Specialization of the HOG Pathway and Its Impact on Differentiation and Virulence of *Cryptococcus neoformans*[□]

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The human pathogenic fungus *Cryptococcus neoformans* has diverged from a common ancestor into three biologically distinct varieties or sibling species over the past 10–40 million years. During evolution of these divergent forms, serotype A *C. neoformans* var. *grubii* has emerged as the most virulent and cosmopolitan pathogenic clade. Therefore, understanding how serotype A *C. neoformans* is distinguished from less successful pathogenic serotypes will provide insights into the evolution of fungal virulence. Here we report that the structurally conserved Pbs2-Hog1 MAP kinase cascade has been specifically recruited as a global regulator to control morphological differentiation and virulence factors in the highly virulent serotype A H99 clinical isolate, but not in the laboratory-generated and less virulent serotype D strain JEC21. The mechanisms of Hog1 regulation are strikingly different between the two strains, and the phosphorylation kinetics and localization pattern of Hog1 are opposite in H99 compared with JEC21 and other yeasts. The unique Hog1 regulatory pattern observed in the H99 clinical isolate is widespread in serotype A strains and is also present in some clinical serotype D isolates. Serotype A *hog1Δ* and *pbs2Δ* mutants are attenuated in virulence, further underscoring the role of the Pbs2-Hog1 MAPK cascade in the pathogenesis of cryptococcosis.

INTRODUCTION

Microbial pathogenesis studies aim to understand how microorganisms infect humans. Often pathogenic microbes cluster with closely related species that are not infectious, providing a unique opportunity to define unique specializations that enable virulence. For example, *Escherichia coli* O157 causes devastating hemolytic renal infection compared with *E. coli* K12, and toxin production by *Vibrio cholera* requires transduction by a filamentous phage. In the pathogenic fungi, a unique species cluster of the genera *Cryptococcus* exhibits novel virulence attributes and presents a similar example, enabling definition of virulence mechanisms in the fungal kingdom.

Cryptococcus neoformans is a basidiomycetous fungal pathogen that causes human infection after inhalation of infectious particles from certain environments, such as soil contaminated with pigeon excreta (Casadevall and Perfect, 1998). *C. neoformans* is an opportunistic pathogen that infects the CNS, causing life-threatening meningoencephalitis (Casadevall and Perfect, 1998; Hull and Heitman, 2002). There are four serotypes of *C. neoformans*: A (var. *grubii*), B and C (var. *gattii*), and D (var. *neoformans*). Based on population genetics studies, the divergence between var. *gattii* and the other varieties occurred ~37 million years ago, and the A and D serotypes diverged ~18 million years ago (Xu *et al.*, 2000).

During evolution, serotype A strains have emerged as the most successful pathogenic clade. More than 95% of clinical isolates worldwide and 99% of isolates from AIDS patients have been identified to be serotype A (Casadevall and Perfect, 1998). Serotype B and C strains are primary pathogens and have further emerged as important clinical isolates in the recent, ongoing Vancouver Island outbreak of *Cryptococcus* infection involving immunocompetent individuals (Speed and Dunt, 1995; Fraser *et al.*, 2003; Hoang *et al.*, 2004). In contrast, serotype D strains are the least virulent variety, representing <10% of clinical isolates, although these are more common in some areas of Europe (Kwon-Chung and Bennett, 1984). Serotype A strains are in general more virulent in animal models of systemic cryptococcosis and meningoencephalitis than serotype D strains, but diversity in genetic background may also contribute. Thus, studying differences between the two serotypes provides an important means to understand the evolution of *C. neoformans* virulence mechanisms.

Thus far, two major signaling pathways, the pheromone-responsive Cpk1 mitogen-activated protein kinase (MAPK) pathway and the cyclical AMP (cAMP) pathway, have been shown to modulate both differentiation (during mating or monokaryotic fruiting), and the production of two critical virulence factors: capsule and melanin (for review see Lengeler *et al.*, 2000). Although the two pathways are largely structurally and functionally conserved in both serotypes, serotype-specific differences have also emerged. For instance, the PKA catalytic subunit Pka1 plays a major regulatory role in the cAMP pathway in serotype A, whereas Pka2 does so in serotype D (Hicks *et al.*, 2004). The mating-type-specific p21-activated protein kinase Ste20α contributes to virulence in serotype A, but not in serotype D (Wang *et al.*, 2002). In contrast, the Ste12 transcription factor contributes to virulence in serotype D, but not in serotype A (Yue *et al.*, 1999; Chang *et al.*, 2000).

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Another example of serotype specificity is that serotype A strains are more resistant to osmotic shock than serotype D strains (Cruz *et al.*, 2000), implying that the high osmolarity glycerol (HOG) pathway may differ between the two serotypes. The HOG pathway has been well characterized in the model yeast *Saccharomyces cerevisiae* (for reviews see Hohmann, 2002; O'Rourke *et al.*, 2002) and is controlled by at least two upstream branches that activate the MAPK Hog1. The main upstream branch is a three-component phosphorelay system composed of the transmembrane osmosensor Sln1, the intermediate protein Ypd1, and the response regulator Ssk1 (Posas *et al.*, 1996; Posas and Saito, 1998). Under normal or hypo-osmolar conditions, Ssk1 is constitutively phosphorylated by the activated Sln1-Ypd1 system and is prevented from interacting with the downstream MAPK kinase kinases (MAPKKKs) Ssk2 and Ssk22. In response to hyper-osmotic stress, the phosphorelay system is inactivated, resulting in dephosphorylation of Ssk1 and activation of Ssk2 and Ssk22 (Maeda *et al.*, 1995). Another upstream branch includes Sho1, which spans the membrane four times and cooperates with Cdc42, Ste20 (a p21-activated kinase), and Ste50, culminating in the activation of the MAPKKK Ste11 upon hyper-osmotic stress (Maeda *et al.*, 1995; Posas and Saito, 1997; O'Rourke and Herskowitz, 1998; Posas *et al.*, 1998). The two branches converge to activate the MAPK kinase (MAPKK) Pbs2 through phosphorylation by any of three MAPKKKs (Posas and Saito, 1997). Activated Pbs2 phosphorylates evolutionarily highly conserved threonine and tyrosine residues on the MAPK Hog1, which then translocates to the nucleus and activates downstream target genes to counteract the eliciting stress (Reiser *et al.*, 1999).

To understand the role of the HOG pathway in the serotype A and D lineages, we used strains H99 and JEC21, which are the most commonly used serotype A and D strains, respectively, and which share ~95% genome sequence identity (Loftus *et al.*, 2005). Here we report that the HOG pathway is uniquely adapted to control differentiation and virulence of the serotype A strain H99, but not of the serotype D strain JEC21. Although the HOG pathway plays both conserved and distinct roles in responding to a wide range of external environmental stresses, including osmotic shock, high temperature, UV irradiation, and oxidative stress between the two strains, it is exclusively used by the serotype A strain H99 as a central signaling pathway to modulate morphological differentiation during mating and production of two crucial virulence factors (melanin and capsule). This functional differentiation appears to result from opposing phosphorylation and localization patterns of the Hog1 protein between the two strains. Furthermore, we discovered that the unique Hog1 regulatory pattern observed in the H99 strain is observed in all serotype A strains tested and also in some serotype D clinical isolates. These findings provide new insight into how the basidiomycetous fungus *C. neoformans* has adapted an evolutionarily conserved signaling pathway to control differentiation and virulence and also reveal a novel paradigm for Hog1 MAPK regulation.

MATERIALS AND METHODS

Strains and Media

The strains used in this study are listed in Table 1. Yeast extract-peptone-dextrose (YPD) and synthetic (SD) media, V8 medium for mating, Niger seed medium for melanin production, agar-based Dulbecco's modified Eagle's (DME) medium for capsule production were all as described (Granger *et al.*, 1985; Alspaugh *et al.*, 1997; Bahn *et al.*, 2004; Hicks *et al.*, 2004). Among clinical serotype A strains, 78.7.98 and 46F.5.02 strains are from Tanzania, BT63 and BT130 are from Botswana, S25C and S25J are from Asia, and 2970 is from

Uganda. MMRL751, MMRL757, and MMRL760 are clinical serotype D strains isolated from HIV patients in Italy. CDC92-27, CDC92-16, and 11, are also clinical serotype D strains. The serotype of each strain was further confirmed by serotype-specific PCR for the *STE20*, *PAK1*, *GPA1*, and *CNA1* genes (unpublished data). NIH12 and NIH433 are clinical and environmental serotype D *MAT α* and *MAT α* strains, respectively, and are the parental strains for B3501 (*MAT α*) and B3502 (*MAT α* , equivalent to JEC20; Heitman *et al.*, 1999).

Identification of 5' and 3' Regions of the HOG1 Gene

Strain H99 was incubated overnight at 30°C in YPD medium, pelleted, lyophilized, and used to isolate total RNA with Trizol (Life Technologies-BRL, Rockville, MD) according to the manufacturer's instruction. The 5' and 3' rapid amplification of cDNA ends (RACE) were performed by GeneRacer kit (Invitrogen, Carlsbad, CA). Each RACE product was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced. The sequence for the *HOG1* gene from strain H99 has been assigned GenBank accession number AY775548.

Complementation of *S. cerevisiae* *hog1 Δ* Mutants with the *C. neoformans* *HOG1* Gene

For constitutive expression of the *C. neoformans* *HOG1* gene in *S. cerevisiae*, the full-length *HOG1* cDNA was amplified by RT-PCR using first-strand cDNA generated from H99 total RNA (SuperScript III, Invitrogen) and primers 12712/12713 (see Supplementary Table 1 for the primer sequences), and cloned into plasmid pTH19 under the control of the *ADH1* promoter (provided by Toshiaki Harashima), creating plasmid pADH-HOG1c. The *ura3* *S. cerevisiae* *hog1/hog1* mutants (from the diploid homozygous deletion mutant collection) and its parental strain BY4743 (diploid of strains BY4741/BY4742; Giaever *et al.*, 2002) were then transformed with plasmids pTH19 and pADH-HOG1c.

Disruption of the HOG1 and PBS2 Genes

The *HOG1* gene was disrupted by biolistic transformation in the congenic *C. neoformans* serotype A strains H99 and KN99a and serotype D strains JEC21 and JEC20 with constructs generated by PCR overlap as previously described (Davidson *et al.*, 2002). The 5' and 3' regions of the *HOG1* gene were PCR-amplified with the following primers: 11793/11794 and 11795/11796 for the 5' and 3' regions, respectively, of the *HOG1* gene in serotype A, and 12415/12416 and 12417/12418 for the 5' and 3' regions, respectively, of the *HOG1* gene in serotype D. M13 forward (M13F) and M13 reverse (M13R) primers were used to generate the *Not*I (Nourseothricin acetyl-transferase) or *Neo*^r (Neomycin phosphotransferase II) dominant selectable markers with template pNATSTM#177 with a unique signature tag (kindly provided by Dr. Jennifer K. Lodge, Saint Louis University School of Medicine) and pJAF1, respectively. The *HOG1* disruption cassettes were generated by PCR overlap using primers 11793/11796 for serotype A and 12415/12418 for serotype D. The gel-extracted *HOG1* disruption cassette was precipitated onto 600 μ g of gold microcarrier beads (0.8- μ m, bioWORLD, Dublin, OH) and biolistically transformed into the prototrophic serotype A and D wild-type strains as described previously (Davidson *et al.*, 2000). Stable transformants were selected on YPD medium containing nourseothricin (100 mg/L) or G418 (200 mg/L). *hog1 Δ* strains were screened by diagnostic PCR for the 5' and 3' junctions and Southern blot analysis using a *HOG1*-specific probe generated by PCR with primers 11799/11800 for serotype A and 12421/12422 for serotype D (unpublished data). Uridine auxotrophic serotype A *hog1 Δ* mutants, YSB114 and YSB115 (Table 1) and the serotype D *hog1 Δ* mutant, YSB241, were generated by inducing spontaneous mutations at the *URA5* gene in strains YSB64, YSB81, and YSB139, respectively, on SD medium containing 5-fluoroorotic acid (5-FOA).

To construct serotype A *hog1 Δ* +*HOG1* reconstituted strains, H99 genomic DNA containing the full-length *HOG1* gene was isolated from a *C. neoformans* H99 bacterial artificial chromosome (BAC) library. The 4.4-kb *Apal*-*Xba*I fragment containing the full-length *HOG1* gene was cloned into pJAF7 (*URA5*), pJAF12 (*Neo*^r), or pJAF13 (*Nat*^r), generating pURAHOG1A, pNEOHOG1A, or pNATHOG1A, respectively. *Mfe*I-digested linearized pURAHOG1A, pNEOHOG1A, or pNATHOG1A DNA was biolistically transformed into strains YSB64, YSB81, or YSB114/YSB115 (Table 1), respectively. Southern blot analysis with the *HOG1*-specific probe described above confirmed targeted integration of each linearized plasmid into the *Mfe*I-site in the 5' UTR (1220-base pairs upstream from ATG) of the native *HOG1* locus through a single cross-over event (unpublished data). To construct serotype D *hog1 Δ* +*HOG1* reconstituted strains, the 3.4-kb fragment containing the full-length *HOG1* gene was PCR-amplified using primers 12866/12867, cloned into plasmid pCR2.1-TOPO (Invitrogen) generating pCR-HOG1D, and sequenced. The *HOG1* insert of pCR-HOG1D was subcloned into plasmids pJAF12 and pJAF13, generating pNEOHOG1D and pNATHOG1D, respectively. *Nde*I-digested linearized pNEOHOG1D and pNATHOG1D DNA was biolistically transformed into strains YSB139 and YSB143 (Table 1), respectively. The targeted reintegration of the *HOG1* gene was confirmed by Southern blot analysis (unpublished data).

The *PBS2* gene was disrupted in the serotype A H99 and KN99a background. The 5' and 3' regions of the *PBS2* gene were PCR-amplified with

Table 1. Strains used in this study

Strain	Serotype	Genotype	Reference
H99	A	<i>MATα</i>	Perfect <i>et al.</i> (1993)
KN99a	A	<i>MATa</i>	Nielsen <i>et al.</i> (2003)
F99	A	<i>MATα ura5</i> (5-FOA ^r)	Wang <i>et al.</i> (2002)
JKH7	A	<i>MATα pka1Δ::URA5 ura5</i>	J. K. Hicks
YSB119	A	<i>MATα aca1Δ::NAT-STM#43 ura5 ACA1-URA5</i>	Bahn <i>et al.</i> (2004)
YSB121	A	<i>MATa aca1Δ::NEO ura5 ACA1-URA5</i>	Bahn <i>et al.</i> (2004)
YSB42	A	<i>MATα cac1Δ::NAT-STM#159</i>	Bahn <i>et al.</i> (2004)
YSB83	A	<i>MATα gpa1Δ::NAT-STM#5</i>	Bahn <i>et al.</i> (2004)
YSB49	A	<i>MATα gpb1Δ::NAT-STM#146</i>	Bahn <i>et al.</i> (2004)
YSB51	A	<i>MATα ras1Δ::NAT-STM#150</i>	Bahn <i>et al.</i> (2004)
YSB64	A	<i>MATα hog1Δ::NAT-STM#177</i>	This study
YSB81	A	<i>MATa hog1Δ::NEO</i>	This study
YSB114	A	<i>MATα hog1Δ::NAT-STM#177 ura5</i> (5-FOA ^r)	This study
YSB115	A	<i>MATa hog1Δ::NEO ura5</i> (5-FOA ^r)	This study
YSB145	A	<i>MATα hog1Δ::NAT-STM#177 HOG1-NEO</i>	This study
YSB250	A	<i>MATα hog1Δ::NAT-STM#177 HOG1^{T171A}-NEO</i>	This study
YSB252	A	<i>MATα hog1Δ::NAT-STM#177 HOG1^{Y173A}-NEO</i>	This study
YSB253	A	<i>MATα hog1Δ::NAT-STM#177 HOG1^{T171A+Y173A}-NEO</i>	This study
YSB308	A	<i>MATα hog1Δ::NAT-STM#177 HOG1^{K49S+K50N}-NEO</i>	This study
YSB148	A	<i>MATa hog1Δ::NEO HOG1-NAT</i>	This study
YSB242	A	<i>MATα hog1Δ::NAT-STM#177 ura5 URA5-ACT1pro-HOG1-FLAG-GFP</i>	This study
YSB123	A	<i>MATα pbs2Δ::NAT-#213</i>	This study
YSB125	A	<i>MATa pbs2Δ::NEO</i>	This study
YSB212	A	<i>MATα pbs2Δ::NAT-#213 PBS2::NEO</i>	This study
YSB112	A	<i>MATα ura5 hog1Δ::NAT-STM#177 pka1Δ::URA5</i>	This study
YSB155	A	<i>MATα hog1Δ::NAT-STM#177 cac1Δ::NEO</i>	This study
YSB153	A	<i>MATa hog1Δ::NAT-STM#177 cac1Δ::NEO</i>	This study
YSB152	A	<i>MATα hog1Δ::NAT-STM#177 gpa1Δ::NEO</i>	This study
YSB151	A	<i>MATa hog1Δ::NAT-STM#177 gpa1Δ::NEO</i>	This study
H99 crg1	A	<i>MATα ura5 crg1Δ::URA5</i>	Wang <i>et al.</i> (2004)
PPW196	A	<i>MATa ura5 crg1Δ::URA5</i>	Wang <i>et al.</i> (2004)
CAP59	A	<i>MATa cap59Δ::HYG</i>	Nelson <i>et al.</i> (2003)
JEC21	D	<i>MATα</i>	Moore and Edman (1993)
JEC20	D	<i>MATa</i>	Moore and Edman (1993)
CDC85	D	<i>MATα pka2Δ::URA5 ura5</i>	Hicks <i>et al.</i> (2004)
CDC101	D	<i>MATa pka2Δ::URA5 ura5</i>	Hicks <i>et al.</i> (2004)
YSB139	D	<i>MATα hog1Δ::NAT-STM#177</i>	This study
YSB143	D	<i>MATa hog1Δ::NEO</i>	This study
YSB241	D	<i>MATα hog1Δ::NAT-STM#177 ura5</i> (5-FOA ^r)	This study
YSB203	D	<i>MATα hog1Δ::NAT-STM#177 HOG1-NEO</i>	This study
YSB206	D	<i>MATa hog1Δ::NEO HOG1-NAT</i>	This study
YSB231	D	<i>MATα hog1Δ::NAT pka2Δ::URA5 ura5</i>	This study
YSB243	D	<i>MATα hog1Δ::NAT-STM#177 ura5 URA5-ACT1pro-HOG1-FLAG-GFP</i>	This study
YSB311	D	<i>MATα hog1Δ::NAT-STM#177 HOG1^{K49S+K50N}-NEO</i>	This study

Each NAT-STM# indicates the Nat^r marker with a unique signature tag.

primers 12087/12088 and 12089/12090, respectively. Nat^r (from pNAT-STM#213) and Neo^r selectable markers were PCR-amplified as described above. The *PBS2* disruption alleles were generated by PCR overlap using primers 12087/12090. The *pbs2 Δ* mutant strains were screened by diagnostic PCR for the 5' and 3' junctions and Southern blot analysis using *Pst*I-digested genomic DNA and a *PBS2* gene-specific probe generated by PCR with primers 12093/12094 (unpublished data).

To construct the *pbs2 Δ* +*PBS2* reconstituted strains, the 4.3-kb fragment containing the full-length *PBS2* gene was PCR-amplified using primers 12858/12859, cloned into plasmid pCR2.1-TOPO to generate plasmid pCR-PBS2, and sequenced. The *PBS2* gene insert of pCR-PBS2 was subcloned into plasmid pJAF12, generating pNEOPBS2. The *Nde*I-digested linearized pNEOPBS2 DNA was biolistically transformed into strain YSB123 (Table 1), targeting to the *Nde*I-site in the 5' UTR (858-base pairs upstream from ATG) of the native *PBS2* locus. The targeted reintegration of the *PBS2* gene through a single cross-over event was confirmed by Southern blot analysis (unpublished data).

Assay for Capsule and Melanin Production

For capsule induction, each strain (a single colony from solid YPD medium) was incubated for 16 h at 30°C in YPD medium, spotted (~3 × 10⁵ cells) onto agar-based DME medium, and further incubated for 24 h at 37°C. After

incubation, capsule was visualized by staining with India ink and observed microscopically. Quantitative measurement of capsule size was performed as previously described (Zaragoza *et al.*, 2003) by microscopically measuring diameters of the capsule and the cell using AxioVision 3.1 software (Zeiss, Thornwood, NY). For melanin production, cells were spotted onto Niger seed medium containing 0.1 or 2% glucose and incubated up to 4 d at 37°C. Melanin production was monitored daily and photographed.

Mating, Cell Fusion, and Confrontation Assays

Mating, cell fusion, and confrontation assays were performed as previously described (Bahn *et al.*, 2004; Hicks *et al.*, 2004). Images of mating and confrontation assays were captured with a Nikon Eclipse E400 microscope equipped with a Nikon DXM1200F digital camera (Melville, NY). Transcript levels of the *Mfa1* pheromone gene during mating were monitored by Northern blot analysis using an *Mfa1*-specific probe and an *ACT1* probe as the loading control as described (Hull *et al.*, 2002).

Sensitivity Test for Stress Responses

Each strain was incubated overnight at 30°C in YPD medium and subcultured in fresh YPD medium to OD_{600 nm} 0.7–0.9. Then cells were washed, serially diluted (1–10⁴ dilutions) in dH₂O, and spotted (2 μ l) onto solid YPD medium

containing 1 or 1.5 M of NaCl or KCl for osmotic shock, or 2 or 3 mM H₂O₂ for oxidative stress. To test sensitivity to UV, cells spotted on solid YPD was exposed to UV for 0.2 (480 J/m²) or 0.3 (720 J/m²) min using a UV Stratalinker (Model 2400, Stratagene, La Jolla, CA). To test temperature sensitivity, plates were incubated at 30, 37, and 40°C. Each plate was incubated for 2 d and photographed.

Western Blot Analysis of Hog1 Phosphorylation

Yeast cells grown to midlogarithmic phase as described above were added to an equal volume of YPD medium containing 1, 2, or 3 M NaCl (final 0.5, 1, or 1.5 M NaCl) and further incubated for indicated amount of time. A portion of culture at each time point was rapidly frozen in a dry ice/ethanol bath, resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 1% [wt/vol] sodium deoxycholate, 5 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 50 mM NaF, 0.1% [wt/vol] SDS, and 1% [vol/vol] Triton X-100) containing a cocktail of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μ g of pepstatin ml⁻¹, 1 mM benzamidin, and 0.001% aprotinin) with 1–1.2 g of acid-washed glass beads (425–600 μ m, Sigma, St. Louis, MO) and disrupted using a FastPrep instrument (FP120; Bio101, Savant Instruments, Farmingdale, NY). Protein concentrations were determined by Bio-Rad Protein Assay reagent (Richmond, CA) and an equal amount of protein (25 μ g) was loaded into a 10% Tris-glycine gel (Novex, Encinitas, CA). Separated proteins were further transferred to Immuno-blot/PVDF membrane (Bio-Rad) and incubated overnight at 4°C with a primary rabbit p38-MAPK specific antibody (Cell Signaling, Beverly, MA) and with a secondary anti-rabbit IgG horseradish peroxidase-conjugated antibody. The blot was developed using the ECL Western Blotting Detection System (Amersham Bioscience, Piscataway, NJ). Subsequently, the blot was stripped and further used for detection of Hog1 with a rabbit polyclonal anti-Hog1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control.

Site-specific Mutagenesis of HOG1

The phosphorylation site HOG1^{T171A}, HOG1^{Y173A}, and HOG1^{T171A+Y173A} mutants were generated by PCR overlap using pNEOHOG1A as a template and the following primers: 13139/13142 and 13140/13141 for HOG1^{T171A}, 13139/13144 and 13140/13143 for HOG1^{Y173A}, and 13139/13146 and 13140/13145 for HOG1^{T171A+Y173A}. Subsequently, each mutated HOG1 allele was produced by overlap PCR using primers 13139 and 13140, cloned into pCR2.1-TOPO, and confirmed by sequencing. Each 3-kb mutated HOG1 allele was further cloned into pJAF7, generating pNEOHOG1^{T171A}, pNEOHOG1^{Y173A}, and pNEOHOG1^{T171A+Y173A}. The catalytically inactive (kinase-dead) HOG1^{KD} mutants (K49S and K50N) were generated by PCR amplification using pNEOHOG1A as the template and primers 13139/14203 and 13140/14202, followed by PCR overlap with primer pair 13139/13140. The overlap PCR product was cloned into plasmid pCR2.1-TOPO, sequenced, and subcloned into plasmid pJAF7 to generate pNEOHOG1^{KD}. To construct each site-directed HOG1 mutant, the *Bsp*IEI-digested linearized plasmids were transformed into the *hog1Δ* strain YSB64, targeting to the *Bsp*IEI-site in the 5' UTR (507-base pairs upstream from ATG) of the native HOG1 locus. To determine the phenotype of the serotype D HOG1^{KD} mutant, the circular pNEOHOG1^{KD} was biolistically transformed and ectopically integrated into the *hog1ΔD* mutant (YSB139). Site-specific or ectopic integration of the plasmids into the *hog1Δ::NAT* allele (Neo^r Nat^r) was confirmed by Southern blot and expression of mutated Hog1 proteins was confirmed by Western blot analysis (unpublished data).

Hog1 Localization Study

To study the subcellular localization of Hog1 in both serotypes, plasmid pACT-HOG1fGFP, where expression of the HOG1-GFP fusion construct was driven by the *ACT1* promoter, was transformed into *ura5 hog1Δ* or *hog1ΔD* mutants (YSB114 and YSB241). First, plasmid pGPD-HOG1fGFP was constructed by the following steps: three genomic DNA fragments were PCR-amplified with primers 12557/12558 for base pairs 1–912 of HOG1, 12559/12568 for base pairs 898–1620 of HOG1 with a FLAG-tag and unique *Not*I site in the C-terminus, and 12569/12580 for 296 base pairs of the HOG1 3' UTR, using pNATHOG1 as the template. With those PCR products combined as template, the 1.9-kb HOG1 gene was subsequently amplified by overlap PCR using primers 12557/12580, cloned into pCRD83 (*GPD1* promoter), and sequenced. The GFP fragments with *Not*I sites on both 5' and 3' ends were PCR-amplified from pGFP (provided by Connie Nichols) using primers 12561/12562, cloned into pCR2.1-TOPO generating pCR-GFP*Not*I, and sequenced. The *Not*I-digested GFP fragment from pCR-GFP*Not*I was further cloned into the pGPD-HOG1fGFP, generating pGPD-HOG1fGFP. Considering the possibility that the *GPD1* promoter is the Hog1 target, plasmid pACT-HOG1fGFP was further constructed by replacing the *GPD1* promoter with the *ACT1* promoter amplified from pJAF1 using primers 13200/13201. Plasmid pACT-HOG1fGFP was biolistically transformed into *ura5 hog1Δ* mutants, YSB114 and YSB241.

Yeast cells harboring pACT-HOG1fGFP were grown as described in Western blot analysis for Hog1 phosphorylation. Cell cultures were fixed with formaldehyde (9.3%) for 10 min. Fixed cells were washed twice with 1× phosphate-buffered saline (PBS), permeabilized with an equal volume of 1×

PBS containing 1% Triton X-100 for 5 min, washed twice again with 1× PBS, and resuspended in 1× PBS. For DAPI staining, an equal volume of cell suspension and DAPI mix (2 μ g/ml DAPI, 1 mg/ml antifade, 40% glycerol) was mixed and microscopically observed with a Zeiss Axioskop 2 equipped with an AxioCam MRM digital camera. GFP, DAPI, or GFP/DAPI merged images were processed by AxioVision 3.1 software (Zeiss).

Virulence Assays

Yeast strains (wild-type [H99], *hog1Δ* [YSB64], *hog1Δ+HOG1* reconstituted [YSB145], *pbs2Δ* [YSB123], and *pbs2Δ+PBS2* reconstituted [YSB212]) were grown in YPD medium at 30°C for 16 h and then subcultured in fresh YPD medium to midlogarithmic phase (OD_{600 nm} = 0.9–1.0). Cells were collected by centrifugation and washed twice with sterile PBS, and the final concentration was adjusted to 2 × 10⁶ CFU/ml with sterile PBS. Female A/Jcr mice (NCI/Charles River Laboratories, Wilmington, MA, 20–24 g) in each test group (10 mice per group, except 9 mice for YSB123) were inoculated with 1 × 10⁵ CFU, in a volume of 50 μ l, via nasal inhalation as previously described (Cox *et al.*, 2000). Mice that appeared moribund, i.e., lethargic, exhibiting rapid weight loss (>15% loss), or in pain, were sacrificed using CO₂ inhalation. Survival data from the murine experiments were statistically analyzed between paired groups using the log-rank test using the PRISM program 4.0 (GraphPad Software, San Diego, CA). The animal protocol used for these experiments was approved by The Duke University Animal Use Committee.

RESULTS

C. neoformans Hog1/p38-MAPK Homolog

A *C. neoformans* homolog encoding a member of the Hog1/p38-like MAPK protein family was identified by BLAST searches of the serotype A (H99) and D (JEC21) genomes. A single gene located on chromosome 3 in both serotype A and D was discovered to encode a protein highly homologous to *S. cerevisiae* Hog1. The genomic structure of the HOG1 gene was analyzed by 5' and 3' RACE and cDNA analysis. In serotype A and D, Hog1 is encoded by 1620- and 1608-base pair ORFs, respectively, both of which are interrupted by 5 introns. The serotype A and D Hog1 proteins share 100% amino acid identity encoded by a 1095-base pair coding region (365 amino acid [aa]). *C. neoformans* Hog1 shares 73, 65, and 48% identity with *Candida albicans* Hog1, *S. cerevisiae* Hog1, and mammalian p38 MAPK, respectively. Protein domain analysis by Pfam HMM search (Washington University, St. Louis, MO, <http://pfam.wustl.edu/hmmsearch.shtml>) revealed that *C. neoformans* Hog1 contains a typical protein kinase signature sequence (20–299 aa) that is highly conserved in other Hog1 or p38 MAPK homologues. In particular, dual phosphorylation residues essential for Hog1 activation are conserved in *C. neoformans* Hog1 (T171 and Y173).

Considering these conserved structural features, we hypothesized that *C. neoformans* Hog1 may be a functional homolog of *S. cerevisiae* Hog1. To test this hypothesis, the *C. neoformans* HOG1 gene was expressed from the *ADH1* promoter in the *S. cerevisiae* *hog1Δ* mutant. Heterologous expression of the *C. neoformans* HOG1 gene completely rescued hyper-osmosensitivity of the *S. cerevisiae* *hog1Δ* mutant in the presence of 1 M NaCl or KCl (Figure 1). Therefore, *C. neoformans* Hog1 is a bona fide homolog of the Hog1/p38-MAPK protein family.

C. neoformans Serotype A and D Hog1 Share Conserved and Distinct Roles in Response to a Variety of Environmental Cues

To elucidate the roles of *C. neoformans* Hog1, isogenic sets of serotype A and D *hog1Δ* mutants (*hog1ΔA* and *hog1ΔD*, respectively) and reconstituted strains (*hog1Δ+HOG1*) were generated. The most common *hog1Δ* mutant phenotype observed in other yeast species is hypersensitivity to a range of environmental stresses, including temperature, osmotic pressure, UV irradiation, or oxidative damage (Degols *et al.*, 1996; Alonso-Monge *et al.*, 2003). Therefore, the sensitivity of *hog1Δ* mutants to these stresses was tested. In general, the *C.*

Figure 1. Expression of the *C. neoformans* HOG1 gene complements the osmosensitive phenotypes of a *S. cerevisiae* *hog1Δ* mutant. The wild-type *S. cerevisiae* diploid strain BY4743 and the homozygous *hog1Δ/hog1Δ* mutant bearing the control plasmid pTH19 (WT or *Schog1Δ*+vector only) or plasmid pADH-HOG1 expressing *C. neoformans* HOG1 from the ADH1 promoter (WT or *Schog1Δ*+*CnHOG1*) were grown overnight at 30°C in SD medium uracil, serially diluted (1–10⁴ dilutions), spotted onto solid SD medium containing 1 M NaCl or KCl, incubated at 30°C for 2 d, and photographed.

neoformans serotype A wild-type (WT) strain H99 was more resistant to a variety of stresses than the serotype D WT strain JEC21 (Figure 2). Strikingly, serotype A and D Hog1 were found to share conserved functions with respect to certain stresses but also to play distinct roles in response to others. The *hog1ΔA* mutant displayed hypersensitivity to high temperature (40°C), but not to 30°C or 37°C (Figure 2), whereas the *hog1ΔD* mutant did not show any temperature-sensitive growth defect. In contrast, both *hog1ΔA* and *hog1ΔD* mutants exhibited hypersensitivity to UV irradiation (a range of 480–720 J/m²) and 1 or 1.5 M KCl (Figure 2) or NaCl (unpublished data) compared with WT and *hog1Δ*+HOG1 strains (Figure 2). In contrast to the dramatic osmosensitivity of *S. cerevisiae* *hog1Δ* mutants, the phenotype of the *C. neoformans* mutants was less pronounced (Figures 1 and 2).

The major difference in stress response between *hog1ΔA* and *hog1ΔD* mutant strains was observed in sensitivity to oxidative stress exerted by hydrogen peroxide (H₂O₂). The *hog1ΔA* mutant showed hypersensitivity to H₂O₂ treatment, similar to *C. albicans* and *Schizosaccharomyces pombe* *hog1Δ* mutants, whereas the *hog1ΔD* mutant was resistant (Figure 2). Noticeably, in most stress response patterns, the *hog1ΔA* mutant exhibited phenotypes similar to the WT serotype D strains, implying that differential regulation of Hog1 may determine patterns of stress-responses between the two divergent strains. Taken together, the HOG pathway in *C. neoformans* responds to multiple stress conditions in a conserved and distinct manner between representative strains of the two serotypes.

Cross-talk between the HOG1 and cAMP Pathways Regulates Capsule and Melanin Production in Serotype A

We further characterized the role of Hog1 in the regulation of two important virulence attributes of *C. neoformans*, capsule and melanin production, both of which are controlled by cAMP signaling (Alspaugh *et al.*, 1997; D'Souza *et al.*, 2001; Alspaugh *et al.*, 2002; Bahn *et al.*, 2004; Hicks *et al.*, 2004). Thus far, no MAPK is known to be involved in these processes. Here we show that Hog1 negatively regulates synthesis of capsule and melanin in the serotype A H99 strain background, but not in the serotype D JEC21 background. The *hog1ΔA* mutant produced more capsule than the WT strain (H99) and *hog1Δ*+HOG1 reconstituted strains, whereas the *hog1ΔD* mutant generated levels of capsule similar to the WT strain (JEC21; Figure 3A).

To determine if Hog1 signals coordinately with the cAMP pathway to regulate capsule production in serotype A, genes encoding the Gα subunit (*GPA1*), adenylyl cyclase (*CAC1*), or the PKA catalytic subunit 1 (*PKA1*) were deleted in the *hog1ΔA* mutant background and capsule production was monitored. The *gpa1Δ*, *cac1Δ*, and *pka1Δ* mutations completely abolished enhanced capsule production of the *hog1ΔA* mutant and the *gpa1Δ hog1Δ*, *cac1Δ hog1Δ*, and *pka1Δ hog1Δ* double mutants were as defective in capsule production as the *gpa1Δ*, *cac1Δ*, and *pka1Δ* single mutants, respectively (Figure 3B). Hog1 may negatively regulate components upstream of Gpa1 or Pka1 itself, or signal in a parallel pathway.

The ability of Hog1 to interact with the cAMP-signaling pathway was also evident in melanin synthesis. Deletion of the *HOG1* gene significantly increased melanin production in the serotype A strain H99, but not in the serotype D strain

Figure 2. Hog1 plays shared and distinct roles in diverse stress responses in divergent *C. neoformans* serotypes. Each *C. neoformans* strain (serotype A WT [H99], *hog1Δ* [YSB64], and *hog1Δ*+HOG1 reconstituted [YSB145] strains; serotype D wild-type [JEC21], *hog1Δ* [YSB139] and *hog1Δ*+HOG1 reconstituted [YSB203] strains) was grown to midlogarithmic phase in YPD medium, 10-fold serially diluted (1–10⁴ dilutions), and 2 μl of each diluted cell suspension was spotted on YPD medium containing 1 or 1.5 M KCl for hyperosmotic shock, or 2 or 3 mM H₂O₂ for oxidative stress. To test temperature and UV sensitivity, cells on solid medium were incubated at 30, 37, and 40°C, and exposed to UV for 0.2 (480 J/m²) and 0.3 min (720 J/m²), respectively. Cells were further incubated for 2 d and photographed.

Figure 3. Hog1 represses melanin and capsule production by counteracting the cAMP-PKA pathway in serotype A, but not in serotype D. (A and B) Capsule production by the serotype A (WT [H99], *hog1* [H99], *hog1*Δ [YSB64], *hog1*Δ+*HOG1* [YSB145], *hog1*Δ *gpa1*Δ [YSB152], *hog1*Δ *cac1*Δ [YSB155], and *hog1*Δ *pka1*Δ [YSB112]) strains and by the serotype D (WT [JEC21], *hog1*Δ [YSB139], and *hog1*Δ+*HOG1* [YSB203]) strains grown at 37°C on solid DME medium for 24 h was visualized by India ink staining and relative capsule size (%) was determined. Asterisk indicates the serotype A *hog1*Δ strain, which has a significantly larger capsule size ($p < 0.05$) than the WT. Bar, 10 μ m. (C) The same isogenic strain series in A and B and the serotype D *hog1*Δ *pka2*Δ (YSB231) strain were grown at 30°C (serotype D) or 37°C (serotype A) on Niger seed medium to induce melanin production for 2 d (0.1% glucose) or 4 d (2% glucose) and photographed.

JEC21, which was most apparent on Niger seed medium containing 2% glucose at 37°C (Figure 3C). Suppression of melanin biosynthesis by Hog1 was further demonstrated by epistasis analysis in which the *hog1* mutation completely restored (or enhanced) melanin production in the serotype A *gpa1*Δ, *cac1*Δ, and *pka1*Δ mutants (Figure 3C). These data indicate that Hog1 negatively modulates a downstream target of Pka1 or a parallel factor controlling melanin synthesis. Epistasis analysis further supports that Hog1 does not play a role in melanin synthesis in serotype D because *pka2*Δ *hog1*Δ double mutants were similar to *pka2*Δ single mutants in melanin production (Figure 3C). Taken together, Hog1

plays an essential role in regulating virulence factors in the serotype A, but not in the serotype D strain, providing further evidence that Hog1 is differentially regulated in the two divergent serotypes.

hog1 Mutation Enhances Mating in Serotype A, But Not Serotype D

We also examined the role of Hog1 in conjunction with another signaling cascade, the MAPK-mediated mating signaling pathway. First the mating ability of *hog1*Δ mutants was assessed. Surprisingly, the *hog1*ΔA mutant, but not *hog1*ΔD, was enhanced in the formation of mating filaments

Figure 4. Hog1 represses pheromone MAPK cascade activated mating in serotype A, but not in serotype D. (A) The following MAT α and MATa strains were cocultured on V8 medium (pH 5.0 for serotype A and pH 7.0 for serotype D) for 1 wk at room temperature in the dark: for serotype A, H99 and KN99a ($\alpha \times a$), YSB64 and YSB81 (*hog1* $\times$ *hog1*), and YSB145 and YSB148 (*hog1*+*HOG1* $\times$ *hog1*+*HOG1*), and for serotype D, JEC21 and JEC20 ($\alpha \times a$), YSB139 and YSB143 (*hog1* $\times$ *hog1*), YSB203 and YSB206 (*hog1*+*HOG1* $\times$ *hog1*+*HOG1*). Representative edges of the mating patches were photographed at $\times 100$ magnification after 2 or 7 d incubation. (B) The serotype A MATa WT (KN99a, the first and third columns) and *hog1* Δ (YSB81, the second and fourth columns) strains were cocultured for 2 wk on V8 medium with the MAT α *gpa1* Δ (YSB83), *cac1* Δ (YSB42), *pka1* Δ (JKH7), *ras1* Δ (YSB51), and *gpb1* Δ (YSB49) strains and photographed.

and cell fusion efficiency compared with the WT and reconstituted strains (Figure 4A). Quantitative measurement of cell fusion efficiency using Nat^r and Neo^r marked control strains (YSB119 and YSB121; Bahn *et al.*, 2004) and *hog1* Δ A mutants (YSB64 and YSB81; Table 1) showed that the *hog1* Δ A mutant is twofold more efficient than WT in cell fusion (unpublished data).

Several lines of evidence indicate that the enhanced mating of the *hog1* Δ A mutant results from hyperactivation of pheromone production by derepression of the Gpb1-activated MAPK pathway. First, unilateral mating defects observed in *ras1* Δ , *gpa1* Δ , *cac1* Δ , or *pka1* Δ mutant strains, but not in the *gpb1* Δ mutant, were almost completely abolished when the mating partner bore the *hog1* mutation (Figure 4B). Second, *hog1* mutations confer a dramatic impact on pheromone production only in serotype A. In confrontation assays using the pheromone-hypersensitive *crg1* Δ mutants, which lack an RGS protein that normally desensitizes the pheromone-response pathway (Nielsen *et al.*, 2003; Wang *et al.*, 2004), *hog1* Δ A mutants were as effective in inducing pheromone-mediated conjugation tubes, but not in responding to pheromone, as *crg1* Δ mutants (compare confrontation of *crg1* Δ MAT α vs. *crg1* Δ MATa or *hog1* Δ A MATa strains in Figure 5A). Hyper-expression of the MF α 1 pheromone gene in *hog1* Δ A mutants was further confirmed by Northern blot analysis (Figure 5B). The *hog1* Δ A mutant produced fivefold more pheromone transcripts than WT (H99) under mating

conditions. Even without an opposite mating partner, the *hog1* Δ A mutant generated $\sim$ threefold more pheromone transcripts than did a WT cross (H99 $\times$ KN99; Figure 5B). In contrast, the *hog1* Δ D mutant produced equivalent levels of MF α 1 pheromone transcripts compared with the serotype D WT strain (JEC21) under mating conditions, further confirming that the *hog1* mutation enhances mating only in the serotype A background (Figure 5B). Taken together, the serotype A Hog1-MAPK cascade negatively regulates mating by repressing the pheromone production pathway.

Opposing Phosphorylation and Localization Patterns of Hog1 in the Serotype A Strain H99 Compared with the Serotype D Strain JEC21 or *S. cerevisiae*

We have shown that Hog1 functions are either shared or distinct between serotype A and D. Because the protein sequence of Hog1 is identical between the two, we examined whether Hog1 is differentially regulated via its phosphorylation pattern. The phosphorylation mechanism of Hog1 is highly conserved in diverse fungi. In general, Hog1 is unphosphorylated under normal or hypo-osmolar conditions, but is rapidly phosphorylated in response to osmotic stress. Phosphorylated Hog1 then translocates to the nucleus where it activates expression of target genes (for review, see Hohmann, 2002).

The phosphorylation pattern of Hog1 in the serotype D strain JEC21 was found to be equivalent to that of *S. cerevisiae*

Figure 5. Mutation of the serotype A *HOG1* gene increases mating pheromone production. (A) The MAT α WT (H99) and *crg1* Δ (H99 *crg1*) strains were confronted with the MAT α WT (KN99a), *crg1* Δ (PPW196), and *hog1* Δ (YSB81) strains, incubated for 7 d at room temperature in the dark, and photographed at $\times 40$ magnification. (B) Northern blot analysis was performed with total RNA isolated from solo- or cocultures of the indicated strain(s) grown for 24 h under mating conditions: for serotype A, WT α (H99), WT α (KN99a), *hog1* α (YSB64), and *hog1* α (YSB81); for serotype D, WT α (JEC21), WT α (JEC20), *hog1* α (YSB139), and *hog1* α (YSB143). The blot was probed with the MF α 1 gene and subsequently probed with an ACT1 probe as a loading control. The bar graph demonstrates the quantitative measurement of MF α 1 induction by phosphorimager analysis. The fold induction in the Y-axis indicates relative MF α 1 expression levels of each culture(s) normalized to ACT1 expression levels and compared with WT α .

siae; Hog1 was rapidly phosphorylated after exposure to 1 M NaCl (Figure 6A). However, in the serotype A strain H99 Hog1 phosphorylation was regulated in a manner opposite to that in the serotype D strain JEC21 or *S. cerevisiae*. Serotype A Hog1 was constitutively phosphorylated under normal conditions and then was rapidly dephosphorylated after exposure to 1 M NaCl (Figure 6A). The Hog1 phosphorylation pattern of serotype A is in sharp contrast with that of *S. cerevisiae*, where the constitutive phosphorylation of Hog1 is lethal (Maeda *et al.*, 1994). The authenticity of Hog1-specific phosphorylation signals was confirmed by the absence of the equivalent Western blot signal in extracts from *hog1* Δ mutant cells (Figure 8A) and an appropriate increase of the size in the anti-Hog1 cross-reacting protein in extracts from a strain expressing an epitope-tagged Hog1 protein (Figure 7).

We performed a detailed analysis of the Hog1 phosphorylation kinetics in serotype A and D over a range of osmolarity for an extended period of time (up to 4 h). In the serotype D strain JEC21 strain, Hog1 was rapidly phosphorylated within 1 min in response to 0.5 or 1 M NaCl. Phosphorylation was maintained for a longer time in 1 M NaCl than in 0.5 M NaCl (Figure 6B), where Hog1 is rapidly dephosphorylated after 1 min. In response to a higher concentration of NaCl (1.5 M), maximal phosphorylation was achieved after 60-min exposure but sustained even up to 4 h (Figure 6B). The phosphorylation pattern of Hog1 in strain JEC21 is almost identical to that in *S. cerevisiae* (Van Wuytswinkel *et al.*, 2000), indicating that the HOG regulatory mechanism is highly conserved between the serotype D strain JEC21 and the model yeast. In the serotype A strain H99, Hog1 was dephosphorylated most rapidly in 1 M NaCl (within 10 min) and maintained in the dephosphorylated

state for up to 4 h. In response to 0.5 M NaCl, Hog1 was slowly and only modestly dephosphorylated by 1 h. In response to 1.5 M NaCl, dephosphorylation of serotype A Hog1 was rather delayed until after 30 min, whereas phosphorylation of serotype D Hog1 was sustained (Figure 6B). These data imply that the functional difference between the serotype A and D Hog1 proteins may result from different phosphorylation kinetics of the two proteins.

The opposite pattern of Hog1 phosphorylation kinetics prompted us to investigate Hog1 localization in each serotype. For this purpose, a serotype A Hog1-GFP C-terminal fusion protein was expressed from the ACT1 promoter in the *hog1* Δ A and *hog1* Δ D mutants. The HOG1-GFP fusion gene was completely functional and complemented to restore the phenotypes of the *hog1* Δ A and *hog1* Δ D mutants to wild-type (unpublished data). By Western blot analysis, the phosphorylation kinetics of the Hog1-GFP fusion protein were identical to wild-type Hog1 in both serotypes (Figure 7A). The finding that serotype A Hog1 can rescue both *hog1* Δ A and *hog1* Δ D mutants further confirms that differential function and regulatory mechanisms impinging on Hog1 result from divergence of upstream or downstream elements.

In agreement with the known Hog1 localization pattern in *S. cerevisiae* (Reiser *et al.*, 1999), in serotype D Hog1 was distributed in both the cytosol and the nucleus under normal conditions and was rapidly (within 5 min) concentrated in the nucleus after exposure to 1 M NaCl (Figure 7B). Thus, nuclear localization of serotype D Hog1 was temporally associated with its dual phosphorylation after osmotic shock. In contrast, in serotype A Hog1 was localized in both the cytosol and the nucleus and any concentration in the nucleus in response to hyperosmotic conditions was transient and moderate compared with serotype D (up to 15

Figure 6. Hog1 exhibits opposite phosphorylation patterns in serotype A compared with serotype D and *S. cerevisiae*. (A) *C. neoformans* serotype A (H99), serotype D (JEC21), and *S. cerevisiae* (Σ strain) strains were grown to midlogarithmic phase and exposed to 1 M NaCl in YPD medium for the time indicated and total protein extracts were prepared for Western blot analysis. The dual phosphorylation status of Hog1 (T171 and Y173) was monitored using antidiagonally phosphorylated p38 antibody (P-Hog1). The same blots were stripped and then probed with polyclonal anti-Hog1 antibody for the Hog1 loading control (Hog1). (B) The Hog1 phosphorylation patterns in serotype A (H99) and D (JEC21) were monitored for a longer time course in different NaCl concentrations (0.5, 1, and 1.5 M NaCl) as described in A. (C) The Hog1 phosphorylation patterns in various clinical and environmental serotype A and D isolates were monitored during osmotic shock (1 M NaCl) as described A. For serotype A, six clinical strains isolated from Tanzania (78.7.98 [first row] and 46F.5.02 [second row]), Botswana (BT63 [third row] and BT130 [fourth row]), Asia (S25C [fifth row] and S25J [sixth row]) were used. For serotype D, B3501, NIH12, NIH433, MMRL757, MMRL760, and CDC92-27 strains were tested. NIH12 and NIH433 are parental clinical and environmental strains, respectively, for B3501 and JEC21. MMRL757 and MMRL760 are clinical serotype D strains isolated from HIV patients in Italy.

min; Figure 7B). After 15 min, Hog1 in serotype A was more evenly distributed in both the cytosol and the nucleus, similar to Hog1 in serotype D under normal conditions. The most striking comparison for Hog1 localization between the two serotypes was found after 30- or 60-min exposure to NaCl (Figure 7B), when the protein was nuclear in serotype D, and nuclear and cytoplasmic in serotype A. Compared

with the obvious phosphorylation-associated localization patterns of serotype D Hog1, the localization of serotype A Hog1 appears to be less-dependent on its phosphorylation status. Taken together, the serotype A strain H99 was found to have unusual Hog1 phosphorylation kinetics and localization patterns, which are distinct from those of the serotype D strain JEC21 or other fungi.

Figure 7. Subcellular localization of Hog1 in serotype A and D *C. neoformans* corresponds to the Hog1 phosphorylation status. (A) Western blot analysis was performed as described in Figure 6 with protein extracts isolated from serotype A and D wild-type strains (H99 and JEC21) and *hog1*Δ mutants containing pACT-HOG1fGFP (constitutive expression of Hog1 (serotype A)-GFP fusion protein from the *ACT1* promoter), YSB242 and YSB243. (B) To determine the subcellular localization of Hog1 in serotype A and D, YSB242 and YSB243 were exposed to 1 M NaCl for the indicated incubation times, fixed, and permeabilized to monitor GFP signals and DAPI staining. Bar, 10 μm.

The Unique Hog1 Phosphorylation Pattern Observed in H99 Is Predominant in Most *C. neoformans* Isolates

To test whether the unique Hog1 phosphorylation kinetics is observed in only H99 (e.g., strain variation) or truly results from serotype differences, Hog1 phosphorylation patterns were monitored in multiple serotype A and D strains. All seven serotype A clinical isolates tested here (two each from Tanzania, Botswana, and Asia [Figure 6C] and one from Uganda [unpublished data]) exhibited the unique Hog1 phosphorylation pattern similar to strain H99.

In serotype D strains, however, variation in the pattern of Hog1 regulation is apparent. First we tested Hog1 phosphorylation patterns in the well-defined JEC21 parental strains, B3501, NIH12, and NIH433. JEC21 is a *MAT*α progeny resulting from ten backcrosses with the parental strain *MAT*α B3502 (=JEC20) that is an f1 sibling of the *MAT*α strain B3501. NIH12 (*MAT*α clinical isolate) and NIH433 (*MAT*α environmental isolate) are the parental strains for B3501 and B3502 (for review see Heitman *et al.*, 1999). Similar to JEC21, in strains B3501, NIH12, and NIH433 Hog1 phosphorylation was activated in response to osmotic shock (Figure 6C). However, strain B3501 and NIH12 exhibited a higher level of basal Hog1 phosphorylation, whereas strain NIH433 showed very low basal Hog1 phosphorylation similar to strain JEC21. We further examined Hog1 phosphorylation patterns in several other clinical serotype D strains. One strain (MMRL757) exhibited the JEC21-like Hog1 phos-

phorylation pattern, whereas the other 5 clinical serotype D strains (MMRL760, CDC92-27 [Figure 6C], 11, MMRL751, and CDC92-16 [unpublished data]) showed an H99-like phosphorylation pattern. Therefore, the Hog1 regulatory kinetics observed in H99 appears to be universal in serotype A, whereas serotype D strains exhibited either pattern.

Pbs2-mediated Phosphorylation Is Required for Hog1 Function

The finding of an atypical Hog1 regulatory mechanism raised the question of whether the upstream MAPK kinase might be unconventional. In *S. cerevisiae*, Hog1 is phosphorylated by the upstream MAPKK Pbs2. Through BLAST searches, we identified Pbs2 homologues in both serotype A and D (95% identity between serotypes) that share 35% identity with *S. cerevisiae* Pbs2. To test whether Pbs2 is responsible for phosphorylation of Hog1, the serotype A *PBS2* gene was disrupted in strains H99 and KN99a. Hog1 phosphorylation patterns were monitored in the *pbs2*Δ mutant during exposure to 1 M NaCl. Pbs2 was found to be necessary for dual phosphorylation of Hog1. Hog1 was constitutively expressed but not phosphorylated in the *pbs2*Δ mutant, with or without osmotic shock (Figure 8A). Next the phenotypes of *pbs2*Δ mutants were compared with those of *hog1*ΔA mutants to examine the impact of phosphorylation status on serotype A Hog1 function. The phenotypes of *pbs2*Δ mutants were indistinguishable from those of *hog1*Δ

Figure 8. Dual phosphorylation mediated by the MAPKK Pbs2 is required for Hog1 function. (A) Hog1 dual phosphorylation was monitored by Western blot analysis in the serotype A WT (H99), *pbs2* Δ mutant (YSB123), and *hog1* Δ mutant (YSB64) grown in YPD medium containing 1.0 M NaCl for the indicated times. (B) Multistress responses of the *pbs2* Δ mutant (YSB123) and *pbs2* Δ +*PBS2* reconstituted strain (YSB212) were compared with those of the WT (H99) and *hog1* Δ mutant (YSB64) as described in Figure 2 (UV [720 J/m²] and H₂O₂ [3 mM]). (C) The MAT α WT, *pbs2* Δ , and *pbs2* Δ +*PBS2* strains were cocultured for 7 d with the MAT α WT (KN99a) or *pbs2* Δ (YSB125) mutant strains under mating conditions and photographed at $\times 100$ magnification as described in Figure 4. (D) Capsule production of the *pbs2* Δ and *pbs2* Δ +*PBS2* strains was visualized and quantitatively measured as described in Figure 3 and compared with the WT and *hog1* Δ mutant strains. Asterisks represent significant increases in the capsule size of the *hog1* Δ and *pbs2* Δ mutants relative to the WT. Bar, 10 μ m. (E) Capsule production of the WT (H99), *hog1* Δ (YSB64), *hog1* Δ +*HOG1* (YSB145), *hog1* Δ +*HOG1*^{T171A} (YSB250), *hog1* Δ +*HOG1*^{Y173A} (YSB252), and *hog1* Δ +*HOG1*^{T171A+Y173A} (YSB253) strains was visualized with India ink and photographed. Bar, 10 μ m.

mutants. Similar to the *hog1* Δ mutant, *pbs2* Δ mutants were hypersensitive to high temperature (40°C), UV irradiation, and oxidative stress (Figure 8B), enhanced in mating (Figure 8C) and cell-cell fusion efficiency (approximately twofold), and hyperactive in capsule (Figure 8D) and melanin production (unpublished data). These data indicate that Pbs2-mediated phosphorylation is essential for Hog1 function.

The importance of dual phosphorylation at residues T171 and Y173 in the function of serotype A Hog1 was further examined by site-directed mutagenesis. The mutated *HOG1* alleles (*HOG1*^{T171A}, *HOG1*^{Y173A}, and *HOG1*^{T171A+Y173A}) were integrated into the original *HOG1* locus in the *hog1* Δ mutant. Expression of each mutant *HOG1* allele was confirmed by Western blot analysis and sequencing clones obtained from RT-PCR (unpublished data). We found that the T171A, Y173A, or T171A+Y173A *HOG1* mutant allele did not complement any *hog1* Δ mutant phenotype, including enhanced capsule (Figure 8E) and melanin production, increased mating efficiency, and high sensitivity to multiple

stresses (unpublished data). Taken together, these findings demonstrate that Pbs2-mediated dual phosphorylation at T171A and Y173A is critical for Hog1 function.

Hog1 Catalytic Activity Is Required for Hog1 Function and Its Dephosphorylation in Response to Osmotic Shock

Next, we addressed whether Hog1 catalytic activity is required for its diverse functions. In *S. cerevisiae*, two conserved lysine residues were found to be essential for Hog1 function and the mutations K52S and K53N abolish catalytic activity (Alepuz *et al.*, 2001). The two equivalent lysine residues are conserved in *C. neoformans* Hog1 (K49 and K50). To examine the role of Hog1 catalytic activity, the K49S+K50N *HOG1* kinase-dead allele (*HOG1*^{KD}) was constructed by site-directed mutagenesis and integrated into the native *HOG1* locus in the *hog1* Δ mutant. We discovered that catalytic activity of Hog1 is required for proper response to a variety of stresses, including osmotic shock, high temperature, oxidative stress (Figure 9A), and UV (unpublished data). Sim-

Figure 9. Hog1 catalytic activity is required for Hog1 function and osmotic stress-induced dephosphorylation in serotype A. (A) Multistress responses of the serotype A (WT [H99], *hog1A* [YSB64], *hog1A+HOG1* [YSB145], and *hog1A+HOG1^{KD}* [YSB308, the K49S+K50N Hog1 kinase-dead mutant]) and serotype D (WT [JEC21], *hog1D* [YSB139], *hog1D+HOG1* [YSB203], and *hog1D+HOG1^{KD}* [YSB311]) strains were assessed as described in Figure 2. (B) Capsule production of each serotype A strain in A was visualized with India ink and photographed. Bar, 10 μ m. (C) The phosphorylation kinetics of the *hog1+HOG1^{KD}* mutants (YSB308 and YSB311) was monitored during osmotic shock (1 M NaCl) as described in Figure 6 and compared with those in the WT (H99 and JEC21) strains.

ilarly, the *HOG1^{KD}* allele was inactive and failed to restore the WT pattern of capsule (Figure 9B) and melanin production and mating (unpublished data) in the *hog1A* mutant. Interestingly, in response to H₂O₂, the *HOG1^{KD}* allele impaired oxidative stress resistance of the *hog1A* mutant (Figure 9A), implying that the catalytically inactive form of Hog1 can repress a factor(s) contributing to oxidative stress resistance. In serotype D, the *HOG1^{KD}* allele was also unable to complement the *hog1D* mutants with respect to osmotic shock and UV irradiation (Figure 9A). Strikingly, however, the unusual hyper-resistance of *hog1D* mutants to H₂O₂ was completely rescued by the *HOG1^{KD}* allele, suggesting a factor(s) contributing to oxidative stress resistance can be repressed by kinase inactive Hog1. Overall, the data indicate that Hog1 requires both dual phosphorylation and catalytic activity for interacting or modulating pathways crucial for differentiation and virulence of *C. neoformans*.

In *S. cerevisiae*, the feedback control of Hog1 (dephosphorylation) is regulated by phosphotyrosine phosphatases (Ptp2 and Ptp3) or phosphoserine/threonine phosphatases (Ptc1; Hohmann, 2002). In particular, Ptp2 is localized in the nucleus and directly activated by Hog1 (Mattison *et al.*, 1999). In *C. neoformans*, we have identified several Ptc or Ptp-types

of phosphatases encoded by both the serotype A and D genomes. Two possible hypotheses can explain osmotic stress-induced Hog1 dephosphorylation. First, it can be initiated by phosphatases that are activated by Hog1 catalytic activity. Second, the dephosphorylation process might be completely independent from Hog1 signaling output and directly activated in response to osmotic shock.

We found a striking difference in the Hog1 phosphorylation pattern between the wild-type and *HOG1^{KD}* mutant (Figure 9C). After osmotic shock, the Hog1^{KD} protein was not dephosphorylated like WT Hog1 and in fact was even more highly phosphorylated. These results demonstrate that Hog1 dephosphorylation is induced by Hog1 catalytic activity, possibly via activation of Hog1-specific phosphatases. Furthermore, the fact that the Hog1^{KD} protein exhibits an osmotic stress-induced phosphorylation pattern like strain JEC21 indicates that serotype A Hog1 can be more phosphorylated by its upstream component during osmotic shock, but its phosphorylation is rapidly reversed by phosphatase activity triggered by Hog1 itself. The finding that basal phosphorylation levels are equivalent between Hog1 and Hog1^{KD} further indicates that the potential phosphatase activity is osmotic stress-inducible and depends on Hog1 cata-

Figure 10. The Hog1 MAPK and Pbs2 MAPKK promote virulence of *C. neoformans*. A/Jcr mice were infected with 10^5 cells of MAT α WT (■: H99), *hog1* Δ (□: YSB64), *hog1* Δ +*HOG1* reconstituted (■: YSB145), *pbs2* Δ (×: YSB123), and *pbs2* Δ +*PBS2* reconstituted strains (○: YSB212) by intranasal inhalation. Percent survival (%) was monitored for 33 d postinfection. Both *hog1* Δ and *pbs2* Δ mutants are significantly less virulent than the WT and their reconstituted strains ($p < 0.001$) and the *pbs2* Δ mutant is less virulent than the *hog1* Δ mutant ($p < 0.003$).

lytic activity. In serotype D, as expected, Hog1^{KD} exhibited similar Hog1 phosphorylation kinetics compared with WT Hog1 within 1 h after osmotic shock (Figure 9C) but sustained phosphorylation up to 4 h, whereas WT Hog1 is rapidly dephosphorylated (unpublished data), indicating that Hog1-specific phosphatase activation triggers feedback regulation of Hog1 after adaptation to osmotic shock.

Hog1 and Pbs2 Are Required for Full Virulence of C. neoformans

The findings that Hog1 and Pbs2 regulate stress responses and production of two known virulence factors in the sero-

type A strain H99 prompted us to investigate their role in virulence. We used a murine cryptococcosis model, in which fungal cells first infect the lungs by intranasal inhalation and then disseminate to the brain, causing meningoencephalitis. *hog1* Δ mutants were attenuated for virulence compared with the WT and reconstituted strains but eventually caused lethal infection (Figure 10). Similar to *hog1* Δ mutants, *pbs2* Δ mutants also exhibited attenuated virulence compared with the WT and reconstituted strains (Figure 10). However, we note that the *pbs2* Δ mutant was also modestly less virulent than the *hog1* Δ mutant, implying that Pbs2 might have an additional target(s) that also contributes to virulence.

DISCUSSION

The main discovery of this study is that the HOG pathway has undergone functional and mechanistic specialization enabling control of multiple stress responses, morphological differentiation, and virulence factors in diverse *C. neoformans* strains. Based on our findings, we propose a working model for the divergence of the *C. neoformans* HOG pathway between the divergent high (e.g., serotype A H99) and low pathogenicity (e.g., serotype D JEC21) strains in Figure 11. In the serotype D strain JEC21, in response to osmotic shock, Hog1 is dually phosphorylated with rapid kinetics similar to those of other model yeasts. Strikingly, the phosphorylation pattern of Hog1 in the serotype A strain H99 is oppositely regulated. The constitutive phosphorylation of Hog1 that we observe under normal growth conditions has never been reported in any other species. Phosphorylation of Hog1 is associated with sustained nuclear localization in serotype D, whereas dephosphorylation of Hog1 is associated with transient and limited nuclear accumulation in serotype A. Studies with a kinase-dead but phosphorylatable Hog1 mutant (*HOG1*^{KD}) reveal that in fact serotype A Hog1 can be further phosphorylated during osmotic shock, but is actually dephosphorylated rapidly as a result of robust activation of Hog1-specific phosphatases. Therefore, in this model, two unique features of Hog1 regulation operate in the clinical

Figure 11. Proposed model for differential regulation of the HOG pathway in *C. neoformans*. In the serotype D strain JEC21, in response to osmotic shock, Hog1 MAPK is rapidly phosphorylated by Pbs2 MAPKK and then translocates to the nucleus to adapt to osmotic changes in a manner equivalent to that observed in budding yeast. In contrast, the HOG pathway is specially adapted to control differentiation and virulence factors as well as osmotic stress in serotype A strains including H99. In this model, Hog1 is constitutively phosphorylated by Pbs2 under normal conditions either by weak upstream activation or lack of pathway repression. After osmotic shock, phosphorylation-primed Hog1 is more rapidly activated than the unphosphorylated form, activates phosphotyrosine (Ptp) or phosphoserine/threonine (Ptc) phosphatase(s) through its catalytic activity, resulting in rapid Hog1 dephosphorylation. Under normal conditions, the constitutively phosphorylated form of Hog1 represses the pheromone-MAPK and cAMP-signaling pathways, indicating that the functions of the HOG pathway are uniquely specialized to control virulence factor production in serotype A and clinical serotype D strains.

serotype A strain H99 compared with the laboratory adapted, less virulent strain JEC21 and other model yeasts: 1) constitutive Hog1 phosphorylation without osmotic shock and 2) osmotic stress-induced Hog1 dephosphorylation by Hog1-specific phosphatase activation.

We propose that this unusual constitutive phosphorylation of Hog1 results in cross-talk with signaling cascades regulating virulence factors of *C. neoformans* serotype A, conferring novel roles for the Hog1 pathway in virulence. Serotype A Hog1 negatively regulates melanin synthesis driven by the cAMP-PKA signaling cascade. The complete restoration of melanin synthesis in *gpa1Δ*, *cac1Δ*, and *pka1Δ* mutants by the *hog1* mutation suggests that Hog1 represses PKA downstream targets for melanin biosynthesis. For capsule production, however, Hog1 may act on an element upstream of Gpa1 or PKA itself because enhanced capsule synthesis by *hog1* mutation was completely blocked by *gpa1*, *cac1*, or *pka1* mutations. It is also conceivable that Hog1 represses a factor(s) such as Ste12 that modulates melanin and capsule synthesis parallel to or in conjunction with the cAMP pathway. At this point, however, it is not clear how Hog1 regulates these virulence factors *in vivo*. One can imagine that *C. neoformans* could tightly regulate Hog1 to control virulence factor production depending on its micro-environment during the course of infection. Nevertheless, considering the prevalence of the unique H99-like Hog1 phosphorylation patterns in many clinical strains, we speculate that the HOG MAPK cascade has been adapted as a global regulatory pathway mediating virulence of this pathogen.

Cross-talk of Hog1 with the pheromone response MAPK pathway is also evident in *C. neoformans* serotype A. Both confrontation assays and Northern blot analysis reveal that pheromone production is derepressed by *hog1* mutations, resulting in enhanced mating with *ras1Δ*, *gpa1Δ*, *cac1Δ*, and *pka1Δ* mutants, but not *gpb1Δ* mutants. Repression of the mating pathway by the HOG pathway has also been reported in *S. cerevisiae*. Mutation of the *HOG1* or *PBS2* gene causes an increase in Fus3 MAPK phosphotyrosine levels and expression of pheromone responsive genes (Hall *et al.*, 1996). Similar to constitutively activated pheromone production observed in *C. neoformans* *hog1Δ* mutants (Figure 5), Hall *et al.* (1996) found that Fus3 is highly phosphorylated in *S. cerevisiae* *hog1Δ* mutants even without osmotic shock. O'Rourke and Herskowitz (1998) provided genetic evidence showing that the Pbs2-Hog1 pathway prevents cross-talk with the pheromone response Fus3/Kss1 MAPK potentially by blocking the Sho1 branch of the HOG pathway. However, the repression mechanism could be divergent between *C. neoformans* and *S. cerevisiae* for several reasons. First, cross-talk between the two pathways was observed only in serotype A *C. neoformans* that shows an opposite Hog1 regulatory pattern compared with *S. cerevisiae*. Second, *C. neoformans* appears to lack a Sho1 homolog, which is known to be required for osmolarity-induced cross-talk in *S. cerevisiae*. Therefore, the detailed mechanism by which Hog1 negatively modulates the mating MAPK pathway in *C. neoformans* serotype A should be further investigated in future studies.

In contrast to the unique phosphorylation patterns of Hog1, the function of the upstream MAPKK, Pbs2, appears to be evolutionarily conserved. Hog1 phosphorylation was completely abolished by *pbs2* mutations and the *pbs2Δ* mutant exhibited phenotypes equivalent to those of the *hog1Δ* mutant. These data suggest that Pbs2 is necessary upstream of MAPKK for Hog1 activation, similar to *S. cerevisiae*. Furthermore, in animal studies, both *hog1Δ* and *pbs2Δ* mutants

were attenuated for virulence. Previous studies have shown that mutants enhanced in melanin or capsule synthesis, such as *prk1Δ* and *crg1Δ* mutants, are hypervirulent (D'Souza *et al.*, 2001; Wang *et al.*, 2004). Yet even though the *hog1Δ* and *pbs2Δ* mutants produce higher levels of melanin and capsule, both exhibit reduced virulence. We speculate that hypersensitivity to diverse environmental stresses counterbalances any virulence increase that would be attributable to enhanced melanin and capsule production.

Considering that virulence was more severely reduced in the *pbs2Δ* mutant compared with the *hog1Δ* mutant, Pbs2 might have other targets related to virulence in addition to Hog1. The protein kinase C-Mpk1/Slr2 MAPK is one potential Pbs2 target, and several lines of evidence indicate that the HOG pathway is also involved in regulation of cell wall integrity. In *S. cerevisiae*, Pbs2 overexpression increases expression of the cell wall-modifying enzyme Exg1, an exo- β -glucanase (Jiang *et al.*, 1995), and several genes involved in cell wall assembly were isolated as multicopy suppressors of the hyperosmosensitive phenotype of HOG pathway mutants (Alonso-Monge *et al.*, 2001). Furthermore, mutation of HOG pathway genes confers increased resistance to cell wall perturbation by Calcofluor white (Garcia-Rodriguez *et al.*, 2000). Additional evidence comes from the finding that the growth defect of the *C. neoformans* *mpk1* mutant at 37°C is suppressed by osmotic stabilization (Kraus *et al.*, 2003). The functional correlations between the Pbs2-Hog1 and Pkc1-Mpk1 pathways should be investigated in future studies.

The question remains how Hog1 is differentially regulated and in particular how serotype A Hog1 is constitutively phosphorylated under normal growth conditions and rapidly dephosphorylated after osmotic shock. The fact that serotype A and D Hog1 are identical at the amino acid level and are functionally interchangeable indicates that upstream signaling components are likely diverged and differentially regulate Hog1 in the two serotypes. By searching for potential components upstream of Pbs2 and Hog1 in the *C. neoformans* serotype A and D genome databases, we discovered two MAPKKK homologous to Ssk2/Ssk22 containing the highly conserved serine/threonine protein kinase catalytic domain in the C-terminus (95% identity between serotypes A and D), implying that elements at or upstream of the Ssk2/22 step in the Hog1 pathway might be divergent between the two serotypes.

In *S. cerevisiae*, at least two upstream branches are known to modulate the Hog1 MAPK cascade: the Sho1- and Sln1-mediated osmosensing pathways. Interestingly, homologues of these two transmembrane osmosensors were not identified in *C. neoformans* although other signaling components from each branch were identified in both serotypes (e.g., Ste50, Cdc42, and Ste11 for the Sho1 pathway; Ypd1 and Ssk1 for the Sln1 pathway). The absence of these transmembrane osmosensors might explain why *hog1* disruption results in only a modest increase in osmosensitivity of *C. neoformans* unlike *hog1* mutations in *S. cerevisiae*. One possible explanation is that the multiple-stress-responsive and global regulatory character of *C. neoformans* Hog1 might require the presence of multiple signal sensors, from which signals converge on the Pbs2-Hog1 MAPK cascade. In support of this idea, six hypothetical proteins were discovered in *C. neoformans* having the same domain structures (both histidine kinase and response regulator receiver domains in a single protein) that are normally observed in other eukaryotic two-component proteins. However, unlike *S. cerevisiae* Sln1, typical transmembrane and external loop structures were not observed in these putative proteins. This raises the possibility that the phosphorelay system diverges function-

ally and structurally, which in turn differentially regulates the Hog1 MAPK cascade in *C. neoformans*. Perhaps analogously, *S. pombe* has sensor histidine kinases, Mak2 and Mak3, which appear to be cytosolic proteins that are functionally and structurally distinct from Sln1 (Buck *et al.*, 2001). It will be interesting to investigate how components upstream of the HOG pathway are evolutionarily diverged between the two serotypes and contribute to serotype- and strain-specific specialization of Hog1.

The impact of *hog1Δ* or *pbs2Δ* mutations on virulence in the serotype A strain H99 appears to result from complex phenotypic outcomes conferred by pathway inactivation. Because a single animal model system that allows parallel virulence analysis for both serotype A and D strains has not been developed thus far, whether differential Hog1 regulation determines the pathogenicity differences between the two serotypes or strains in the same serotype remains to be established. This issue can be solved by identifying upstream and downstream components governing the pathway specialization described above and then by performing animal studies using a congenic set of strains with the component(s) interchanged. However, several observations we made in this study potentially correlate the differential Hog1 regulation with virulence variations in *C. neoformans* strains. First, the unique control of the HOG pathway observed in the serotype A strain H99 that enables cells to regulate essential virulence factors was the predominant pattern observed in most *C. neoformans* strains. Among 16 serotype A and D strains tested here (with the exception of the lab strains JEC21 and B3501), 13 strains (all 8 serotype A and 5 serotype D strains) exhibit H99-like Hog1 regulation represented by constitutive Hog1 phosphorylation under normal conditions whereas only two strains (NIH433 and MMRL757) showed the JEC21-like pattern of Hog1 regulation. Second, the serotype D B3501 strain that shows increased levels of basal Hog1 phosphorylation was found to be indeed more virulent than JEC21 (personal communication, Kirsten Nielsen). In particular, the difference in basal levels of Hog1 phosphorylation between B3501 and JEC21 provides a unique opportunity to identify upstream component(s) responsible for constitutive Hog1 phosphorylation using the meiotic map recently reported for these related serotype D strains (Marra *et al.*, 2004). Moreover, it will be of interest to further compare the virulence potential of other serotype A and D strains exhibiting distinct Hog1 phosphorylation patterns in future studies.

These studies will enable a definition of the molecular events that underlie Hog1 signal cascade pathway adaptation that occurred between serotype D environmental and laboratory isolates and true clinical isolates, and also during the divergence of serotype A and D that gave rise to the most successful pathogenic clade.

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