an increased expression of *BRN1* in aerial hyphae and spores.

Fig. 5. Measurement of Chk1 activity during conidial germination on an inductive surface (plastic petri dish) and in suspension. **A**, Western blots were hybridized with anti-phospho-P42/44 and anti-P42/44 antibodies. Total protein was visualized using Ponceau stain. Data are typical of two independent experiments. **B**, Expression of *CHK1*, tetrahydroxynaphthalene reductase *BRN1*, scytalone dehydratase *SCD1*, endoglucanase *EG6*, and putative appressoria-specific gene *MAS3*, quantified using real time polymerase chain reaction (PCR). The top panel shows the results of real-time quantitative PCR analysis performed on RNA isolated from the same fungal samples used for the immunoblots in A. The results of two additional independent experiments are shown in the lower two panels. Each bar represents the average of three replicate PCR reactions on the same RNA sample. **C**, Localization of Chk1^{WT}-GFP fusion protein during different stages of germination. Upper panels show germination on plastic surface, and the lower panels show germination in suspension. Micrographs were taken using the confocal microscope. Different shades of gray represent green fluorescent protein fluorescence, with darker shades indicating stronger fluorescence.

DISCUSSION

Extensive research has been conducted to determine functions of pathogenicity MAPK in filamentous fungi, but the role

of phosphorylation in MAPK activation has not been assessed systematically. In *C. heterostrophus*, functional Chk1 is required for formation of appressoria following sensing of an inductive surface. We therefore tested localization and phos-

Fig. 6. Environmental and physiological conditions affecting Chk1 phosphorylation. **A**, Mycelium grown in complete medium (CM) shake culture was shifted from 25 to 38°C for 15 min and was then harvested. **B**, Sorbitol was added to the fungal shake cultures (CM) to a final concentration of 1 M. The cultures were harvested after 10 and 25 min of incubation. Western blots in **A** and **B** were hybridized to anti-phospho-P42/44 antibodies. **C**, Chk1 phosphorylation and protein quantity were assessed in fungal mycelium under different physiological conditions. Growth in CM shake culture, in an air-exposed colony on CM-agar plate, in aerial hyphae with conidia, and during conidial germination (in water) on a plastic surface or in suspension. **D**, Quantitative polymerase chain reaction assay of *CHK1* and *BRN1* gene expression in the indicated samples.

phorylation of Chk1 in the course of conidial germination and appressoria formation, as compared with germination in suspension. Surprisingly, phosphorylation of Chk1 was very similar in all the samples tested during the entire incubation period. On the other hand, Chk1 protein, very abundant in ungerminated conidia, accumulated in appressoria and mycelial knots in the course of germination. In some appressoria, even greater Chk1 accumulation was observed in nuclei. These findings imply that activity of Chk1 during formation of appressoria is associated with its local concentration and subcellular localization rather than with changes in phosphorylation. We have observed that appressoria formation is not a synchronous process, occurring in each hypha independently of the others. Therefore, when sampling gene expression and phosphorylation in a large population, activation would occur over several hours. This makes it unlikely that, in these experiments, we have overlooked a transient increase in Chk1 phosphorylation, although one can never formally exclude the possibility of a phosphorylation change that is very rapid and transient, avoiding detection entirely. Significant Chk1 phosphorylation was detected in cultures grown under different conditions, indicating that basal Chk1 activity is required for normal cellular homeostasis. In the absence of plant defensin treatment, constitutive phosphorylation of the Chk1 ortholog Gpmk1 was reported in *Fusarium graminearum* (Ramamoorthy et al. 2007). We followed the expression of the melanin biosynthesis genes *BRN1* and *SCD1*, the predicted appressorium-expressed gene *MAS3*, and cellulase *EG6* in the course of conidial germination. Our findings suggest two possible scenarios for the regulation of these genes. In the first scenario, during appressoria maturation, Chk1 phosphorylates one or more as-yet-unidentified transcription factors, which upregulate expression of the target genes. We have previously identified a transcription factor gene, *CMR1*, that depends on Chk1 for its expression and, in turn, regulates several melanin biosynthesis genes, including *BRN1* and *SCD1* (Eliahu et al. 2007). According to the second scenario, basal Chk1 signaling leads to appressoria formation. During appressoria maturation, Chk1 activation builds up, possibly through a self-activation mechanism manifested by increased Chk1 nuclear localization. In mature appressoria, new sets of genes, including *BRN1*, *SCD1*, and *MAS3*, are expressed, which might be important to attain penetration into the host plant or set the stage for subsequent invasive growth. According to both scenarios, target gene expression reflects Chk1 activity, either directly or indirectly.

As shown in *Xenopus* cells, phosphorylated ERK translocates to the nucleus by two mechanisms, slow passive diffusion of the monomeric form or fast active transport as a dimer (Adachi et al. 1999). Phosphorylation of ERK increases their propensity to form homodimers, either with another phosphorylated or unphosphorylated ERK (Khokhlatchev et al. 1998). Overexpression of *ERK* in *Xenopus* cells increased its active nuclear import, suggesting that a higher concentration of MAPK in the cytosol increases the probability of dimer formation (Adachi et al. 1999). We can speculate that, in the absence of an appressorium-related signal, phosphorylated Chk1 passively enters nuclei and activates cytosolic as well as nuclear targets, whereas in the process of appressoria maturation, the activity of Chk1 is elevated through its increased synthesis in appressoria, followed by dimerization and active transport to nuclei. According to Khokhlatchev and associates (1998), MAPK homodimers may be formed when only one of the molecules is phosphorylated, thus further diminishing the importance of phosphorylation. Kinase activity of unphosphorylated Chk1 in the nucleus is not expected to be different from the activity of the phosphorylated molecule, providing the same output in phosphorylation of nuclear target proteins.

Fungal pathogens including *C. heterostrophus*, *M. grisea*, and *Colletotrichum lagenarium* rely on thigmotropic sensing to recognize a surface suitable for appressoria formation. Fus3/Kss1 and Slr2 fungal homologs together with cAMP-dependent signaling have been implicated in different aspects of appressoria formation. Several lines of evidence suggest that pathogenicity MAPK are involved in advanced stages of appressoria maturation rather than in initial surface sensing. *M. grisea pmk1* mutants were unable to form mature appressoria but still retained their ability to respond to cAMP and thigmotropic surface signals by increased germ-tube hooking and swelling (Xu and Hamer 1996). Similarly, *Colletotrichum lagenarium cmk1* mutant conidia supplemented with yeast extract were able to germinate and to respond to inductive surfaces by formation of swollen appressoria-like structures (Kojima et al. 2002; Takano et al. 2000). Moreover, interaction of Pmk1 with its upstream MAPK kinase Mst7 was detected in maturing appressoria but not at the phase when swelling and hooking of germ tubes precedes appressorium formation (Zhao and Xu 2007). In *C. heterostrophus*, the evidence for enhanced Chk1 activation in maturing appressoria comes from the timing of expression of its downstream genes, which occurs mostly after 4 hpi, when numerous appressoria have already formed. It is likely that basal Chk1 activity is required for initiation of appressoria formation, since *chk1* deletion mutants do not form any structures resembling appressorial initials (Lev et al. 1999).

C. heterostrophus growing in shake culture and on agar plates implements different developmental programs, as exposure to air triggers formation of hydrophobic aerial hyphae and conidiation. *Chk1* deletion mutants have an autolytic, conidia- and pigment-deficient appearance when grown on plates, indicating that Chk1 activity is crucial for normal colony development. Microscopic examination of the colony cross-section revealed increased accumulation of Chk1-GFP in conidia. Expression of Chk1-dependent *BRN1* is dramatically induced in mycelium and even more in conidia of the air-exposed colony. The observation that Chk1 is phosphorylated to the same degree in shake culture and in the plate-grown colonies again implies that Chk1 activity is regulated mainly by its local concentration but not by changes in phosphorylation. It is possible that basal Chk1 activity is required for normal development of aerial hyphae, but to initiate conidia formation, increased synthesis of Chk1 occurs in conidiophores.

An increase in temperature or the addition of 1 M sorbitol caused fast changes in Chk1 phosphorylation. In yeast, addition of 1 M sorbitol decreased the extent of Slr2 phosphorylation in response to cell wall-damaging agents (de Nobel et al. 2000). On the other hand, Slr2 was activated in yeast cells grown at 37°C or following a shift from 23 to 39°C (Kamada et al. 1995). Interestingly, the increase in Chk1 phosphorylation was accompanied by a decrease in Mps1 phosphorylation and vice versa. In *M. grisea*, the same tendency has been described, namely, phosphorylation of Mps1 increased following inactivation of the Pmk1 MAPK cascade (Zhao et al. 2005). It is possible that the same signaling component activating *C. heterostrophus* Mps1 in response to mild stress concomitantly represses the phosphorylation of Chk1.

In summary, kinase activity and phosphorylation of Chk1 are necessary for its function. The extent of Chk1 phosphorylation changes in response to some environmental conditions. In general, however, the extent of phosphorylation remains relatively stable, even while large changes in downstream gene expression are induced. Our data suggest that activation of Chk1 signaling during appressoria maturation is provided by its increased accumulation in appressoria and appressorial nuclei to phosphorylate nuclear targets. This mode of MAPK

activity regulation is similar to yeast Fus3 regarding increased transcription and synthesis in stimulated cells and intense nuclear translocation. The difference, though, is that Fus3 is phosphorylated in response to the stimulus, whereas Chk1 is phosphorylated continuously. This mechanism of activation implies that signaling components other than MAPK kinase kinase and MAPK kinase may play a pivotal role in determination of Chk1 activity. Thus the upstream kinases of the cascade would be necessary but not sufficient for functional activation. Possible additional levels of signaling are accumulation of Chk1 in developing appressoria by transport of its mRNA, transport of the protein, increased synthesis, or decreased degradation. The activation mechanism proposed in this study differs from previously described models, and it will be of interest to see how general it is among other fungi and higher eukaryotes. The activation patterns found here appear specifically adapted to the pathogenic lifestyle, because the accumulation of Chk1 occurs during maturation of appressoria, just prior to penetration into the leaf.

MATERIALS AND METHODS

Culture conditions and analysis of transformants.

Wild-type (C4, C5) and mutant strains were cultured on complete medium (CM). For selection with hygromycin B (50 µg/ml) (Calbiochem, La Jolla, CA, U.S.A.) and nourseothricin (120 µg/ml) (Werner BioAgents, Jena, Germany) selection, CM was prepared without salts. For germination experiments, conidia were harvested from CM-agar plates grown at 25°C under continuous white illumination. Conidia were suspended in water and were filtered through nylon mesh to remove mycelial debris. For germination on an inductive surface, 50 ml of a 10⁴ conidia/ml suspension were transferred to empty 15-cm plastic petri dishes (Nunc, Roskilde, Denmark) and were incubated in the light at 22 to 24°C for the indicated time periods. A different type of culture dish (15 cm) (Greiner Bio-One, Monroe, NC, U.S.A.) was much less effective in induction of the target genes. To prevent changes in MAPK phosphorylation and gene expression during sample collection, we added TRI-reagent to the petri dishes and then scraped the conidia from the plastic surface. For germination in suspension, conidia in water were gently stirred with a magnetic stirrer to avoid their attachment to the bottom of the flask. For the temperature-shift experiment, the mycelium was grown in shake

CM culture at 25°C overnight. Some amount of the medium was removed from the culture and preheated to 38°C. Mycelium was filtered and resuspended in preheated medium and was incubated for 15 min at 38°C. For the experiment with sorbitol, mycelium was grown in shake CM culture (25°C) for 2 days. Sorbitol powder was dissolved in used culture medium to the final concentration of 1 M. Filtered mycelium was added to the solution and was incubated with shaking for 10 and 25 min.

CHK1 replacement constructs and transformant verification.

The vector for deletion of *CHK1* was assembled from the following fragments: 620-bp 5' flank (amplified with primers FBsp120I-s and *PaeI*-a), 963-bp nourseothricin resistance cassette including the *Streptomyces noursei* nourseothricin acetyltransferase-encoding gene (GenBank X73149), 953-bp 3' flank amplified with primers FNheI-s and *SacI*-a, and pBluescript cloning vector (Stratagene, La Jolla, CA, U.S.A.) (Fig. 1A; Table 1). For transformation, the resulting vector was amplified by PCR with standard primers M13F and M13R, using BIO-X-ACT Long high fidelity DNA polymerase (BIO-21049; Boline, Luckenwalde, Germany). Polyethylene glycol-mediated transformation into fungal protoplasts was performed as described previously (Lu et al. 1994; Turgeon et al. 1993).

Genomic DNA was isolated from the fungal mycelium using the Extract-N-Amp plant PCR kit (XNAP2; Sigma, St. Louis). The deletion of *CHK1* in nourseothricin-resistant colonies was confirmed by PCR amplification, crossing the junction point of the vector with genomic DNA formed following a homologous recombination event. Primer pairs used for PCR were DCOver and NAT-a and *NheI*-s and *HindIII*-a (Fig. 1A and B; Table 1).

Construct Chk1^{WT} was assembled from the following fragments: 1.9-kb *CHK1* promoter and coding sequence synthesized with primers FBsp120I-s and *HindIII*-a, GFP-encoding gene with terminator of nopaline synthase (from vector HBTsGFP (S65T)-NOS, accession EF090408), hygromycin-resistance cassette, 953-bp 3' flank amplified with primers FNheI-s and *SacI*-a, and pBluescript vector. To construct vectors Chk1^{AEF} and Chk1^{K49R}, the 5' flank of the *CHK1*^{WT} plasmid was replaced with the same fragment containing corresponding mutations. Two primer pairs used to synthesize mutated *CHK1* variants are listed in Table 1. Nourseothricin-resistant *chk1* deletion

Table 1. Primers used in this study

Gene/construct	Primer name	Primer sequence ^a	Primer name	Primer sequence ^a
Chk1-DCO-NAT	FBsp120I-s	taaggGCCAGCCAGTGCCAGAACA	PaeI-a	ataacgcGCTGCCGCTTCCTGCTGGTG
	FNheI-s	TTGATGCTAGCTATACAAGAGTGCG	SacI-a	attgaGCTCTCTTAACCTCCGAGGCTCC
Chk1 ^{WT}	FBsp120I-s	taaggGCCAGCCAGTGCCAGAACA	HindIII-a	atcaagctTCGCATAATCTCCTGGTAG
Chk1 ^{AEF}	AEF-s	gTtCGTTGCCACTCGTTGGTACCG	AEF-a	TCaGcCATGAAACCCGAGTTGTCCTC
Chk1 ^{K49R}	K49R-s	gGAAGATTACGCCCTTTGACCAC	K49R-a	TAATGGCAACCTTCTGGCCC
Chk1 ^{WT} , Chk1 ^{AEF} , Chk1 ^{K49R}	NheI-s	AtCGCTAgCATGCCACCAGCAGGAA	HindIII-a	atcaagctTCGCATAATCTCCTGGTAG
transformants verification		GCG		
Chk1-DCO-NAT transformants	DCOver	TGGAAAGCGGAAGCGGAAGCG	NAT-a	AGCCGTGTCGTCAAGAGTGGTC
verification				
Chk1 ^{WT} , Chk1 ^{AEF} , Chk1 ^{K49R}	DCOver	TGGAAAGCGGAAGCGGAAGCG	HYG-a	CGTCGCGGTGAGTTCAGGCAT
transformants verification				
<i>CHK1</i> -GFP expression	3s	GCGCATCTCAGTCGAGGACG	GFP	CCTTCAGCTCGATGCGGTTACCC
Actin, control for <i>CHK1</i> -GFP	ACT2284-s	TTCTCCACCACTGCCGAGCG	ACT2855-a	GCGGTGAACGATGGAGGGAC
expression verification				
<i>CHK1</i> , real-time PCR	CHK1-RLT-s	AGGCAAGGACTACCACCACCA	CHK1-RLT-a	CCTGGCTCGTCATCTGGGTC
<i>BRN1</i> , real-time PCR	BRN1-s	TGGGTGGCCGCATTATCCTCA	BRN1-a	GAAGCAGACGACGCGGGCAAT
<i>MAS3</i> , real-time PCR	MAS3-RLT-s	GAGTATCATCGCCGCCACTG	MAS3-RLT-a	CCACCGAAAGGTCCAGCACG
<i>EG6</i> , real-time PCR	EG6-M-s	CAGAATGAAGTTCTCCGCTGCT	EG6-2-a	CGGCAGAGGAGAAAGCACCTGGT
				TGC
<i>ACT1</i> , real time PCR	ACT1-RLT-s	TCAAGATCATCGTCTCTCCC	ACT1-RLT-a	GGACCCTCTCGTCGTACTC

^a The lowercase letters represent sequence with no homology to template DNA, whereas homologous regions are shown in uppercase.

strain was used for transformation with linearized Chk1^{WT}, Chk1^{AEF}, and Chk1^{K49R} vectors. Resulting hygromycin-resistant transformants were verified using primers DCOVER and HYG-a, *NheI*-s and *HindIII*-a, and 3s and GFP (Fig. 1A and B).

RNA isolation and RT-PCR.

RNA was isolated from mycelia ground in liquid nitrogen followed by extraction with TRI-reagent (Sigma), using the manufacturer's protocol. Routinely, 2 µg of RNA was treated with RQ1 DNase (M610A; Promega, Madison, WI, U.S.A.) and was then used for cDNA synthesis with oligo(dT)₁₇V primer in a total reaction volume of 25 µl (protocol supplied with Moloney murine leukemia virus RT, M170A; Promega,). The resulting cDNA was diluted eightfold, and 2 µl of the solution was used as a template for subsequent PCR (performed with 5× PCR reaction mix; Larova, Teltow Germany) or real-time quantitative (q)PCR. qPCR was performed using ABsolute Blue (Abgene, Epsom, U.K.) SYBR green reaction mix and 125 nM of each primer. Thermal cycling was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Gene-expression values were normalized per actin as a reference gene. Calculation of gene A in sample 2, the relative gene expression R_A, was performed as follows:

$$R_A = \frac{E_A^{\text{refC}_{IA} - C_{IA2}}}{E_{\text{ACT}}^{\text{refC}_{IACT} - C_{IACT2}}}$$

where refC_{IA} is the average cycle threshold (C_t) value of all PCR reactions for A and refC_{IACT} is the average C_t value of all reactions for actin, E_A is qPCR efficiency for gene A, and E_{ACT} is qPCR efficiency for actin, calculated according to Liu and Saint (2002). Efficiency was assumed to be the same for all samples. All amplifications were performed in triplicates.

Phospho-MAPK detection.

Total protein was isolated from mycelia by grinding in liquid nitrogen, followed by extraction with TRI-reagent (Sigma) following the manufacturer's protocol. Alternatively, for temperature shift and sorbitol experiments, total protein was isolated from ground mycelium with NP-40 buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1% NP-40; 1 mM EDTA) containing 2.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, and 10 mM sodium fluoride precipitated in acetone and resuspended in Laemmli sample buffer. Samples were separated by SDS-PAGE on precast 10% gels (25201; Pierce, Rockford, IL, U.S.A.), were transferred to nitrocellulose membranes and blocked with 3% bovine serum albumin, and were probed with anti-P42/44 (9102; Cell Signaling, Beverly, MA, U.S.A.) or anti-phospho P42/44 (9101; Cell Signaling) antibodies and detected by enhanced chemiluminescence. Ponceau Red (Sigma) staining was used as a control for loading of the lanes.

Microscopy.

Micrographs were taken using a Leica DMIRE2 or Zeiss LSM 510 META laser scanning microscope. For nuclear staining, spores and germ tubes were stained with DAPI (4',6'-diamidino-2-phenylindole) solution (1 µg/ml) for 5 min.

Identification of *C. heterostrophus* MAS3 and MAS1.

We searched the genome of *C. heterostrophus* for homologs of *M. grisea* MAS3 (*GAS1*) and MAS1 (*GAS2*). The gene showing greatest homology to MAS3 was used in expression studies (accession EU159530). Predicted *C. heterostrophus* Mas3 is a 346-amino acid protein that shares 68.9% identity with hypothetical protein EAT82061.1 of *Phaeosphaeria nodorum* and 33.5% identity with *M. grisea* Mas3 (*Gas1*). *C.*

heterostrophus Mas3 contains a signal peptide with a predicted cleavage site between positions 22 and 23 and an unusual C-terminal domain rich in glycine, alanine, and threonine. Predicted ChMas1 is a 323-amino acid protein sharing 78.6% identity with Mas1 of *Pyrenophora tritici-repentis*. It has a signal peptide with predicted cleavage site between positions 17 and 18.

ACKNOWLEDGMENTS

This study and S. Lev's postdoctoral fellowship were supported by the Israel Science Foundation (grant 773/04); H. Tal was supported by a Technion graduate student fellowship. We thank B. G. Turgeon for providing us with the nourseothricin resistance plasmid.

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