Mating-Type-Specific and Nonspecific PAK Kinases Play Shared and Divergent Roles in Cryptococcus neoformans

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Cryptococcus neoformans is an opportunistic fungal pathogen with a defined sexual cycle involving fusion of haploid MATα and MATa cells. Virulence has been linked to the mating type, and MATα cells are more virulent than congenic MATa cells. To study the link between the mating type and virulence, we functionally analyzed three genes encoding homologs of the p21-activated protein kinase family: STE20α, STE20α, and PAK1. In contrast to the STE20 genes that were previously shown to be in the mating-type locus, the PAK1 gene is unlinked to the mating type. The STE20α, STE20α, and PAK1 genes were disrupted in serotype A and D strains of C. neoformans, revealing central but distinct roles in mating, differentiation, cytokinesis, and virulence. ste20α pak1 and ste20α pak1 double mutants were synthetically lethal, indicating that these related kinases share an essential function. In summary, our studies identify an association between the STE20α gene, the MATα locus, and virulence in a serotype A clinical isolate and provide evidence that PAK kinases function in a MAP kinase signaling cascade controlling the mating, differentiation, and virulence of this fungal pathogen.

Cryptococcus neoformans is a pathogenic fungus that infects the central nervous system in immunocompromised individuals (7). This heterothallic basidiomycete is distributed worldwide and has evolved into four distinct serotypes (A, B, C, and D), all of which infect humans and which have been classified into three different varieties. Serotypes A (Cryptococcus neoformans var. grubii) and D (Cryptococcus neoformans var. neoformans) are distributed worldwide in association with pigeon droppings; cause the majority of infections in human immuno- deficiency virus-infected and other immunocompromised hosts; and, based on population genetics studies, diverged from a common ancestor ~18.5 million years ago (56).

C. neoformans has a defined sexual life cycle involving haploid cells of MATα and MATa mating types (20, 21). Mating involves cell fusion and leads to the production of heterokaryotic hyphal filaments. The tips of the hyphal filaments differentiate to form basidia, in which nuclear fusion and meiosis occur, and long chains of basidiospores are then produced by budding from the basidia (2). In response to nitrogen starvation and mating pheromone, MATα strains, but not MATa strains, can also form filaments and sporulate by a process known as haploid or monokaryotic fruiting (53, 55). The basidiospores produced by mating or fruiting may represent the infectious propagules (47), which are inhaled and then spread as yeast cells via the bloodstream to infect the brain.

The MATα mating type has also been linked to virulence in C. neoformans. MATα strains are more prevalent in the environment, most clinical isolates are MATα, and MATα strains are more virulent than congenic MATa strains in animal models (22, 23).

The MATα locus was previously identified and found to contain two mating-type-specific genes: MFα1 and STE12α. The MFα1 gene encodes a mating pheromone that stimulates conjugation tube formation, cell fusion, and mating (11, 37; W.-C. Shen, R. C. Davidson, G. M. Cox, and J. Heitman, submitted for publication). The STE12α gene encodes a homolog of the Ste12/Cph1 transcription factor that regulates mating, filamentation, or virulence in Saccharomyces cerevisiae and Candida albicans (31–33, 54). Recent studies have revealed that the MAP kinase cascade that controls mating in C. neoformans is composed of both mating-type-specific and non-specific components. For example, the G protein β subunit Cbp1 (52, 53) and the MAP kinase Cpk1 (R. C. Davidson, G. M. Cox, J. R. Perfect, and J. Heitman, submitted for publication) are not mating type specific, whereas mating-type-specific elements include the MEK kinase Ste11α (9) and the recently identified p21-activated protein kinase (PAK) homologs Ste20α and Ste20α (30).

The PAK family of protein kinases is conserved from yeast to humans, and its members play central roles in cell signaling and development as effectors of Rho-type p21 GTPases (10, 14, 24, 34). PAK kinases share a highly conserved C-terminal catalytic domain and a CRIB (for Cdc42-Rac interactive binding) regulatory domain. In addition, the Cla4 and Cla4-like kinases contain an amino-terminal pleckstrin homology (PH) domain. PH domains mediate binding to membrane phosphoinositides and may also interact with proteins (29). In the ascomycetous yeasts S. cerevisiae and Schizosaccharomyces pombe, the PAK kinases Ste20 and Cla4 (Skl1/Pak1 and Shk2/Pak2 in S. pombe) act as effectors of the Cdc42 GTPase and regulate mating, the cell cycle, and morphogenesis, in part by activating MAP kinase signaling (12, 17). In other fungi, PAK kinases have been linked to virulence (C. albicans), hyphal maturation (Ashbya gossypii), and filamentation (C. albicans and Yarrowia lipolytica) (5, 18, 25, 27, 48).
We have identified three PAK kinase homologs, Ste20α, Ste20a, and Pak1, in C. neoformans. Interestingly, we recently showed that the STE20 genes reside in the mating-type loci; therefore, the STE20 gene exists as two mating-type-specific alleles, STE20α and STE20a, which are related but divergent (30). In contrast, we find that the third PAK kinase homolog, Pak1, is not mating type specific. Mutations in the serotype A STE20α gene resulted in a modest defect in mating, defects in cytokinesis and growth at 39°C, and attenuated virulence. Serotype D ste20α and ste20a mutants exhibited a similar cytokinesis defect; however, they grew normally at 37°C and were fully virulent. ste20 mutants were sterile in bilateral crosses. Mutations in the PAK1 gene also conferred defects in mating and haploid differentiation but did not affect growth or cytokinesis. pak1 mutant strains of both serotypes A and D were attenuated for virulence. ste20α pak1 and ste20a pak1 strains were inviable, indicating that these mutant combinations are synthetically lethal. Taken together, our studies reveal that the Ste20 and Pak1 kinases share an overlapping essential function but also have distinct roles in mating, differentiation, cytokinesis, and virulence.

**MATERIALS AND METHODS**

**Strains, media, and bioinformatic transformation of C. neoformans.** The C. neoformans serotype A strains H99 (MATa), M049 (H99 ade2), and F99 (H99 ura5) and the congeneric serotype D MATa and MATa strains JEC20 and JEC21 and their auxotrophic derivatives have been described (37, 46, 53) and are listed in Table 1. Yeast-pesto-dextrose (YPD) and yeast nutrient base media, synthetic (SD) medium, V8 agar for mating, filament agar, Niger seed for melanin production, low-iron medium plus 56 μM ethylenediamine-di(o-hydroxyphenylacetic acid), and serum-free Dulbecco’s modified Eagle’s medium for capsule induction were prepared as previously reported (3). Transformation reactions were conducted using established methods (49). Transformants were selected on SD medium lacking adenine or uracil and containing 1 M sorbitol.

**TABLE 1. Strains used in this study**

| Strain | Genotype | Reference |
|--------|----------|-----------|
| H99    | MATa     | 42        |
| M049   | MATa ade2 | 42        |
| F99    | MATa ura5 | 51        |
| PPW54  | ste20a::ADE2 ade2 | This study |
| PPW55  | ste20a::ADE2 ade2 ura5 | This study |
| PPW63  | ste20a::ADE2 ura5 STE20a + URA5 | This study |
| PPW91  | ste20a::UR5 ura5 | This study |
| PPW96  | ste20a::ura5 ura5 | This study |
| PPW151 | ste20a::URA5 STE20a + URA5 | This study |
| CSB1   | pak1::URA5 ura5 | This study |
| CSB2   | pak1::ura5 ura5 | This study |
| CSB3   | PAK1 ura5 ura5 | This study |

**Serotype D**

| JEC20 (B-4476) | MATa | 23 |
| JEC21 (B-4500) | MATa | 23 |
| JEC34 | MATa ura5 | J. Edman |
| JEC43 | MATa ura5 | J. Edman |
| JEC50 | MATa ade2 | J. Edman |
| JEC155 | MATa ura5 ade2 | J. Edman |
| JEC156 | MATa ura5 ade2 | J. Edman |
| CSB5 | MATa ste20a::ADE2 ade2 | This study |
| CSB7 | MATa ste20a::UR5 ura5 | This study |
| CSB8 | MATa pak1::UR5 ura5 ura5 | This study |
| CSB9 | MATa pak1::UR5 ura5 ura5 | This study |
| CSB10 | MATa pak1::UR5 ura5 ura5 | This study |
| CSB12 | MATa ste20a::URA5 ade2 ura5 | This study |
| CSB23 | MATa pak1::ura5 ura5 | This study |
| CSB48 | MATa ste20a::ura5 ura5 | This study |
| KBL156-1 | MATa ste20a::ADE2 ade2 ura5 | This study |

**Isolation of the C. neoformans STE20/PAP genes.** Generators primers, 5′-GT NGGATAACCARCATG (2090) and 5′-YTCGNGGCATCCGAT (2092), were used to amplify a partial clone of the STE20 kinase gene by PCR using the following parameters: 94°C, 40 s; 39°C, 1 min; 72°C, 1 min: 40 cycles. C. neoformans cDNA from H99 (200 ng) was used as the template. A 420-bp PCR product of the expected sizes was excised, cloned, verified by sequencing, and used as a probe to isolate the corresponding genomic locus.Genomic DNA from strains H99, JEC20, and JEC21 was also used in touchdown PCR amplification with the same primer set and the following parameters: 94°C, 30 s; 60°C with a temperature increment of −1°C every cycle for 20 cycles, 1 min; 72°C, 45 s; and an additional 20 cycles of 94°C, 30 s; 40°C, 1 min; and 72°C, 45 s (see also reference 30). Open reading frames containing the serotype A STE20a and PAK1 genes were isolated on 9 kb HindIII and 11 kb EcoRI-PstI fragments, respectively, from serotype A (H99) size-selected DNA libraries. Reverse transcription (RT)-PCR and 5′ and 3′ rapid amplification of cDNA ends (RACE) (Gibo BRL) were performed to verify predicted intron-exon junctions and to determine transcription initiation and polyadenylation sites. Similar methods were employed to isolate and characterize the serotype D STE20 gene by PCR using size-selected DNA libraries. The STE20a gene was previously isolated as a 2.5-kb PstI fragment from a partial library of the serotype D JEC20 strain (30). The serotype D PAK1 gene was identified in sequence traces generated by the Stanford Genome Technology Center Cryptococcus neoformans Genome Project. Primers based on the predicted sequence were used to amplify and sequence the PAK1 open reading frame from serotype D JEC21 genomic DNA. Predicted intron-exon junctions were confirmed by amplification of the PAK1 sequence from a JEC21 cDNA library.

**Generation of STE20 and PAK1 disruption mutants.** To disrupt the STE20a gene in serotype A, two different disruption alleles, ste20a::ADE2 and ste20a::URA5, were constructed by inserting the ADE2 or URA5 gene at a unique Hpal site within the coding region. Primers 5′-AGGACATCTATCAGCAGAT (2576) and 5′-CTGTAAGGTAACTAACTT (2579) were used to amplify the ste20a disruption allele, which was used to transform strain M049, or following SnuBI digestion to transform strain F99, generating strains PPW54 (ste20a::ADE2) (6 out of 132 Ade+ isolates) and PPW91 (ste20a::URA5) (5 out of 132 Ura+ isolates). To disrupt the serotype D STE20a gene, the URA5 gene was inserted at a unique BglII site within the coding region to generate a ste20a::URA5 disruption allele. Similarly, the ADE2 gene was inserted at a unique EcoRV site within the coding region of the serotype D STE20a gene. The ste20a::URA5 and ste20a::ADE2 alleles were transformed into strain JEC155 (MATa ura5 ade2) or JEC156 (MATa ura5 ade2), respectively, generating strains CSB12 (MATa ste20a::URA5 ura5 ade2) (16%) and KBL156-1 (MATa ste20a::ADE2 ade2 ura5) (8 out of 79).

**Generation of PAK1 disruption mutants.** To disrupt the serotype A ste20a mutant strains was accomplished by first generating ura5 mutant versions by selection on 5-fluoroorotic acid (5-FOA)
medium. These _ura5_ strains were then transformed with the _STE20a_ gene physically linked to the _C. neoformans_ _URA5_ (ste20a::ADE2 ura5 _URA5_) (38). Similarly, the _ste20a::ADE2 ura5 STE20a_ _URA5_ reconstituted strain (strain PPW63) was also constructed. Reconstitution of the _STE20_ gene was achieved by transformation with the wild-type _PAK1_ gene and incubation on 5-FOA medium, selecting for integration of _PAK1_ at the _STE20_ locus. Finally, the newly reconstituted _PAK1_ _UR5_ strain (CSB2) was transformed with the _URA5_ gene to restore _Ura^+_, prototrophic strain CSB3. For all deletions and reconstitutions, transformants were screened by PCR and confirmed by Southern hybridization analysis.

**Mating, haploid differentiation, melanin, and capsule formation.** The mating competence of the _PAK_ mutant strains was determined by crossing each strain to a tester strain of the opposite mating type. Crosses were prepared by growing the strains overnight in YPD medium at 30°C to 10^7_ cells/ml, mixing together 200 μl of each mating pair, and spotting 5 μl of each mating mixture onto V8 agar medium. Similarly, mutant strains were tested for filamentation; the strains were grown in YPD medium overnight at 30°C to 10^7_ cells/ml, 1 ml was washed and resuspended in 500 μl of H_2O, and 5 μl of cells was spotted onto filament agar medium with 0.5% glucose (55). In confrontation tests, strains were streaked in a single straight line with the opposite mating partner in proximity without physical contact (53). For each assay, the plates were incubated at ambient temperature for a period of 2 (confrontation assays) to 14 (filamentation) days in the dark and examined microscopically for basidiospore formation or filaments. Melanin production was examined by inoculating the strains on Nager seed agar medium with incubation at 37°C for 3 days. Capsule was induced by inoculating the strains in low iron medium plus 56 μM ethylenediamine-dl-hydroxyphenylacetic acid for 1 week (50) or in Dulbecco’s modified Eagle’s medium (15) for 3 days at 30°C.

**Overexpression analysis and two-hybrid interactions.** _C. neoformans_ plasmids expressing the _GBP1_ (pGAL7-GBP1), _CPK1_ (pGAL7-CPK1), and _activated STE11a_ (pGPD-STE11a-1) genes were transformed into the serotype _A_ _ste20a::ADE2 ura5_ (PPW55) mutant. The _CPK1_ allele is missing the final exon of 15 amino acids that spans sequences not conserved in other MAP kinases. _ste20a::ADE2 ura5_ _GBP1_, _ste20a::ADE2 ura5_ _CPK1_, and _ste20a::ADE2 ura5 STE11a::CPK1_ transformants were mated to the _ste20a::URA5 strain CSB5 on V8 mating agar medium for 7 days. _C. neoformans_ plasmids expressing the _STE11a_ (pGAL7-STE11a), _CPK1_ (pGPD1-CPK1), and _activated STE11a_ (pGPD1-STE11a-1) genes were transformed into serotype _A_ _MATa_ _ste20a::ADE2 ura5_ (CSB48) and _pok1::ADE2 ura5_ (CSB23). To test for filamentation, transformants were inoculated on filament agar containing 2% galactose or 0.5% glucose and incubated for 14 days at 24°C in the dark.

For two-hybrid interactions, the _S. cerevisiae_ reporter strain P169-4A was cotransformed with plasmids pGBD-Cdc42 and pGAD-STE20a, pGBD-CDC42 and pGAD-PAK1, pGBD-CDC42 and pGAD424, pGBT9 and pGAD-STE20a, pGBT9 and pGAD-PAK1, and pGBT9 and pGAD424. Transformants were selected on SD-TRP-Leu medium, and the transformed strains were tested on SD-TRP-Leu-Ade and SD-TRP-Leu-His plus 3-aminotriazol media. The transformants were selected on SD-Trp-Leu medium, and the transformed strains were tested for _STE20_ and _PAK1_ expression by PCR analysis.

Animal models of cryptococcal meningitis. Virulence tests were performed using established rabbit and murine models of cryptococcal meningitis (3, 36, 39, 41, 53). Three or four male New Zealand White rabbits weighing 2.5 to 3 kg were administered either hydrocortisone acetate (2.5 mg/kg of body weight) or betamethasone acetate (0.15 mg/kg) intramuscularly 1 day prior to inoculation and then daily for the entire test period. One day after the initial steroid treatment, the animals were anesthetized with xylazine and ketamine intramuscularly and inoculated intracisternally with 0.3 ml of cell suspension (3 x 10^7/ml) for each strain. Animals were anesthetized on days 4, 7, and 10 following infection, and 50 μl of cerebrospinal fluid (CSF) was withdrawn, serially diluted, and plated onto YPD medium. The colonies were counted, and the mean number of CFU was plotted with the standard error of the mean indicated. A standard Student _T_ test was performed to establish _P_ values. The virulence test was performed twice and yielded similar results.

In the murine infection model, 50 μl containing 5 x 10^7_ serotype _A_ yeast cells was used to infect 10-4- to 6-week-old female _A/Jer_ mice (10 mice per strain) by nasal inhalation. The mice were anesthetized by phenobarbital injection, and yeast cells were then pipetted directly into the nostrils. In the tail vein injection model, groups of 10-4- to 6-week-old female DBA mice obtained from National Cancer Institute/Charles River laboratories were directly injected in the lateral tail vein with 10^7_ cells of the serotype _D_ strains to be tested. The mice were monitored twice daily, and those that appeared to be sick or in pain were sacrificed by CO_2_ inhalation. The number of surviving mice was plotted against time, and _P_ values were calculated by the Kruskal-Wallis test. In addition, to examine cell morphology and capsule formation in vivo, mice infected with the serotype _A_ strains H99, PPW91 (ste20a::URA5), PPW151 (ste20a::STE20a), CSB1 (pok1::URA5), and CSB3 (reconstituted _PAK1_) were sacrificed 10 days after infection. Brain samples were harvested and examined microscopically for _C. neoformans_ capsule formation.

**Nucleotide sequence accession numbers.** The GenBank accession numbers are as follows: _STE20a_ (A), AF162330; _STE20a_ (D), AF315635; _STE20a_ (A), AF315636; _STE20a_ (D), AF315638; _PAK1_ (A), AF931150; and _PAK1_ (D), AF931151.

**RESULTS**

Identification of _C. neoformans_ _PAK_ kinases homologs. As previously described, the mating-type-specific _PAK_ kinase homologs _Ste20a_ and _Ste20a_ were identified in _C. neoformans_ by low-stringency PCR using primers designed for conserved catalytic regions (VAIKOM and YWMAPE). Here, we used the same PCR-based approach to identify a third _PAK_ kinase homolog, which was named Pak1. The corresponding genes were cloned from both serotype _A_ and serotype _D_ strains (30) (see Materials and Methods). In total, six _PAK_ kinase genes have been identified from four _C. neoformans_ strains encompassing two varieties and both mating types.

The _PAK_ kinases are defined by two conserved domains: an N-terminal regulatory CRIB domain and a C-terminal catalytic domain. The _Cryptococcus_ _PAK_ kinases also contain these...
conserved domains (Fig. 1A, C, and D). For example, the serotype A Ste20α homolog has 48 to 53% identity in the CRIB domains and 57 to 61% identity in the catalytic domains with those of human PAK1, *S. pombe* Shklp1/Paklp1, and *S. cerevisiae* Cla4 and Ste20. As noted earlier, a subgroup of PAK kinases also contain a PH domain in the N terminus. Members of this subgroup include Cla4 from *S. cerevisiae* and Shk2p/Pak2p from *S. pombe*. In contrast to the previously described *C. neoformans* Ste20 kinases, the *C. neoformans* Pak1 kinase does not contain a PH domain (Fig. 1A and B).

In contrast to the STE20 genes, the PAK1 gene is present in both MATα and MATα cells and is located on a different chromosome than the mating-type locus. First, in Southern blotting, a *PAK1* gene probe hybridized to identical DNA fragments from the congenic serotype D strains JEC20 and JEC21 (not shown). When chromosomes from *MATα* and *MATα* strains were separated by pulsed-field gel electrophoresis, the STE20 gene-specific probes hybridized to the 1.8-Mb chromosome from their respective mating types (Fig. 2A), as reported previously (30). In contrast, *PAK1* gene probes hybridized to a smaller, ∼1.4-Mb chromosome, indicating the PAK1 gene is not linked to the mating-type locus (Fig. 2A).

**Disruption of the *C. neoformans* STE20α, STE20α, and PAK1 genes.** To establish the respective functions of the PAK kinase homologs Ste20α, Ste20α, and Pak1 in *C. neoformans*, serotype- and/or mating-type-specific disruption mutations were generated for each gene by inserting the *ADE2* or *URA5* marker gene into a unique restriction site in the N-terminal half of the gene (Fig. 2B) (see Materials and Methods). Linear DNA containing each gene disruption allele was biolistically transformed into auxotrophic serotype A or D recipient strains, and the efficiency of obtaining mutant strains was within the range of 5 to 16%. By this approach, *ste20α* and *pak1* mutations were obtained in the serotype A strain H99, and *ste20α*, *ste20α*, and *pak1* mutations were isolated in the congenic serotype D strains (JEC21 and JEC20). Thus, neither *Ste20* nor *Pak1* is essential in *C. neoformans* in either serotype or mating type.

We took three approaches to ensure that the observed phenotypes resulted from the introduced gene disruptions. First, reconstituted strains were constructed by two methods. Either a wild-type copy of the original gene with a physically linked marker (e.g., *STE20α URA5*) was introduced ectopically, or the disrupted allele was replaced with the wild-type gene at the endogenous locus by recombination and replacement of the integrated *URA5* marker and selection for growth on 5-FOA medium (*PAK1*). Second, in all cases, multiple independent mutants were obtained and tested to ensure common phenotypes. Third, for serotype D strains, genetic crosses were conducted to establish whether the mutant phenotypes were linked to the gene disruption.

**ste20 mutants exhibit cytokinesis and temperature-sensitive growth defects.** Because *S. cerevisiae* mutants lacking the PAK kinase Cla4 exhibit a cytokinesis defect, we examined whether *ste20α* or *pak1* mutations confer a similar phenotype in *C. neoformans*. Whereas *pak1* mutants showed no obvious cytokinesis defect, microscopic examination revealed that cells from each of the *ste20α* mutants exhibited an altered cell morphology at elevated temperature, consistent with defects in polarity establishment and cytokinesis. Wild-type *C. neoformans* strains typically grow as budding yeast. In contrast, *ste20α* mutant cells formed elongated buds that failed to separate, and abnormally wide mother-bud necks were frequently observed (Fig. 3A). Cell shape in *C. neoformans* is maintained by an actin cytoskeleton, and F-actin dynamics are similar in *S. cerevisiae* and *C. neoformans* (19). F-actin localization was compared in wild-type, *ste20α* mutant, and *ste20α* STE20α reconstituted strains grown at 39°C. In cells from each strain, the F-actin cytoskeleton was visualized as discrete patches and cables, culminating in the formation of a cap structure in small buds that had not yet undergone the apical-to-isotropic growth switch. However, F-actin remained polarized to the tips of elongated *ste20α* mutant buds, indicating a defect in the switch from polar to isotropic growth (Fig. 3A). Similar cytokinesis defects were observed with the serotype D *ste20α* and *ste20α* mutants (not shown). Taken together, these results suggest that, similar to Cla4 in *S. cerevisiae*, the *C. neoformans* Ste20 PAK kinases have a conserved role in cell polarity and cytokinesis.

The *ste20α* mutation was also found to confer a temperature-sensitive growth defect in serotype A strains. Because the cytokinesis and cell polarity defects of the *ste20α* mutants were more severe at higher growth temperatures, we tested whether these defects had an impact on cell viability. The serotype A and D *ste20α* and *pak1* mutants and reconstituted strains were analyzed by serial dilution spotting assays at different temperatures. This analysis revealed that the serotype A *ste20α* mutants are viable at 30 or 37°C but inviable at 39°C (Fig. 3B). This temperature-sensitive growth defect was observed in multiple independent mutants with two different *ste20α* disruption alleles and was complemented by reintroduction of the wild-type *STE20α* gene (Fig. 3B and not shown). No growth defect was observed for the serotype A *pak1* mutant at 39°C (Fig. 3B). Similarly, no growth defect was observed with the serotype D *ste20α*, *ste20α*, and *pak1* mutants at 37°C, the maximal growth temperature for these congenic serotype D strains (Fig. 3B). Differences in the sequence or function of Ste20 could be one mechanism that contributes to the higher maximal growth temperature of the serotype A strain H99 compared to the congenic serotype D strains.

**PAK kinases function in *C. neoformans* mating.** Based on previous studies of model yeasts, we hypothesized that the *C. neoformans* PAK kinases would have a role in mating and filamentation. Mating assays were conducted by coinoculating prototrophic serotype D wild-type and mutant strains at opposite mating types on V8 mating medium. Under these conditions, wild-type mating partner cells fuse, and the resulting heterokaryons produce abundant heterokaryotic filaments, basidia, and basidiospores within 2 to 3 days (Fig. 4A). Similarly, abundant filamentation and basidiospore production were also observed when *ste20α* or *pak1* mutants were crossed to wild-type strains (unilateral crosses) (Fig. 4 and data not shown). In contrast, in bilateral crosses (*ste20α × ste20α* and *α pak1 × α pak1*) no filamentation was observed after 3 days and only the *α pak1 × α pak1* mating exhibited residual filaments at 7 days (Fig. 4A). Interestingly, in the *ste20α* bilateral mutant matings, some filaments could be observed 1 day after mating. However, these filaments were much shorter (“stubby”) than those observed in wild-type matings, exhibited extensive branching, and were quickly overgrown by vegetative cells (not shown). Although *ste20α* and *ste20α* mutants were mating defective with
FIG. 1. *C. neoformans* PAK kinase homologs. (A) Predicted domain structures of the *C. neoformans* serotype A and D PAK kinases. Both the Ste20 and Pak1 kinases contain a CRIB domain (stippled boxes) and a kinase domain (solid boxes). In addition, the Ste20 kinases have an N-terminal PH domain (hatched boxes). (B) Amino acid sequence alignment of the *C. neoformans* Ste20 PH domains. S.c., *S. cerevisiae*. (C) Amino acid sequence alignment of the CRIB domains of the *C. neoformans* Ste20 and Pak1 kinases with *S. cerevisiae* Ste20 and Cla4 kinase CRIB domains. (D) Amino acid sequence alignment of the kinase domains of *C. neoformans* and *S. cerevisiae* PAK kinase catalytic domains. For panels B to D, identical amino acids are boxed and darkly shaded, and conservative substitutions are lightly shaded.
FIG. 2. Mapping and disruption of the \textit{STE20\alpha}, \textit{STE20a}, and \textit{PAK1} genes. (A) Chromosomes from the serotype A wild-type \textit{MATa} (H99) and serotype D wild-type \textit{MAT\alpha} and \textit{MATa} (JEC20 and JEC21) strains were separated on CHEF gels (39). The gels were stained with ethidium bromide (left), transferred to nitrocellulose, and sequentially probed with the \textit{STE20\alpha}, \textit{STE20a}, and \textit{PAK1} genes. \textit{S. cerevisiae} chromosomes served as size markers. (B) Schematic representation of the \textit{STE20\alpha}, \textit{STE20a}, and \textit{PAK1} gene disruptions. Gene-specific primers flanking the inserted \textit{ADE2} and \textit{URA5} marker genes were used to amplify genomic DNA from the wild-type (H99), \textit{ste20\alpha}/H9251 mutant (PPW54), and \textit{ste20\alpha}/H9251 \textit{STE20\alpha}/H9251 reconstituted (PPW63) strains. Southern hybridization analysis with the \textit{STE20\alpha} and \textit{PAK1} genes as probes was used to verify the genotypes of the \textit{STE20\alpha} wild-type and \textit{ste20\alpha} mutant strains (JEC20 and KBL156-1) and of the \textit{PAK1} wild-type (H99), \textit{pak1} mutant (CSB1), and \textit{PAK1} reconstituted (CSB3) strains. The \textit{pak1} mutant strain was reconstituted by replacing the \textit{pak1::URA5} allele with the wild-type \textit{PAK1} gene, resulting in the reappearance of the 2.5-kb wild-type \textit{PAK1} fragment. Restriction sites are as follows: A, \textit{ApaI}; RV, \textit{EcoRV}; P, \textit{PstI}; E, \textit{EcoRI}; S, \textit{SmaI}. 

\[ \text{Restriction sites are as follows: A, ApaI; RV, EcoRV; P, PstI; E, EcoRI; S, SmaI.} \]
FIG. 3. Ste20α mutants exhibit defects in cytokinesis and a serotype-specific growth defect at 39°C. (A) Mutation of the STE20α kinase gene confers a cytokinesis defect. Serotype A MATα STE20α wild-type (H99), ste20α mutant (PPW54), and ste20α STE20α reconstituted (PPW63) strains were grown in YPD medium for 24 h at 30°C and then shifted to 39°C for 4 h. The cells were fixed and stained with rhodamine-conjugated phalloidin to visualize actin (bottom row) or visualized by DIC microscopy (top row). The cells were photographed at ×1,000 magnification. (B) Serotype A ste20α mutants (top) but not serotype D ste20α mutants (bottom) exhibit temperature-sensitive growth. The wild-type (H99) (WT), ste20α mutant (PPW54), ste20α STE20α reconstituted (PPW63), pak1 mutant (CSB1), and PAK1 reconstituted (CSB3) strains were grown overnight at 30°C, counted, serially diluted (10-fold), inoculated as 5-μl aliquots on YPD medium, and incubated at 30, 37, or 39°C for 60 h. The isogenic serotype D strains were JEC20 [WT (a)], JEC21 [WT (α)], CSB5 (ste20α), and CSB7 (ste20α).
each other, both mated normally with pak1 mutant strains (Fig. 4A and data not shown). These findings implicate Ste20 and Pak1 as playing important roles in mating, most likely involving filamentation following cell fusion.

**PAK kinases are necessary for haploid fruiting.** In response to nitrogen deprivation and desiccation, haploid MATα strains undergo asexual filamentation and sporulation (haploid fruiting). We tested whether the *C. neoformans* PAK kinases play a role in haploid fruiting. The wild-type serotype D MATα strain underwent robust haploid fruiting when incubated on filament agar for 14 days, whereas the congeneric ste20α and α pak1 mutant strains failed to form filaments and produced no basidia or basidiospores (Fig. 4B), indicating roles for both Ste20α and Pak1 in haploid fruiting.

Because haploid fruiting of MATα cells is enhanced by mating pheromones, one possible explanation for the inability of ste20α and α pak1 mutant strains to haploid fruit is a defect in pheromone sensing or response. To test this, confrontation assays were conducted by placing strains of opposite mating types in close proximity on assays were conducted by placing strains of opposite mating pheromone sensing or response. To test this, confrontation assays were conducted by placing strains of opposite mating types. To determine if the Ste20 and Pak1 proteins interact with Cdc42, the Cdc42 protein was fused to the Gal4 DNA binding domain and portions of the Ste20α (amino acids 164 to 327) and Pak1 (amino acids 1 to 139) proteins encompassing the CRIB domains were fused to the Gal4 activation domain. Coexpression of the resulting GAL4BD-Cdc42 and GAL4AD-Ste20 or GAL4AD-Pak1 fusion proteins activated Gal4-dependent expression of the ADE2, HIS3, and lacZ reporter genes (Fig. 5C). Thus, Cdc42 physically interacts with both Pak1 and Ste20α, supporting a model in which the PAK kinases are activated by Cdc42.

**Ste20 and Pak1 have a shared role essential for viability.** In *S. cerevisiae*, cla4 and ste20 mutations are synthetically lethal, indicating that the two kinases share an overlapping essential function. We took two approaches to test if the Ste20 and Pak1 kinases have a similar shared essential function in *C. neoformans*. First, we attempted to delete the serotype A *PAK1* gene in a ste20α::ADE2 ura5 strain using a pak1::URA5 disruption allele. No pak1 mutants were identified from ~400 Ura− colonies. Because gene disruptions typically occur at 2 to 16% in *C. neoformans*, we would have expected to isolate between 8 and 64 pak1 mutants if the ste20α pak1 double mutant were viable.

To further test this hypothesis, genetic crosses were performed with the *C. neoformans* serotype D ste20α and pak1 mutants. The ste20α::ADE2 ura5 mutant strain KBL156-1 and the MATα pak1::URA5 ade2 mutant strain CSB5 were crossed, and basidiospores were micromanipulated and germinated on YPD medium at 24°C. The resulting meiotic segregants were then replica plated onto selective medium to follow segregation of the ste20α and pak1 mutations (Fig. 6A). In addition, DNA was isolated and analyzed by PCR with primers specific to the STE20α, STE20α, and *PAK1* genes (Fig. 6B). From

**FIG. 4.** Ste20α and Pak1 kinases function in mating and haploid fruiting. (A) Isogenic serotype D MATα wild-type (JEC21), ste20α mutant (CSB7), and α pak1 mutant (CSB9) strains were each mixed with a wild-type MATα strain (JEC20), ste20α mutant (CSB8), or MATα pak1 (CSB10) strain and incubated on V8 mating medium for 7 days at 24°C. The edges of mating patches were photographed at ×40 final magnification in all images. (B) Isogenic serotype D MATα wild-type (JEC21), ste20α (CSB7), and α pak1 (CSB9) mutant strains were incubated on filament agar for 14 days at 24°C in the dark, and the edges of growth patches were photographed at ×40 final magnification. (C) Congenic serotype D wild-type, ste20α, ste20α, and pak1 mutant strains of opposite mating types (JEC21, JEC20, CSB7, CSB5, CSB9, and CSB10) were grown in confrontation as lines of cells ~2 mm apart on filament agar for 72 h at 24°C in the dark. The opposing interfaces were photographed at ×200 final magnification (×200 final magnification for the inserts).
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FIG. 5. Ste20α kinase functions in a MAP kinase pathway for mating. (A) Plasmids expressing the GAL7-CPK1-1 fusion gene or the
constitutively active STE11α-1 allele were transformed into the serotype A MATa ste20α ura5 mutant strain PPW96. Prototrophic GAL7-CPK1-1
and STE11α-1 transformants were mated with the serotype D ste20α mutant (CSB5) on V8 medium for 1 to 3 weeks at 24°C and photographed
at ×40 final magnification. (B) ste20α::ura5 ura5 (CSB88) and a pak1::ura5 ura5 (CSB23) mutant strains with or without the STE12α gene
expressed from the GAL7 promoter were inoculated on filament agar containing 0.5% galactose. The plates were incubated for 7 days in the dark
at 24°C and photographed at ×40 final magnification. (C) Cdc42 interacts with Ste20α and Pak1 in the two-hybrid system. Two-hybrid
protein-protein interaction assays were conducted with strain PJ69-4A containing plasmids expressing the Gal4 activation and DNA binding
domains either alone or fused to Cdc42 or to regions of Ste20α or Pak1 encompassing the CRIB domains. The cells were grown on SD-Leu-Trp
synthetic medium (Control) or SD-Leu-Trp-His plus 3-AT medium (Binding) to monitor protein-protein interaction-dependent expression of the
Gal4-driven GAL-HIS3 reporter gene. The cells were grown for 72 h at 30°C. Similar results were obtained with the GAL7-CPK1-1 reporter gene
(not shown). β-Galactosidase expressed from the GAL-lacZ reporter was assayed in Miller units and yielded 667 units for Cdc42 and Pak1, 141
units for Cdc42 and Ste20α, and 40 to 55 units in control strains. +, present; −, absent.

several ste20α × α pak1 crosses, a total of 60 viable basidiospores were obtained and yielded similar results. One such
mating that gave rise to 13 viable meiotic segregants out of 18
spores is depicted in Fig. 6A. Of the 13 segregants, five were
MATa ste20α::ADE2, four were MATα pak1::URA5, and four
were wild type MATα (Fig. 6A). No ste20α pak1 colonies were
recovered, indicating that these mutations are synthetically
lethal. A similar analysis was performed with the ste20α and a
pak1 mutant strains. No ste20α pak1 progeny were obtained,
indicating that the ste20α pak1 mutant combination is also
synthetically lethal. We note that the inability to recover these
double mutants is not attributable to linkage, because the
genes are located on different chromosomes (Fig. 2A). In
conclusion, both Ste20α and Ste20α share an essential function
with Pak1.

STE20α plays a serotype-specific role in virulence. The viru-
ulence of C. neoformans has been linked to growth at 37 and
39°C and mating type. We tested the role of the C. neoformans
PAK kinases in virulence in two animal models of cryptococ-
cosis (3, 39, 41). First, the serotype A wild-type strain H99, the
ste20α mutant, and the ste20α STE20α reconstituted strains
were inoculated intrathecally into steroid-immunosuppressed
rabbits. The survival of cryptococcal cells in the central nervous
system was determined by serially diluting and plating CSF
onto YPD agar medium. While wild-type cells persisted in the
central nervous system at similar levels over the 10-day course
of infection (10^6 cells/ml), the persistence of the ste20α mutant
was significantly reduced (10^2 cells/ml) (Fig. 7A). In compari-
on, the reconstituted ste20α STE20α strain was partially re-
stored for virulence (10^4 cells/ml) (Fig. 7A).

Similar results were obtained in the murine inhalation
model. The average survival of A/Jcr mice infected with the
wild-type serotype A strain H99 was 28 days, and 100% leth-
ality occurred by day 34 (Fig. 7B). Virulence was attenuated in
the ste20α mutant (P < 0.001 versus the wild type); 80% of
infected mice survived to day 37, the average survival was 42
days, and 100% lethality occurred by day 51. Virulence was
largely restored to wild type in the ste20α STE20α reconsti-
tuated strain in the murine model (P = 0.082 versus the wild
type), and the average survival was 31 days, with 100% lethality
occurring by day 40 (Fig. 7B).

We also examined the virulence of serotype D ste20α and
ste20α mutants in a murine tail vein injection model of cryp-
tococcosis in DBA mice lacking the C5 component of the
complement system. In contrast to the serotype A ste20/H9251 mutant strains in the A/Jcr murine inhalation model, three independent serotype D ste20/H9251 mutants and the ste20a mutant strain were fully virulent in the DBA murine tail vein injection model. Mice infected with the wild-type MAT/H9251 JEC21 strain survived an average of 22 days, whereas those infected with the ste20/H9251 mutant survived on average 19 days, with 80% lethality occurring by day 31 with both the wild type and the ste20/H9251 mutant (Fig. 7D) (P/H11005 0.59). Similarly, the survival curves for mice infected with the MATa wild-type strain JEC20 and the congenic ste20a mutant were essentially superimposable (Fig. 7E) (P/H11005 0.44). Thus, neither Ste20 kinase is required for virulence in the congenic serotype D laboratory strains, whereas the Ste20a kinase contributes to virulence in the serotype A strain H99.

Pak1 kinase is required for virulence. We next examined the role of the Pak1 kinase in virulence. In the rabbit model of cryptococcal meningitis, survival of the serotype A pak1 mutant in CSF was significantly reduced by day 10 following infection (10³ cells/ml), while the PAK1 reconstituted strain was fully virulent (10⁶ cells/ml) (Fig. 7A). Similarly, in the murine model, the pak1 mutant strains were significantly attenuated (Fig. 7C and F) (P < 0.001). With the serotype A pak1 mutant strain, 100% lethality was delayed until day 46 compared to day 30 for the wild-type strain (Fig. 7C). All mice infected with serotype D pak1 mutant strains were still alive 114 days postinfection, indicating that these strains are nearly avirulent (Fig. 7F) (P < 0.001). Fifty percent lethality of mice infected with the MATα pak11 and the MATα pak1 mutant strains occurred by days 132 and 144 postinfection, respectively. These viru-
lence defects were attributable to the pak1 mutation; virulence was restored to the wild-type level in the serotype A PAK1 reconstituted strain (Fig. 7C) \((P = 0.32\) compared to the wild type), and independent pak1 mutants in both mating types in the serotype D strains were dramatically attenuated (Fig. 7F).

In summary, the Pak1 kinase is required for virulence in both serotypes of *C. neoformans*.

**The ste20α mutation impairs capsule production in vivo.** A large polysaccharide capsule and the pigment melanin are two well-established virulence factors of *C. neoformans*. We examined whether the effects of the ste20α or pak1 mutation on virulence in serotype A are attributable to defects in capsule or melanin production. Capsule size was found to be reduced in animals infected with the serotype A ste20α mutant, but not in those infected with the pak1 mutant. This capsule defect was apparent in both CSF yeast cells stained with India ink from infected rabbits (day 3 postinfection) (not shown) and India ink-stained brain smears from mice (day 10 postinfection) (Fig. 8). The capsule defect was not complemented in all cells of the ste20α STE20α reconstituted strain (Fig. 8), which may explain why virulence was not fully restored (Fig. 7A and B).

Interestingly, a significant proportion of the serotype A ste20α mutant cells isolated from infected animals exhibited a non-yeast cell morphology in that the cells were elongated and

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**FIG. 7. Virulence of *C. neoformans* ste20α, ste20α, and pak1 mutant strains.** (A) Isogenic serotype A wild-type (H99), ste20α mutant (PPW91), ste20α STE20α reconstituted (PPW151), pak1 mutant (CSB1), and PAK1 reconstituted (CSB3) strains were inoculated intracisternally into immunosuppressed rabbits. CSF was withdrawn on days 4, 7, and 10 postinfection, and the number of surviving yeast cells was determined by plating serially diluted CSF on YPD medium. The mean value of cell counts for each strain was plotted (the error bars indicate the standard error of the mean). (B and C) Serotype A strains used for panel A were also inoculated into A/Jcr mice (10 mice per strain) by inhalation, and survival was monitored daily and plotted. (D, E, and F) Similarly, wild-type (JEC21 and JEC20) and ste20α (CSB7), ste20α (CSB5), α pak1 (CSB9), and a pak1 (CSB10) mutant serotype D strains were inoculated by tail vein injection into DBA mice (10 mice per strain), and survival was plotted against time.
encapsulated. These “filaments” lacked clamp connections associated with mating and haploid fruiting. In addition, the filamented cells contained complete septa but failed to separate. These results suggest that the ste20a mutant cells have adopted a pseudohypha-like growth mode in vivo (Fig. 8). Normal budding yeast cell morphology was restored in the ste20a STE20a reconstituted strain (Fig. 8). In summary, ste20a mutant strains exhibited defects in two known cryptococcal virulence factors, capsule and high-temperature growth, and exhibited an interesting filamentous morphology in vivo. By comparison, the ste20 and pak1 mutant strains exhibited no melanin defect, and the pak1 mutant strains had no capsule defect and grew normally at elevated temperature, failing to provide a clear link between these factors and the attenuated phenotype of pak1 mutants.

**DISCUSSION**

In summary, we have functionally characterized the STE20a, STE20a, and PAK1 genes, which encode homologs of the Ste20/PAK kinase family in *C. neoformans*. Importantly, the STE20a and STE20a genes are mating type specific and reside in the *MATa* and *MATa* mating-type loci, whereas the PAK1 gene is not mating type specific and is present on a different chromosome. Genetic studies of kinase mutant strains reveal that these kinases play overlapping roles in mating, fruiting, and virulence. The Ste20 kinases also play a unique role in cytokinesis and, in serotype A, growth at elevated temperature. Finally, the observation that ste20 and pak1 mutations are synthetically lethal further indicates that these kinases share an essential function required for viability. These studies illustrate how mating-type-specific and nonspecific components of a conserved MAP kinase pathway contribute to cell-type-specific signaling.

Ste20 kinases have similarities to both Ste20 and Cla4 of *S. cerevisiae*. In the yeast *S. cerevisiae*, the Ste20 and Cla4 kinases partially overlapping functions in the regulation of morphogenesis and mating (10). The Ste20 and Cla4 kinases both interact with and are activated by the Cdc42 GTPase (26, 43), but the Ste20 kinase is uniquely adapted to associate with the Gβγ subunit complex during responses to pheromone and mating (28). Interactions between Cdc42 and Ste20 are necessary for pseudohyphal differentiation (26), and the small GTPase Ras2 promotes filamentous growth, in part by signaling upstream of Cdc42 and Ste20 (38). Similarly, the Ras1 homologs function in pheromone signaling in *S. pombe* and *C. neoformans* (1, 35).

The *C. neoformans* Ste20a, Ste20a, and Pak1 kinases have levels of amino acid sequence identity and structural and functional properties similar to those of both yeast kinases. *C. neoformans* ste20a, ste20a, and pak1 mutants are mating impaired, a phenotype exhibited by ste20 mutants in many but not all *S. cerevisiae* strains (10, 24). The STE20a and STE20a genes are contained within the *MATa* and *MATa* loci that determine mating type (30). On the other hand, the Ste20a and Ste20a kinases contain a PH domain that is a unique feature of the Cla4 kinases of both *S. cerevisiae* and *C. albicans*, and the *C. neoformans* ste20a and ste20a mutants both exhibit cytokinesis defects similar to those of *S. cerevisiae* cla4 mutants. We have designated the Ste20a and Ste20a kinases Ste20 homologs.
based on the links to MAP kinase signaling and the MAT loci, but given a role in cytokinesis and the presence of a PH domain, they also share features with Cla4-related kinases.

**Ste20α and Pak1 kinases function in MAP kinase signaling.** Epistasis tests indicate that the Ste20α and Pak1 kinases function in the MAP kinase pathway regulating mating. Overexpression of the G protein β subunit Gpb1 failed to suppress the mating defect of ste20α mutant strains. These findings are consistent with a model in which Ste20α functions downstream from Gpb1. Overexpression of the MAP kinase Cpk1 or the dominant-active Ste11α-1 mutant restored mating of ste20α and pak1 mutant strains, indicating that Ste11α and Cpk1 may function downstream of the PAK kinases.

Overexpression of the MAP kinase Cpk1 or an activated form of the Ste11α kinase failed to restore haploid fruiting of the pak1 and ste20α mutant strains. In contrast, overexpression of the Ste12α transcription factor homolog did restore fruiting of a pak1 and ste20α mutants. Our interpretation is that partial activation of the MAP kinase cascade is insufficient to bypass the upstream components for fruiting, whereas overexpression of Ste12α can bypass the requirement for Ste20α or Pak1, either because the action of Ste12 is more robust or because it functions in more than one pathway. Our findings support models in which the Ste20α and Pak1 kinases have partially redundant roles in activating the MAP kinase pathway. We also found that the Cdc42 GTPase physically interacts with the CRIB domain of the Ste20α kinase. These findings provide evidence that a Cdc42-Ste20α/Pak1-Ste11α-Cpk1 pathway functions in mating and fruiting. Further genetic and biochemical studies will be required to define the point of action of the Ste20 and Pak1 kinases in the MAP kinase and other signaling and morphogenesis pathways and to establish the mechanisms by which these related kinases are activated to phosphorylate their substrates.

**The Ste20α kinase plays divergent roles in the virulence of serotype A and D strains.** The findings that MATα strains (i) are significantly more prevalent in both environmental and clinical isolates, (ii) are more virulent than MATα strains, and (iii) undergo haploid fruiting have focused interest on the genes encoded by the MATα locus and their association with pathobiology in cryptococcosis. Our studies have identified the STE20α gene as a novel component of the MATα locus that is associated with virulence in serotype A strains, and STE20α represents the first gene in the MATα locus to be linked to virulence in serotype A strains. The Ste20α kinase controls production of the polysaccharide capsule, a known virulence factor, and also contributes to virulence by regulating growth at mammalian body temperatures.

Most interestingly, the ste20α mutant exhibited a pseudohypha-like morphology in vivo (Fig. 8), suggesting that a role in normal budding yeast growth may be important for virulence. Our observations may also be related to a recent report of an unusual C. neoformans isolate that produced hypha-like extensions and was obtained from a feline nasal granuloma (6). This unusual serotype A MATα isolate also exhibited a temperature-sensitive growth defect and produced elongated cells at 37°C in vitro (6), strikingly similar to our findings with the ste20α mutant serotype A isolate reported here. These findings suggest that this clinical isolate may harbor a mutation in the Ste20α kinase or another element of the signaling cascade.

In contrast to the functions of the Ste20α kinase in the virulence of serotype A strains, serotype D strains lacking Ste20α were fully virulent. Strains lacking the second PAK kinase homolog, Pak1, were attenuated for virulence in both serotypes. Although the STE20 and PAK1 genes share some functions in both serotypes, the Pak1 kinase may have evolved to supplant the role of the Ste20α kinase in virulence during the divergence of serotype A and D strains from a common progenitor. Alternatively, this difference in the role of the Ste20α kinase in virulence in serotype A compared to serotype D strains may be attributable to divergent roles of other signaling cascades. In previous studies, the Ste12α transcription factor encoded by the MATα locus was found to be required for virulence in the serotype D strain JEC21 but not for virulence in the serotype A strain H99 (8, 57). The protein kinase A pathway plays a central role in the virulence of serotype A strains of C. neoformans (3, 4, 13) but is largely dispensable for pathogenesis of congenic serotype D strains (C. A. D’Souza and J. Heitman, unpublished data). Taken together, these observations suggest that the relative contributions of different signaling cascades to virulence may change as pathogens evolve into closely related but distinct varieties or species.

**PAK kinases play conserved roles in morphogenesis and virulence.** From studies of model yeasts, it is clear that PAK kinases are required for morphogenic events, such as bud emergence and apical growth. Cla4 has an additional role in the switch from apical to isotropic growth in S. cerevisiae (reviewed in reference 44), and the Ste20 homolog Shk1/Pak1 is essential for polar growth in S. pombe (35, 40). However, recent studies have shown that PAK kinases also play an important role in morphogenesis in other fungi. In Y. lipolytica and C. albicans, PAK kinase homologs are required for the dimorphic transition from budding yeast to filamentous growth (18, 25, 27, 48). In C. albicans, this switch is also associated with virulence, and both Cst20 (a Ste20 homolog) and CaCla4 (a Cla4 homolog) are required for virulence in mouse models. However, while cst20/cst20 mutants are only partially attenuated for virulence and can still switch from yeast to hyphae in vivo, cla4/cla4 mutants are completely avirulent and are unable to switch in vivo. In addition, cla4/cla4 mutants exhibit morphological defects. These results parallel our findings that deletion of the STE20 and PAK1 genes confers different phenotypes with respect to the virulence and morphology of C. neoformans.

Recently, a Cla4 homolog in the cotton pathogen A. gossypii was found to be required for hyphal maturation and septation (5). In A. gossypii, early filaments switch from a slow- to a fast-growing form in a poorly understood process called hyphal maturation (5), cla4 mutant strains are unable to switch to the fast-growing form, and as a consequence, the filaments produced are short and undergo premature apical branching. Interestingly, this phenotype is strikingly similar to our observations of the stubby mating filaments produced by an ste20α × ste20α bilateral mutant cross in C. neoformans. It is possible that Ste20 is required for a similar process in C. neoformans and that dikaryotic filaments lacking both ste20α and ste20a are unable to properly signal to the growing hyphal tip to accelerate growth.

Organization of the mating-type locus and links to viru-
ience. Our studies reveal that the MAT locus in \textit{C. neoformans} is organized in a specialized and unique fashion in which components of the MAP kinase signaling cascade itself, the Ste20a and Ste20a genes, are encoded by the MATa and MATa alleles. These studies and others of the structure of the mating-type loci in \textit{C. neoformans} may provide insight into the evolution of virulence and asexual and sexual reproduction. We propose that the components of the MAP kinase cascade encoded by the MATa locus are evolving to play specialized roles in haploid fruiting and virulence, properties known to be associated with the MATa locus. Because other components of the MAP kinase cascade are encoded by genes present in both MATa and MATa cells, the specialization of MATa-encoded components to regulate fruiting and virulence may have resulted in an incompatibility with non-mating-type-specific signaling components such that mating of MATa strains became impaired. As a consequence, MATa strains would become sexually isolated and restricted to asexual reproduction, which may favor virulence by preventing outcrossing events. Our studies provide tools to address this and other models at the molecular level.

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