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The *Cryptococcus neoformans* MAP kinase Mpk1 regulates cell integrity in response to antifungal drugs and loss of calcineurin function

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Summary

Cell wall integrity is crucial for fungal growth, development and stress survival. In the model yeast *Saccharomyces cerevisiae*, the cell integrity Mpk1/Slt2 MAP kinase and calcineurin pathways monitor cell wall integrity and promote cell wall remodelling under stress conditions. We have identified the *Cryptococcus neoformans* homologue of the *S. cerevisiae* Mpk1/Slt2 MAP kinase and have characterized its role in the maintenance of cell integrity in response to elevated growth temperature and in the presence of cell wall synthesis inhibitors. *C. neoformans* Mpk1 is required for growth at 37°C *in vitro*, and this growth defect is suppressed by osmotic stabilization. *C. neoformans* mutants lacking Mpk1 are attenuated for virulence in the mouse model of cryptococcosis. Phosphorylation of Mpk1 is induced in response to perturbations of cell wall biosynthesis by the antifungal drugs nikkomycin Z (a chitin synthase inhibitor), caspofungin (a β -1,3-glucan synthase inhibitor), or FK506 (a calcineurin inhibitor), and mutants lacking Mpk1 display enhanced sensitivity to nikkomycin Z and caspofungin. Lastly, we show that calcineurin and Mpk1 play complementing roles in regulating cell integrity in *C. neoformans*. Our studies demonstrate that pharmacological inhibition of the cell integrity pathway would enhance the activity of antifungal drugs that target the cell wall.

Introduction

The fungal cell wall is a dynamic structure that is constantly remodelled during growth, development and during stress (reviewed by Cabib *et al.*, 2001; Klis *et al.*, 2002). A signal transduction pathway that monitors the cell wall and promotes cell wall remodelling has been described in detail in the yeast *Saccharomyces cerevisiae* (reviewed by Heinisch *et al.*, 1999). The core of the signal transduction pathway responsible for maintaining cell integrity in yeast is a mitogen-activated protein (MAP) kinase cascade. Upstream components of the pathway include membrane sensors that detect stresses to the cell wall (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998) and the β -1,3-glucan synthase subunit Rho1 (Kamada *et al.*, 1996). Rho1 is responsible for activating protein kinase C (PKC), which in turn activates the Mpk1/Slt2 MAP kinase cascade (Torres *et al.*, 1991; Lee *et al.*, 1993). Cell integrity signalling results in the increased expression of numerous genes, many of which encode integral cell wall

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proteins or enzymes involved in cell wall biosynthesis (Jung and Levin, 1999). An analogous MAP kinase pathway that monitors cell wall integrity and promotes cell wall remodelling under stress conditions has been described in a variety of fungi (Navarro-Garcia *et al.*, 1995; Zaitsevskaia-Carter and Cooper, 1997; Xu *et al.*, 1998; Fox and Smulian, 1999). Importantly, this MAP kinase pathway has been linked to virulence of both plant and animal fungal pathogens, suggesting that cell wall integrity is important for virulence (Diez-Orejas *et al.*, 1997; Xu *et al.*, 1998).

The enzymes that synthesize fungal cell wall components have emerged as attractive targets for the development of new antifungal drugs for two reasons: the cell wall is essential and the enzymes that synthesize cell wall components are not found in human cells. Emphasis on the development of cell wall-directed antifungals has increased on account of the inherent selectivity and fungicidal nature of these compounds (reviewed by Georgopapadakou, 2001). Three classes of cell wall synthesis inhibitors have entered preclinical studies for possible use against systemic fungal infections, and these agents target chitin synthase (nikkomycins), β -1,3-glucan synthase (echinocandins) and mannoproteins (pradimicins/benanomycins) (Georgopapadakou, 2001). Echinocandins are the only class of cell wall inhibitor still in development, with caspofungin receiving approval for use in treating refractory infections caused by *Aspergillus* species (reviewed by Hoang, 2001). Echinocandins are not effective against all human pathogenic fungi. For example, caspofungin is relatively ineffective against the important human pathogen *Cryptococcus neoformans* (Del Poeta *et al.*, 2000).

Cryptococcus neoformans is a human pathogenic fungus that causes life-threatening infections in immunocompromised hosts, especially patients with AIDS and organ transplant recipients (Casadevall and Perfect, 1998). Virulence factors for *C. neoformans* include the production of a polysaccharide capsule, melanin synthesis and the ability to grow at 37°C (Fromtling *et al.*, 1982; Kwon-Chung *et al.*, 1982; Odom *et al.*, 1997; Waugh *et al.*, 2002). Growth at 37°C is a stressful condition that activates the cell integrity MAP kinase pathway in *S. cerevisiae* (Kamada *et al.*, 1995), suggesting the possibility that a similar cell integrity MAP kinase pathway in *C. neoformans* could be important for virulence. Furthermore, a cell integrity MAP kinase pathway in *C. neoformans* might be important for responses to antifungal drugs that target the cell wall biosynthetic machinery.

Here, we report the identification and characterization of the *C. neoformans* homologue of the cell integrity MAP kinase Mpk1/Slt2. We determined the role of this MAP kinase, named Mpk1, in growth at elevated temperature, virulence and the response to antifungal drugs. We found that *C. neoformans* Mpk1 was required for growth at 37°C *in vitro*, and that this growth defect can be suppressed by osmotic stabilization. *C. neoformans* mutants lacking Mpk1 are attenuated for virulence in the mouse model for cryptococcosis, but are not avirulent. Phosphorylation of Mpk1 is induced in response to perturbations of cell wall biosynthesis by Calcofluor white, nikkomycin Z and caspofungin. Mpk1 is important for surviving exposure to nikkomycin Z and caspofungin. Lastly, we have observed a relationship between the calcium/calmodulin-dependent protein phosphatase calcineurin and Mpk1 in regulating cell integrity in *C. neoformans*. Taken together, our studies highlight the importance of the cell integrity pathway for pathogenesis and as a potential antifungal drug target.

Results

Identification of the *C. neoformans* cell integrity MAP kinase homologue Mpk1

To identify the *C. neoformans* homologue of the cell integrity MAP kinase, BLAST searches of the *C. neoformans* genome database (<http://www.tigr.org>) were performed using the amino acid sequence of *S. cerevisiae* Slt2/Mpk1 and other MAP kinases. Sequence traces that produced high BLAST scores using Slt2/Mpk1 but not with other *S. cerevisiae* MAP kinase

sequences were assembled into a 2.6 kb contig containing the entire *C. neoformans* *MPK1* gene. The *MPK1* open reading frame (ORF) and location of introns was determined by rapid amplification of cDNA ends (RACE), revealing a predicted protein with significant homology to other fungal MAP kinases, including *S. cerevisiae* Slt2/Mpk1 (60% identity), *Schizosaccharomyces pombe* Spm1 (60%), *Candida albicans* Mkc1 (59%) and *Pneumocystis carinii* Mkp1 (57%). These MAP kinases are involved in regulating cell wall integrity in their respective organisms (Martin *et al.*, 1993; Navarro-Garcia *et al.*, 1995; Zaitsevskaya-Carter and Cooper, 1997; Xu *et al.*, 1998; Fox and Smulian, 1999).

MPK1 is required for growth at elevated temperature in C. neoformans

The function of *MPK1* in promoting cell wall integrity at elevated temperature was analysed by gene disruption. An *mpk1Δ::URA5* DNA construct was used to generate the mutant strain by biolistic transformation and homologous recombination, as described using the serotype D strain JEC43 as the recipient (*Experimental procedures*; Davidson *et al.*, 2002). The mutant strain was analysed by Southern blot and polymerase chain reaction (PCR), which showed that the desired double cross-over event occurred at the *MPK1* locus without ectopic integrations (Fig. 1A; Davidson *et al.*, 2002). The resulting *mpk1Δ* strain exhibited a growth defect at 37°C (Fig. 1B and C), which was remediated by providing 1 M sorbitol as an osmotic support (Fig. 1C). Reintroduction of the wild-type *MPK1* gene was accomplished by transforming the *mpk1Δ* mutant (PK1) with a PCR product containing the wild-type *MPK1* gene (see *Experimental procedures*) and selecting for gene replacement events on media containing 5-fluoroorotic acid (5-FOA). Two transformants were transformed with the *URA5* gene to generate uracil prototrophs (PK4 and PK5). Reintroduction of the wild-type *MPK1* gene complemented the *mpk1* mutation and restored the ability to grow at 37°C (Fig. 1B). This phenotype is similar to the phenotype of *S. cerevisiae* *slt2Δ* mutants (Fig. 1C; Kamada *et al.*, 1995), suggesting that *C. neoformans* *MPK1* is important for cell wall integrity at elevated temperature.

The mpk1Δ mutant is attenuated for virulence in the mouse model for cryptococcosis

The ability to grow at elevated temperature is essential for the virulence of *C. neoformans* (Odom *et al.*, 1997; Fox *et al.*, 2001; Waugh *et al.*, 2002). Because the *mpk1Δ* mutant was unable to grow at 37°C, we hypothesized that Mpk1 would be required for virulence in the mouse model of cryptococcosis. C5 complement-deficient DBA mice were infected with wild-type and *mpk1Δ* strains by lateral tail vein injection and monitored daily for survival. The survival of mice infected with the wild-type strain (JEC21) was significantly reduced compared with mice infected with the *mpk1Δ* mutant strain ($P < 0.001$, Fig. 2A). All 10 mice infected with wild type died by day 100, whereas all 10 mice infected with the *mpk1Δ* mutant survived to day 100, and six mice were still alive on day 125. One caveat to this experiment is that molecular events other than the targeted gene disruption can occur during strain construction. To ensure that the *mpk1Δ* mutation confers a virulence defect, two strains were isolated in which the *mpk1* mutant allele was replaced with the *MPK1* wild-type gene at its endogenous locus, and uracil prototrophy was restored by ectopic integration of the *URA5* gene (see *Experimental procedures*). Mice were infected with the wild-type strain JEC21, the *mpk1Δ* mutant and the two mutant strains reconstituted with the wild-type *MPK1* gene. This analysis confirmed that the *mpk1Δ* mutant is attenuated compared with wild type, and introduction of the wild-type *MPK1* gene restored virulence in both reconstituted strains ($P < 0.001$ for both compared with the *mpk1Δ* mutant, Fig. 2B). We note that the relative virulence of the reconstituted strains was greater than that of strain JEC21, which served as a prototrophic wild-type control, and we attribute this to other molecular changes that occurred during either passage of strain JEC21 or the construction of the reconstituted isolates. In summary, disruption of the *MPK1* gene attenuates virulence, and this defect is complemented by reintroduction of the wild-type *MPK1* gene.

The *mpk1Δ* strain was not avirulent, as has been observed with other *C. neoformans* mutants that are unable to grow at 37°C *in vitro*. After 150 days, the brains of the six surviving mice that received the *mpk1Δ* strain were cultured for *C. neoformans*, and four of these mice were culture positive. Three of the four isolated strains were unable to grow at 37°C in the absence of osmotic support (Fig. 2C). The fourth strain (BC-4) may have a mutation that suppresses the growth defect at 37°C *in vitro*. PCR analysis showed that all four strains still contain the *mpk1Δ* allele (not shown). Consistent with our observations, the cell integrity MAP kinase Mkc1 is important for full virulence of *C. albicans* in the mouse model for candidiasis, but strains lacking *MKC1* are not avirulent (Diez-Orejas *et al.*, 1997). Taken together, these results suggest that the cell integrity MAP kinase pathway is important for full virulence of *C. neoformans*, but an intact cell integrity pathway is not an absolute requirement for producing infection.

Perturbation of the cell wall induces Mpk1 phosphorylation

MAP kinase activation requires dual phosphorylation of conserved threonine and tyrosine residues within the activation loop (reviewed by Cobb and Goldsmith, 1995). To monitor Mpk1 activation, we used a commercially available antiphospho-p44/p42 antibody that can be used to recognize the dually phosphorylated form of Mpk1. Previous studies have established the specificity of this antibody in detecting the dually phosphorylated form of *S. cerevisiae* Slt2/Mpk1 (Martin *et al.*, 2000; de Nobel *et al.*, 2000; Harrison *et al.*, 2001). Calcofluor white (CFW) is a fluorescent dye that hinders normal cell wall assembly and has been used to induce activation of the cell wall integrity MAP kinase pathway in *S. cerevisiae* (Pringle *et al.*, 1989; de Nobel *et al.*, 2000). We used CFW to induce activation of *C. neoformans* Mpk1. As shown in Fig. 3A, CFW induces the phosphorylation of Mpk1. The band corresponding to phospho-Mpk1 was absent in *mpk1Δ* mutant cells. An antibody recognizing the PSTAIRE epitope present in cyclin-dependent kinases served as a loading control.

The echinocandin caspofungin is a specific inhibitor of β -1,3-glucan synthase, an essential enzyme involved in the synthesis of the fungal cell wall (Douglas *et al.*, 1994; 1997). A single essential gene, *FKS1*, encodes β -1,3-glucan synthase in *C. neoformans* (Thompson *et al.*, 1999). Echinocandins possess *in vitro* activity against *Candida* species and other clinically important fungi, but are relatively ineffective against *C. neoformans* (Del Poeta *et al.*, 1997; 2000; Douglas *et al.*, 1997; Nelson *et al.*, 1997). *C. neoformans* is sensitive to caspofungin, but only at high concentrations that are not clinically achievable. We detected Mpk1 phosphorylation after treatment with 6 $\mu\text{g ml}^{-1}$ caspofungin for 20 min (Fig. 3B), indicating that inhibition of β -1,3-glucan synthase activates the cell integrity MAP kinase pathway in *C. neoformans*. Nikkomycin Z is a potent inhibitor of chitin synthases, which are also essential for the synthesis of the fungal cell wall (Gaughran *et al.*, 1994; Cabib *et al.*, 2001). Nikkomycin Z is active against a variety of clinically important fungi *in vitro* (Hector, 1993; Kim *et al.*, 2002). As shown in Fig. 3B, treatment of *C. neoformans* with 25 $\mu\text{g ml}^{-1}$ nikkomycin Z resulted in the phosphorylation of Mpk1 after 180 min, indicating that the inhibition of chitin synthesis activates the cell integrity MAP kinase pathway.

Enhanced sensitivity of the *mpk1Δ* mutant to nikkomycin Z and caspofungin

The cell integrity MAP kinase pathway is required for survival during exposure to a variety of agents, including cell wall-degrading enzymes, Calcofluor white, caffeine, vanadate and others (Heinisch *et al.*, 1999; Martin *et al.*, 2000; de Nobel *et al.*, 2000). To test whether *C. neoformans* Mpk1 is required for survival during treatment with caspofungin or nikkomycin Z, we analysed wild-type and mutant cells for growth in the presence of these drugs. Figure 4A shows that the sensitivity to nikkomycin Z is dramatically increased in the absence of Mpk1, which is consistent with our observation that nikkomycin Z exposure results in the phosphorylation of Mpk1. Cells lacking Mpk1 showed an increase in caspofungin sensitivity

(Fig. 4B). Caspofungin exhibits synergistic antifungal activity with the calcineurin inhibitor FK506 against *C. neoformans* *in vitro* (Del Poeta *et al.*, 2000); therefore, we assessed the importance of Mpk1 in the response to caspofungin treatment in the absence of calcineurin function. Figure 4B shows that a *cnb1Δ mpk1Δ* double mutant strain that lacks both the calcineurin B regulatory subunit and the Mpk1 kinase is more sensitive to 4 $\mu\text{g ml}^{-1}$ caspofungin than the *cnb1Δ* mutant alone, indicating that Mpk1 provides protection against cell wall perturbation in the absence of calcineurin.

Loss of calcineurin function activates FKS1 expression in an Mpk1-dependent manner

The *FKS1* gene encodes a component of the β -1,3-glucan synthase complex in *C. neoformans*, which is the essential cell wall-synthesizing enzyme targeted by caspofungin (Thompson *et al.*, 1999). In *S. cerevisiae*, the *FKS2* gene, which encodes an alternative subunit of β -1,3-glucan synthase, is induced when the cell integrity MAP kinase pathway is activated (Jung and Levin, 1999). To test whether the gene encoding β -1,3-glucan synthase is a target of the cell integrity MAP kinase pathway in *C. neoformans*, expression of *C. neoformans* *FKS1* was examined in wild-type, *cnb1Δ*, *mpk1Δ* and *cnb1Δ mpk1Δ* mutant backgrounds by Northern analysis. As shown in Fig. 5, *FKS1* expression is markedly induced in the *cnb1Δ* mutant, whereas in the *cnb1Δ mpk1Δ* double mutant, there is no such induction. These results implicate the transcriptional regulation of *FKS1* as a downstream target of the Mpk1 cell integrity pathway in *C. neoformans*.

Inhibition of calcineurin by FK506 causes Mpk1 phosphorylation and FKS1 induction

The role of calcineurin in promoting cell wall integrity at elevated temperature has been established in *S. cerevisiae* (Zhao *et al.*, 1998). Two results reported here suggest a relationship between calcineurin function and the cell integrity MAP kinase pathway that exists in *C. neoformans*. First, disrupting the cell integrity MAP kinase pathway exacerbates the caspofungin sensitivity of the *cnb1Δ* mutant (Fig. 4B). Secondly, loss of calcineurin function results in the induction of the *FKS1* gene in an Mpk1-dependent manner (Fig. 5). To test whether loss of calcineurin function results in a change in the phosphorylation state of Mpk1, we analysed protein extracts from a wild-type strain, a *cnb1Δ* mutant and a wild-type strain treated with the calcineurin inhibitor FK506 by immunoblot with antiphospho-p44/p42 antibodies. Phosphorylation of Mpk1 increased slightly by 60 min and was significantly increased at 180 min after treatment with 5 $\mu\text{g ml}^{-1}$ FK506 (Fig. 6A). A concomitant increase in *FKS1* transcription was observed with similar kinetics to the Mpk1 phosphorylation, with a slight increase in transcript levels at 60 min after FK506 exposure and a greater increase at the 180 minute time point (Fig. 6A). Mpk1 was phosphorylated in the *cnb1Δ* mutant, which also showed the highest level of *FKS1* transcript. The target of FK506 is the immunophilin FKBP12, encoded by the *FRR1* gene in *C. neoformans* (Cruz *et al.*, 1999). FKBP12 and FK506 form a protein–drug complex that inhibits calcineurin (Liu *et al.*, 1991). The *C. neoformans* *frr1-3* allele contains a mutation that alters the hydrophobic drug-binding pocket of FKBP12, and thus abolishes the ability of FKBP12 to inhibit calcineurin in concert with FK506 (Cruz *et al.*, 1999). As shown in Fig. 6B, no induction of phospho-Mpk1 was observed in the *frr1-3* mutant strain, indicating that the effect of FK506 on Mpk1 activation is specific for calcineurin inhibition.

Discussion

Fungal growth, development and the ability to survive environmental stress are dependent on the integrity of the cell wall. The goal of this study was to determine the role of the MAP kinase Mpk1 in the response to perturbations of cell wall integrity in the pathogenic fungus *C. neoformans*. Here, we report the identification of Mpk1, the *C. neoformans* homologue of the cell wall integrity MAP kinase. We show that Mpk1 is important for growth at elevated

temperature and for virulence in the mouse model for cryptococcosis. Furthermore, *C. neoformans* Mpk1 is activated by phosphorylation in response to treatment with Calcofluor white, the chitin synthase inhibitor nikkomycin Z or the β -1,3-glucan synthase inhibitor caspofungin. Moreover, Mpk1 provides an additional measure of protection against caspofungin in the absence of calcineurin function. Lastly, we show that inhibition of calcineurin function by FK506 results in the activation of Mpk1 and induction of *FKS1*, a downstream target of the cell integrity MAP kinase pathway. Taken together, our results show that *C. neoformans* possesses a conserved mechanism for regulating cell integrity in response to perturbations of the cell wall.

The yeast *S. cerevisiae* is an excellent model system to study the genetic and biochemical mechanisms of MAP kinase pathways (reviewed by Gustin *et al.*, 1998). In recent years, significant progress has been made in the understanding of the MAP kinase pathway involved in the maintenance of cell integrity in *S. cerevisiae* (Heinisch *et al.*, 1999). A detailed view of the components of this pathway is now available, including the upstream regulatory components, the downstream targets of the pathway and the other cellular processes that are influenced by this pathway (Heinisch *et al.*, 1999). One important aspect of our study was to examine the link between the cell integrity pathway and virulence mechanisms and antifungal drug responses in the human pathogenic fungus *C. neoformans*. In addition to the MAP kinase characterized here, the identification of only one other component of the pathway in *C. neoformans* (Rho1) has been reported (Tanaka *et al.*, 1999; Chang and Penoyer, 2000). The results reported here identify the Mpk1 MAP kinase pathway as an important mechanism for both virulence and the antifungal drug response, and they establish a functional link between the Mpk1 signalling pathway and calcineurin in *C. neoformans* (Fig. 7). A remarkable aspect of the studies reported here is that the functions of Mpk1 are conserved between *S. cerevisiae* and *C. neoformans*. This conservation in function is striking given that *S. cerevisiae* is an ascomycete and *C. neoformans* is a basidiomycete, and the two have been diverging over $\approx$ 500 million years of evolution. Moreover, in many cases, the physiological functions of highly conserved signalling molecules are quite distinct in these divergent yeasts. For example, the protein phosphatase calcineurin is required for growth at 37°C, mating and hyphal elongation in *C. neoformans*, whereas calcineurin is not required for high temperature growth, mating or pseudohyphal filamentous growth in *S. cerevisiae* (reviewed by Fox and Heitman, 2002). Thus, our finding that the Mpk1 cell wall integrity pathway serves similar roles in these divergent yeasts now allows us to use previous studies in the model budding yeast as a guide to further studies in this fungal pathogen.

The ability to grow at physiological temperatures is a prerequisite for any pathogenic fungus. Mutations in the genes encoding the *C. neoformans* calcineurin catalytic and regulatory subunits result in the inability to grow at physiological temperature, and strains harbouring these mutations are avirulent in the mouse model for cryptococcosis (Odom *et al.*, 1997; Fox *et al.*, 2001). *C. neoformans* *MPK1* is also required for growth at elevated temperature (37°C), but the ability to grow at this temperature is restored when osmotic support is supplied to the medium. This suggests that the growth defect results from the inability to alter the cell wall in a manner required for growth at 37°C, as has been shown in *S. cerevisiae* (Lussier *et al.*, 1997). That the *mpk1* Δ mutant is not avirulent suggests that osmotic support sufficient to sustain growth at elevated temperature may be available *in vivo* (Fig. 2A). All four strains cultured from mouse brains infected with the *mpk1* Δ mutant still contained the *mpk1* Δ allele, and three of these isolates were unable to grow at 37°C in the absence of osmotic support *in vitro* (Fig. 2B), providing additional evidence that the 37°C growth defect is partially ameliorated under certain *in vivo* conditions.

The development of antifungal drugs that target the cell wall has been emphasized in recent years for several reasons, including the fungal-specific nature of their targets

(Georgopapadakou, 2001). Notably, the β -1,3-glucan synthase inhibitor caspofungin has recently been approved for use in treating certain fungal infections (Hoang, 2001). Caspofungin is effective against *C. neoformans* only at clinically unachievable concentrations, highlighting the importance of understanding how this pathogenic fungus responds to drugs that target the cell wall synthesis machinery. *C. neoformans* Mpk1 is activated in response to a variety of perturbations to the cell wall, including Calcofluor white treatment and inhibition of chitin synthesis by nikkomycin Z (Fig. 3A and B). Although caspofungin is relatively ineffective against *C. neoformans*, we show that the activating dual phosphorylation of Mpk1 does occur in the presence of caspofungin. Mpk1 is phosphorylated in response to caspofungin after only 20 min, whereas Mpk1 phosphorylation is not observed after nikkomycin Z treatment until 180 min. This may reflect differences in how the drugs enter the cell or the timing at which the respective enzymes inhibited by these drugs are needed during the cell cycle. Our results are consistent with the observation that β -1,3-glucan synthesis is inhibited in the presence of caspofungin (Feldmesser *et al.*, 2000). Several explanations have been proposed to explain the ineffectiveness of caspofungin against *C. neoformans* (Thompson *et al.*, 1999; Del Poeta *et al.*, 2000; Feldmesser *et al.*, 2000). β -1,3-glucan is an essential component of the *C. neoformans* cell wall, but a reduction in β -1,3-glucan may be tolerable if other cell wall components are available to compensate for this reduction. In support of this notion, *C. neoformans* strains with no calcineurin function are extremely sensitive to caspofungin (Fig. 4B, Del Poeta *et al.*, 2000).

Calcineurin is a Ca^{2+} calmodulin-dependent phosphatase that has been implicated in cell wall metabolism in model yeasts (Zhao *et al.*, 1998; Sugiura *et al.*, 2002). Specifically, calcineurin regulates the expression of an alternative β -1,3-glucan synthase subunit (Fks2) in response to growth at 39°C in *S. cerevisiae* (Zhao *et al.*, 1998). Furthermore, calcineurin is essential in *S. cerevisiae* strains lacking components of the cell integrity pathway (Garrett-Engele *et al.*, 1995). Electron microscopy studies revealed that *C. neoformans* strains lacking calcineurin have an altered cell wall (D. Fox and J. Heitman, unpublished results). The findings reported here support the notion that calcineurin plays an important role in cell wall metabolism in *C. neoformans*. Inhibition of calcineurin by FK506 results in activation of Mpk1 and the induction of a downstream component of the cell integrity pathway, *FKS1*. A mutant with an altered FKBP12 that cannot bind FK506 to inhibit calcineurin does not accumulate activated Mpk1 in the presence of FK506, indicating that the effect of FK506 on the phosphorylation state of Mpk1 is specific for calcineurin inhibition by the FKBP12–FK506 complex.

Although the function of Mpk1 in promoting growth at 37°C in *S. cerevisiae* and *C. neoformans* is conserved, several important differences exist with respect to the relationship between calcineurin and the Mpk1-mediated cell integrity pathway. First, although both fungi possess an Mpk1-dependent component in the regulation of β -1,3-glucan synthase genes, loss of calcineurin function results in the induction of *FKS1* expression in *C. neoformans*, whereas full *FKS2* induction in *S. cerevisiae* requires calcineurin function (Zhao *et al.*, 1998). Secondly, calcineurin is essential in *S. cerevisiae* strains lacking a functional cell integrity pathway (Garrett-Engele *et al.*, 1995), but *C. neoformans* *cnb1Δ mpk1Δ* mutants are viable (Figs 3–5) and *mpk1Δ* mutants are not hypersensitive to calcineurin inhibitors (data not shown). These differences may reflect differences in the requirements for regulating cell wall integrity in pathogenic fungi compared with a non-pathogenic model yeast. The specific nature of the cell wall defect in the absence of calcineurin in *C. neoformans* remains to be determined. Systematic identification of protein complexes in *S. cerevisiae* showed that the calcineurin regulatory subunit interacts with Kre6, which is a component of β -1,6-glucan synthase (Roemer *et al.*, 1993; Ho *et al.*, 2002). If such an interaction exists in *C. neoformans*, disrupting this interaction may account for the cell wall defects leading to activation of Mpk1 and the altered response to caspofungin in the absence of calcineurin function.

Experimental procedures

Strains and growth media

Cryptococcus neoformans strains used were as follows: JEC21 (*MAT α*), JEC43 (*MAT α ura5*), DSF11 (*MAT α cnb1 Δ ::NAT*), PK1 (*MAT α mpk1 Δ ::URA5 ura5*), PK2 (*MAT α cnb1 Δ ::NAT mpk1 Δ ::URA5*), PK4 (*MAT α MPK1 ura5 URA5*), PK5 (*MAT α MPK1 ura5 URA5*) and C21F3 (*MAT α frt1-3*). *S. cerevisiae* strains used were W303 (*trp1-1 leu2-3112 ura3-1 ade2-1 his3-11 can1-100*) and DLY4914 (*W303 mpk1 Δ ::HIS3*). All strains were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or SD media lacking uracil (Sherman, 1991). Where indicated, sorbitol was added to media at a concentration of 1 M. Caspofungin (MK-0991) was generously provided by Merck and Co., nikkomycin Z and Calcofluor white were obtained from Sigma, and FK506 was generously provided by Fujisawa.

Identification, disruption and reconstitution of MPK1

The *MPK1* gene was identified as a sequence trace from the *C. neoformans* genome project, The Institute for Genomic Research (<http://www.tigr.org>), funded by the NIAID/NIH under grant number AI48594-01. PCR primers JOHE7288 (5'-ACTAGGCGTGCCATTGTTTAC-3') and JOHE7289 (5'-GCGGACTGGGCAGGAGAAGC-3') were used to amplify a 2.4 kb product containing the entire *MPK1* gene. The *MPK1* ORF and the location of introns were confirmed by rapid amplification of cDNA ends (RACE) using the SMARTTM RACE cDNA amplification kit (Clontech). The *MPK1* cDNA sequence was deposited in GenBank under accession number AY212947. The *MPK1* gene from strain JEC43 was disrupted with the *URA5* cassette using a modified PCR overlap procedure described by Davidson *et al.* (2002). Transformations were performed as described previously (Toffaletti and Perfect, 1994) using a Bio-Rad model PDS-1000/He biolistic particle delivery system. Transformants were selected on defined media lacking uracil (Sherman, 1991) with 1 M sorbitol for osmotic support, and the presence of the disruption allele was confirmed by Southern analysis of genomic DNA (Davidson *et al.*, 2002) and by PCR (see below). Strains PK4 and PK5 were constructed by transforming the *mpk1 Δ* mutant (PK1) with a PCR product containing the wild-type *MPK1* gene. Transformants were grown on media containing 1 $\mu\text{g ml}^{-1}$ 5-fluoroorotic acid (5-FOA) to select for a gene replacement event. Two 5-FOA-resistant isolates were then transformed with a plasmid containing the *URA5* gene to generate uracil prototrophs (PK4 and PK5).

Virulence assay

In vivo testing of *C. neoformans* strains was performed in 4-to 6-week-old female C5 complement-deficient DBA mice (NCI/Charles River Laboratories) using lateral tail vein injections. Strains to be analysed were precultured in YPD at 25°C for 24 h, washed with phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of 10^7 cells ml^{-1} . Ten mice were infected with 10^6 cells of each strain in a volume of 100 μl via lateral tail vein injection as described previously (Cruz *et al.*, 2000; Fox *et al.*, 2001).

Growth assay

The sensitivity of *C. neoformans* strains to caspofungin and nikkomycin Z was assayed by growth inhibition as described previously (de Nobel *et al.*, 2000). Cells were precultured in YPD at 25°C for 16–20 h before dilution to an OD₆₀₀ of 0.025. Growth was continued in the presence of caspofungin or nikkomycin Z in flat-bottomed 96-well microtitre plates at 25°C without shaking. Growth was determined after 48 h as the OD₆₀₀ of each culture after resuspension.

Northern analysis

Strains for Northern analysis were precultured at 25°C for 16–20 h, diluted to an OD₆₀₀ of 0.2 and incubated for 2–4 h before drug addition. Total RNA was prepared using acid phenol as described previously (Ausubel *et al.*, 1992). Total RNA (20 µg) was separated by electrophoresis in 1.2% formaldehyde agarose gels and transferred to Nytran SuPerCharge nylon membranes (Schleicher and Schuell). Fragments of the *FKS1*, *GPD1* and *ACT1* genes were amplified by PCR, labelled with [α -³²P]-dCTP using the Rediprime™ random prime labelling system (Amersham Pharmacia Biotech) and used as probes for Northern hybridization.

Protein extraction and Western blots

All strains for protein extraction were precultured at 25°C for 16–20 h, diluted to an OD₆₀₀ of 0.2 and incubated for 2–4 h before drug addition. Cells were centrifuged and frozen in dry ice before protein extraction. Protein extracts were prepared from cell pellets by glass bead homogenization in 0.5 ml of lysis buffer [50 mM Tris, pH 7.5, 2 mM MgCl₂, 1× protease inhibitor cocktail set IV (Calbiochem), 1× phosphatase inhibitor sets I and II (Calbiochem), and 10% glycerol] with five 1 min bursts with cooling on ice. Extracts were centrifuged at 14 000 g for 10 min at 4°C and the supernatants were collected. Protein concentrations were determined with the Bio-Rad protein assay.

For Western blots, 50 µg of total extract was separated by 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were probed with antiphospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody (New England Biolabs) at a 1:4000 dilution to detect active Mpk1. As a control for loading, anti-PSTAIRE antibody (Santa Cruz Biotechnology) was used at a 1:2000 dilution. Primary antibodies were detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and ECL detection reagents (Amersham Pharmacia Biotech).

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Fig. 1. The *mpk1Δ* mutant displays an osmotically remediable growth defect at 37°C

A. Confirmation of *mpk1Δ* gene disruption by PCR. Genomic DNA was isolated from wild-type and *mpk1Δ* strains and used as a template for PCR using primer sets 1, 2 and 3. Primer sets 1 and 2 anneal to sequences deleted in the *mpk1Δ* allele. M, marker.

B. The *mpk1Δ* mutant displays a growth defect at 37°C. Strains were inoculated on YPD media and incubated for 48 h at the indicated temperatures. Strains used were wild type (JEC21), the *mpk1Δ* mutant (PK1) and an *mpk1Δ* mutant reconstituted with the wild-type *MPK1* gene (PK4).

C. The temperature-sensitive growth defects of the *C. neoformans mpk1Δ* mutant and the *S. cerevisiae slt2Δ* mutant are osmotically remediable. Fivefold serial dilutions of cell suspensions of the *S. cerevisiae slt2Δ* mutant (DLY4914, row 1), *S. cerevisiae* wild type (W303, row 2), *C. neoformans* wild type (JEC21, row 3) and the *C. neoformans mpk1Δ* mutant (PK1, row 4) were inoculated on YPD or YPD + 1 M sorbitol and incubated at the indicated temperatures for 48 h.

Fig. 2. *mpk1Δ* mutants are attenuated for virulence in the mouse model of cryptococcosis

A. C5 complement-deficient DBA mice (10 each) were injected via the lateral tail vein with 10^6 cells of a wild-type strain (JEC21, open squares) and the *mpk1Δ* mutant (PK1, closed diamonds). Mice were monitored daily for survival over a period of 125 days.

B. C5 complement-deficient DBA mice (10 each) were injected via the lateral tail vein with 10^6 cells of wild-type strain JEC21 (open squares), two *mpk1Δ* mutant strains reconstituted with the wild-type *MPK1* gene (PK4, open triangles; and PK5, open circles) and the *mpk1Δ* mutant (PK1, closed diamonds). Mice were monitored daily over a period of 120 days.

C. *mpk1Δ* mutant strains cultured from infected animals still display the *in vitro* growth defect at 37°C. *C. neoformans* was cultured from the brains of four out of the six mice that survived to 150 days after receiving injections of *mpk1Δ* cells in (A). The strains recovered from these four mice (BC1–4) were inoculated on YPD with wild-type (JEC21) and *mpk1Δ* mutant (PK1) cells and incubated at the indicated temperature for 48 h.

Fig. 3. Perturbation of the cell wall induces Mpk1 phosphorylation

A. *C. neoformans* Mpk1 is phosphorylated after Calcofluor white treatment. Exponentially growing strains were untreated or treated with 10 $\mu\text{g ml}^{-1}$ Calcofluor white (CFW) for 2 h. Protein was extracted and subjected to immunoblot analysis using antiphospho-p44/p42 antibodies. Anti-PSTAIRE antibodies were used as a loading control. Strains used were wild type (JEC21) and *mpk1Δ* (PK1).

B. Nikkomycin Z and caspofungin induce Mpk1 phosphorylation. An exponentially growing wild-type strain was treated with 6 $\mu\text{g ml}^{-1}$ caspofungin or 25 $\mu\text{g ml}^{-1}$ nikkomycin Z, and the phosphorylation state of Mpk1 was determined by immunoblot analysis.

Fig. 4. Enhanced sensitivity of the *mpk1* Δ mutant to nikkomycin Z and caspofungin. Cells were precultured in YPD at 25°C for 16–20 h before dilution to an OD₆₀₀ of 0.025. Growth was continued in the presence of nikkomycin Z or caspofungin in flat-bottomed 96-well microtitre plates at 25°C without shaking. Growth was determined after 48 h as the OD₆₀₀ of each culture after resuspension. Experiments were repeated four times, and data are represented as the mean and standard error of the mean

A. Strains used were wild type (JEC21) and the *mpk1* Δ mutant (PK1).

B. Strains used were wild type (JEC21) and the *mpk1* Δ (PK1), *cnb1* Δ (DSF11) and *cnb1* Δ *mpk1* Δ (PK2) mutants.

Fig. 5.

Loss of calcineurin function activates *FKS1* expression in an Mpk1-dependent manner. Total RNA was extracted from cultures in the logarithmic phase of growth, and the level of *FKS1* gene expression was analysed by Northern blot using a fragment of the *FKS1* gene as a probe. An *ACT1* gene fragment was used as a loading control. Strains used were wild type (JEC21) and the *mpk1*Δ (PK1), *cnb1*Δ (DSF11) and *cnb1*Δ *mpk1*Δ (PK2) mutants.

Fig. 6. Inhibition of calcineurin by FK506 causes Mpk1 phosphorylation and *FKS1* induction
 A. Cultures of wild-type strain JEC21 in the logarithmic phase of growth treated with 5 $\mu\text{g ml}^{-1}$ FK506 for 0, 20, 60 and 180 min and an untreated *cnb1Δ* mutant strain were assessed for both Mpk1 phosphorylation and *FKS1* expression. Proteins were extracted and subjected to immunoblot analysis using antiphospho-p44/p42 antibodies. Anti-PSTAIRE antibodies served as a loading control. *FKS1* gene expression was analysed by Northern blot using a fragment of the *FKS1* gene as probe and an *ACT1* gene fragment as the loading control.
 B. Cultures of wild-type strain JEC21 and an *frr1-3* mutant strain (C21F3) were treated with 5 $\mu\text{g ml}^{-1}$ FK506 for 180 min, and protein extracts were separated by SDS-PAGE and assessed for Mpk1 phosphorylation as in (A).

Fig. 7.

Schematic diagram of the *C. neoformans* cell integrity pathway. Chitin synthase (Chs1) and β -1,3-glucan synthase (Fks1) contribute to the maintenance of normal cell wall integrity, a condition under which the cell integrity MAP kinase pathway is not activated. Nikkomycin Z and caspofungin inhibit *C. neoformans* chitin synthase and β -1,3-glucan synthase, respectively, leading to activation of the cell integrity MAP kinase Mpk1. The steps leading to Mpk1 activation may include the activity of cell wall sensors, the β -1,3-glucan synthase subunit Rho1 and protein kinase C. The Mpk1 pathway is important for survival of *C. neoformans* cells treated with nikkomycin Z or caspofungin. In the absence of calcineurin, Mpk1 partially protects *C. neoformans* cells from caspofungin treatment. Inhibition of calcineurin by FK506 leads to activation of Mpk1, possibly because of a cell wall defect caused by the lack of calcineurin function.