PAK Kinases Ste20 and Pak1 Govern Cell Polarity at Different Stages of Mating in *Cryptococcus neoformans*

Connie B. Nichols, James A. Fraser, and Joseph Heitman*

Departments of Molecular Genetics and Microbiology, Medicine, and Pharmacology and Cancer Biology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710

Submitted May 6, 2004; Revised July 14, 2004; Accepted July 15, 2004

Monitoring Editor: Peter Walter

Sexual identity and mating are linked to virulence of the fungal pathogen *Cryptococcus neoformans*. Cells of the α mating type are more prevalent and can be more virulent than α cells, and basidiospores are thought to be the infectious propagule. Mating in *C. neoformans* involves cell-cell fusion and the generation of dikaryotic hyphae, processes that involve substantial changes in cell polarity. Two p21-activated kinase (PAK) kinases, Pak1 and Ste20, are required for both mating and virulence in *C. neoformans*. We show here that Ste20 and Pak1 play crucial roles in polarized morphogenesis at different steps during mating: Pak1 functions during cell fusion, whereas Ste20 fulfills a distinct morphogenic role and is required to maintain polarity in the heterokaryotic mating filament. In conclusion, our studies demonstrate that PAK kinases are necessary for polar growth during mating and that polarity establishment is necessary for mating and may contribute to virulence of *C. neoformans*.

INTRODUCTION

The ability to generate cell polarity, or subcellular asymmetry, is important for all eukaryotic cells. In multicellular eukaryotes, cell polarity is critical for embryogenesis, axon migration during neuronal development, and communication between lymphocytes and the immune system. In unicellular organisms, such as the budding yeast *Saccharomyces cerevisiae*, cell polarization is essential for mitotic cell division and mating (Johnson, 1999).

In *S. cerevisiae*, polarity is generated through reorganization of the actin cytoskeleton and is directed by the small GTPase Cdc42. Ste20 and Cla4, members of the highly conserved p21-activated kinase (PAK) family, act as downstream effectors of Cdc42 in a variety of morphogenetic processes. Ste20 functions upstream of mitogen-activated protein (MAP) kinase signaling pathways mediating pheromone response, invasive growth, osmosensing, and cell wall integrity (Elion, 2000). In addition, Ste20 has been implicated in polarisome activation (Goehring et al., 2003). Cla4 functions in the organization and maintenance of septins at the mother-bud junction (Cvrckova et al., 1995; Weiss et al., 2000; Dobbeltaere et al., 2003; Schmidt et al., 2003; Versele and Thörner, 2004). Ste20 and Cla4 also share overlapping essential functions in budding and cytokinesis and have recently been shown to function in mitotic exit (Cvrckova et al., 1995; Holly and Blumer, 1999; Weiss et al., 2000; Hofken and Schiebel, 2002; Jensen et al., 2002; Seshan et al., 2002).

We previously identified two PAK kinase homologues from the human fungal pathogen *Cryptococcus neoformans*, Ste20 and Pak1 (Wang et al., 2002). Ste20 and Pak1 each contain conserved N-terminal Cdc42-Rac interactive binding (CRIB) regulatory and C-terminal catalytic domains that characterize PAK kinases. In addition, Ste20 contains a pleckstrin homology domain that is found in a subgroup of PAK kinases that includes *S. cerevisiae* Cla4. *C. neoformans* is a dimorphic fungus that is the causative agent of cryptococcosis, a life-threatening fungal meningitis (Mitchell and Perfect, 1995). *C. neoformans* isolates are classified into four subtypes based on capsular antigens (serotype A var. grubii, serotype D var. neoformans, and serotype B and C var. gattii). Serotype A and D strains account for the majority of cryptococcal infections worldwide and occur most commonly in immunocompromised patients. In contrast, serotype B and C strains are primary pathogens and were recently found to be the causative agent of a cryptococcal outbreak on Vancouver Island, Canada (Speed and Dunt, 1995; Stephen et al., 2002; Fraser et al., 2003).

Several factors contribute to virulence of *C. neoformans*, including the ability to produce melanin and a polysaccharide capsule, growth at high temperature, and mating-type. *C. neoformans* is a budding yeast with a bipolar mating-type system. In response to external stimuli, including pheromones and nitrogen deprivation, α and α cells fuse and produce a dikaryotic hyphae. When filamentation ceases, a basidium forms at the filament tip where karyogamy, meiosis, and postmeiotic divisions occur. Multiple basidiospores, the meiotic progeny, bud from the surface of the basidium at four positions to result in four long chains of protruding basidiospores. The life cycle is completed when basidiospores germinate into budding yeast cells (Hull and Heitman, 2002; Wickes, 2002).

Because of their small size, basidiospores or desiccated yeast cells are thought to be the infectious propagules that enter the alveoli of the lung. In the clinic, strains of the α mating-type are more prevalent than strains of the α mating-type, and in serotype D α cells are more virulent than α cells in animal models of cryptococcosis (Kwon-Chung and Bennett, 1978; Kwon-Chung et al., 1992). The MAT locus of *C. neoformans* is unusually large, spans >100 kb, and encodes proteins involved in signal transduction.
Mating and Polarity in *C. neoformans*

### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serotype A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSB1</td>
<td>MATa pak1::URA5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB51</td>
<td>MATa pak1::ura5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>F99</td>
<td>MATa ura5</td>
<td>Wang et al., 2001</td>
</tr>
<tr>
<td>H99</td>
<td>MATa</td>
<td>Perfect et al., 1980</td>
</tr>
<tr>
<td>JF99</td>
<td>MATa ura5</td>
<td>This study</td>
</tr>
<tr>
<td>JF219</td>
<td>MATa ste20a::NAT ura5</td>
<td>This study</td>
</tr>
<tr>
<td>JF265</td>
<td>MATa pak1::NAT</td>
<td>This study</td>
</tr>
<tr>
<td>JF267</td>
<td>MATa pak1::NAT ura5</td>
<td>This study</td>
</tr>
<tr>
<td>KN99a</td>
<td>MATa</td>
<td>Nielsen et al., 2003</td>
</tr>
<tr>
<td>PPW91</td>
<td>MATa ste20a::URA5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>PPW96</td>
<td>MATa ste20a::ura5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td><strong>Serotype D</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSB5</td>
<td>MATa ste20a::ADE2 ade2</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB8</td>
<td>MATa pak1::URA5 ura5 ade2</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB9</td>
<td>MATa pak1::URA5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB10</td>
<td>MATa pak1::URA5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB11</td>
<td>MATa ste20a::URA5 ura5 ade2</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>KLB156-1</td>
<td>MATa ste20a::ADE2 ura5 ade2</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB15</td>
<td>MATa ste20a::URA5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB21</td>
<td>MATa ste20a::ADE2 ura5 ade2 lys1</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB23</td>
<td>MATa pak1::ura5 ura5 ade2</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>CSB25</td>
<td>MATa pak1::ura5 ura5</td>
<td>This study</td>
</tr>
<tr>
<td>CSB27</td>
<td>MATa pak1::URA5 ura5 lys1</td>
<td>This study</td>
</tr>
<tr>
<td>CSB48</td>
<td>MATa ste20a::ura5 ura5</td>
<td>Wang et al., 2002</td>
</tr>
<tr>
<td>RDC5</td>
<td>MATa cpk1::ADE2 ade2</td>
<td>R. Davidson</td>
</tr>
<tr>
<td>JEC20</td>
<td>MATa</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC21</td>
<td>MATa</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC31</td>
<td>MATa lys2</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC34</td>
<td>MATa ura5</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC43</td>
<td>MATa ura5</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC50</td>
<td>MATa ade2</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC53</td>
<td>MATa ura5 lys1</td>
<td>J. Edman</td>
</tr>
<tr>
<td>JEC155</td>
<td>MATa ura5 ade2</td>
<td>J. Edman</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRCD83</td>
<td><em>C. neoformans</em> vector</td>
<td>R. Davidson</td>
</tr>
<tr>
<td>pCBN12</td>
<td>serotype A <em>Pcgr</em>-STE20a</td>
<td>This study</td>
</tr>
<tr>
<td>pCBN19</td>
<td>serotype D <em>Pcgr</em>-STE20a</td>
<td>This study</td>
</tr>
<tr>
<td>pCBN20</td>
<td>serotype D <em>Pcgr</em>-STE20a</td>
<td>This study</td>
</tr>
<tr>
<td>pCBN23</td>
<td>serotype D <em>Pcgr</em>-PAK1</td>
<td>This study</td>
</tr>
<tr>
<td>pCBN34</td>
<td>serotype A <em>Pcgr</em>-PAK1</td>
<td>This study</td>
</tr>
<tr>
<td>pCBN47</td>
<td>serotype A <em>Pcgr</em>-STE20a</td>
<td>This study</td>
</tr>
</tbody>
</table>

(Ste20, Ste11), polar growth and organelle transport (Myo2), and transcription (Ste12 and Sxi1) (Lengeler et al., 2002). Because of the link between mating type and virulence, the genes contained within the MAT locus are of considerable interest.

Our initial analysis of the *C. neoformans* PAK kinases provided additional evidence supporting a link between mating-type and virulence in *C. neoformans*. The genes encoding Ste20α and Ste20α are located in the MAT locus, *ste20* mutants are unable to mate, and the virulence of a clinical isolate deleted for *STE20α* was attenuated in animal models of cryptococcosis. However, we found that Pak1 is also required for mating and virulence in *C. neoformans*. To explore the links between mating and virulence, we have further analyzed the roles of Ste20 and Pak1 during mating in *C. neoformans*. We find that both Ste20 and Pak1 mediate cell polarity during mating. However, the roles of Ste20 and Pak1 are functionally and temporally distinct from each other; Pak1 is required during the initial fusion between α and a cells, whereas Ste20 maintains polarity during growth of the dikaryotic filament.

**MATERIALS AND METHODS**

**Strains and Media**

*C. neoformans* strains used in this study are listed in Table 1. Strains were grown on YPD medium, synthetic dextrose medium, and V8 medium (Sherman 1991; Kwon-Chung and Bennett 1992).

**Molecular Biology**

Standard methods were performed as described by Sambrook et al. (1989). *C. neoformans* genomic DNA for Southern blot analysis was prepared as described previously (Pitkin et al., 1996). Biologic transformations were performed as described previously (Tofaletti et al., 1993; Davidson et al., 2000a).

**Overexpression Analysis**

The serotype A *STE20α*, *STE20α*, and *PAK1* and serotype D *STE20α*, *STE20α*, and *PAK1* open reading frames were amplified by polymerase chain reaction (PCR) with BamHI sites at each end. The PCR products were subcloned into pCRII-TOPO and sequenced. After digestion with BamHI, each open reading frame was subcloned into BamHI digested *C. neoformans* vector pRCD85 generating plasmids pCBN12 (serotype A *STE20α*), pCBN19 (serotype A *STE20α*), pCBN34 (serotype A *PAK1*), pCBN19 (serotype D *STE20α*), and pCBN23 (serotype D *PAK1*). Ura– strains PPW96, JF219, CSB51, CSB48, KLB156-1, and CSB23 were transformed with each plasmid, including pRDC85, which served as a control. Transformants were
analyzed in bilateral crosses with the appropriate mating partner and for growth at 37 or 39°C (for ste20a transformants).

**Northern Blot Analysis**

Equal numbers (2 × 10⁶ cells) of JEC21, a cpk1 (RDC5), ste20a (CSB5), and a pak1 (CSB9) cells were grown on separate V8 plates alone or in coculture with equal numbers (2 × 10⁶ cells) of JEC20, ste20a (CSB5), or a pak1 (CSB10) cells for 24 h. Cells were harvested, lysophilized overnight, and RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA). Northern blots were performed according to standard protocols (Ausubel et al., 1992) by using 10 μg of total RNA per sample. The MFA, Mfa, and ACT1 probes were amplified by PCR using primers specific to MFA (MFA 5′- CCGTTAATACGATACCAAAC-3′, MFA 3′- JOHE10449 CGGTTAATACGATACCAAAC-3′) and PAK1 3′ (JOHE10449 CGGTTAATACGATACCAAAC-3′, MFA 5′- CCGTTAATACGATACCAAAC-3′, and PAK1 3′- JOHE10449 CGGTTAATACGATACCAAAC-3′), with the overlap construct used to transform KN99a to create JF265 (MATa pak1::NAT-ura5) and JF99 to create JF267 (MATa pak1::NAT-ura5). Putative deletion strains were confirmed by PCR and Southern blot analysis.

**RESULTS**

**Pak1 Is Required for Cell Fusion During Mating in C. neoformans**

Mating in *C. neoformans* is initiated when haploid α and a cells sense and respond to pheromone and fuse. The resulting dikaryon produces abundant dikaryotic filaments, basidia, and basidiospores (Kwon-Chung, 1976). Mating filament production is highly reduced in bilateral α pak1 x a pak1 and ste20a x ste20a mutant crosses (Wang et al., 2002). Decreased filamentation during mating could stem from a variety of reasons, including defects in cell fusion or filament morphogenesis. For example, cpk1, ste7, and ste11 MAP kinase cascade mutants all exhibit decreased filamentation during mating due to a defect in cell fusion (Davidson et al., 2003). Our previous epitope analysis placed Ste20 upstream of the Ste11-Ste7-Cpk1 MAP kinase cascade (Wang et al., 2002). We therefore tested whether ste20 mutants exhibit a similar fusion defect.

To address this question, bilateral mutant crosses were conducted between serotype D ste20a, ste20a, a pak1, and a pak1 strains. Each strain was genetically marked to allow selection of prototrophic isolates formed by cell fusion after 48 h of incubation on mating medium. No prototrophic isolates were isolated from the bilateral pak1 mutant cross, whereas abundant prototrophic isolates were produced from both the wild-type control cross and the bilateral ste20 mutant cross (Figure 1A). Hence, Pak1 is required for cell fusion during mating but Ste20 is not. Ste20 likely functions at a later stage in mating, possibly during filament morphogenesis or hyphal elongation.

We also examined fusion in unilateral crosses between pak1 mutant and wild-type strains to determine whether one copy of PAK1 was sufficient for fusion. The number of prototrophic isolates produced from either pak1 unilateral cross was only modestly decreased compared with wild type, demonstrating that one copy of PAK1 suffices for fusion (unpublished data). This is in contrast to cpk1, ste7, and ste11 mitogen-activated protein kinase (MAPK) cascade mutants, which exhibit a fusion defect even in unilateral crosses with a wild-type mating partner (Davidson et al., 2003).

**Pheromone Induction Is Normal in PAK Kinase Mutants**

The Mfa pheromone genes are induced in response to nutrient limitation and coculture with a cells via the Ste11-Ste7-Cpk1 MAPK cascade (Davidson et al., 2000b, 2003; Shen et al., 2002). If Pak1 were to function as the sole activator of the MAPK cascade then we would expect to observe a defect in Mfa pheromone expression in a pak1 cells during coculture with a pak1 cells. Mfa expression was examined by Northern blot analysis by using RNA extracted from α wild-type and α cpk1 cells cocultured with a wild-type cells and α pak1 cells cocultured with a pak1 cells. Consistent with previous observations, Mfa expression was dramatically induced by 24 h when α cells were cocultured with a cells (Shen et al., 2002) (Figure 1B). Mfa induction was absent in a cpk1 cells cocultured with wild-type a cells (Davidson et al., 2003), but
it was induced when $\alpha$ pak1 cells were cocultured with $\alpha$ pak1 cells (Figure 1B). Wild-type levels of $MFa$ also were observed in $\alpha$ pak1 cocultured with $\alpha$ pak1 and ste20a strains alone, $\alpha$ and $\alpha$ cpk1 strains cocultured with wild-type $a$, $\alpha$ pak1 cocultured with $\alpha$ pak1, and ste20a cocultured with ste20a. Cells were incubated on V8 medium for 24 h at room temperature, RNA was prepared, and $MFa$ gene expression was assessed by Northern blot analysis with an $MFa$1 gene probe. $C.\ neoformans$ ACT1 was used as a loading control.

**pak1 Mutants Fail to Polarize in Response to Pheromone**

In *S. cerevisiae*, both $\alpha$ and $a$ cells respond similarly to pheromone by arresting in G1, reorienting growth toward the pheromone gradient, and fusing with each other (Elion, 2000). In *C. neoformans*, $\alpha$ and $a$ cells respond differently when confronted with pheromone; $\alpha$ cells produce conjugation tubes, whereas $a$ cells become enlarged and refractile, and only occasionally produce conjugation tubes (Moore and Edman, 1993; Davidson et al., 2000b; Wang et al., 2000).

Previously, we demonstrated that $\alpha$ pak1 and $a$ pak1 mutants failed to respond morphologically when confronted with mating pheromone, but both are able to produce and secrete pheromone (Wang et al., 2002). To address the role of the PAK kinases in pheromone-induced polarized growth, the initial morphological changes that $\alpha$ and $a$ cells undergo before and during fusion were monitored using filipin, a fluorescent antibody that binds to 3-$\alpha$-hydroxysterols (Wachtler et al., 2003). Sterols have recently been shown to be enriched in areas of polarized growth and mating projections in *Schizosaccharomyces pombe* (Bagnat and Simons, 2002; Wachtler et al., 2003). Here, we examined the localization pattern of sterols in actively growing wild-type cells. In *C. neoformans*, filipin was localized to areas undergoing active polarized growth during vegetative growth: bud tips and septa (Figure 2A). Next, coculture experiments were performed with wild-type and pak1 mutant strains. Cells of opposite mating type were mixed and incubated for 4–24 h on V8 mating medium, harvested, and live cells were stained with filipin. By 4 h of incubation, morphological changes were discernible in wild-type cells (Figure 2B). Whereas budding continued in some cells, the majority of cells (>50%) formed protrusions that stained intensely with filipin (Figure 2B).
The protrusions elongated into tubes and grew until fusion occurred with a cell of the opposite mating type (Figure 2, C–E). Actin localized to the tips of the *C. neoformans* mating protrusions, as detected in samples that were fixed and stained with rhodamine-conjugated phalloidin (Figure 2G). In contrast to wild-type cells, only budding cells were observed in pak1 mutant cells at the 4-h time point (Figure 2F). Inspection of cocultured cells from later time points revealed that some fusion events did occur between pak1 mutant and wild-type C. neoformans cells. This indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.

**Characteristics of pak1 Mutants**

Although reduced compared with wild-type, bilateral pak1 crosses do produce filaments. The lack of recombinant progeny indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.

**Filaments Lacking Pak1 Are Mononucleate**

Although reduced compared with wild-type, bilateral pak1 crosses do produce filaments. The lack of recombinant progeny indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.

**Characteristics of pak1 Mutants**

Although reduced compared with wild-type, bilateral pak1 crosses do produce filaments. The lack of recombinant progeny indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.

**Filaments Lacking Pak1 Are Mononucleate**

Although reduced compared with wild-type, bilateral pak1 crosses do produce filaments. The lack of recombinant progeny indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.

**Filaments Lacking Pak1 Are Mononucleate**

Although reduced compared with wild-type, bilateral pak1 crosses do produce filaments. The lack of recombinant progeny indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.

**Filaments Lacking Pak1 Are Mononucleate**

Although reduced compared with wild-type, bilateral pak1 crosses do produce filaments. The lack of recombinant progeny indicates that the majority of filaments produced in bilateral pak1 crosses are either dysfunctional, mononucleate, or both. Indeed, microscopic examination of the bilateral pak1 filaments revealed several abnormalities. Wild-type and bilateral pak1 filaments were incubated for 7 d on microscope slides covered in V8 agar medium. The slides were fixed and stained with calcofluor and Sytox Green to visualize clamp cells and nuclei, respectively.
5A). Two nuclei were occasionally observed in the leading filament cell as a result of replication of the single nucleus. Mononucleate filaments containing no or unfused clamp cells also are observed in several other physiological settings including conjugation tubes, asexual filaments (haploid fruiting), and diploid filamentous growth. Previously, we demonstrated that pak1 mutant cells are unable to undergo haploid fruiting, excluding this as a source of the mononucleate filaments observed (Wang et al., 2002). It is also unlikely that the mononucleate filaments are diploids because diploid fusion products were not recovered in our assay (Figure 1A). A more likely explanation is that the mononucleate filaments arise from pak1 cells that form mating projections but fail to fuse with a partner. In this model, Pak1 also is required for the physical fusion between \( /H9251 \) and \( a \) cells.

In \( C. neoformans \), both dikaryotic and mononucleate (either haploid fruiting or diploid) hyphae produce basidia and basidiospores. Neither macroscopic nor microscopic examination of the bilateral pak1 filaments revealed the presence of normal basidia or basidiospores. Instead, the filaments terminated with deformed basidia that lacked basidiospores (Figures 5A and 8A) but did occasionally produce yeast-like cells or blastospores (Figure 5B). These observations suggest that Pak1 may play additional roles in basidia formation, meiosis, and basidiospore development.

**Ste20 Is Required to Maintain Polarity**

In contrast to the few filaments produced by bilateral pak1 crosses, bilateral ste20 mutant crosses produced abundant yet deformed filamentous structures that were rapidly overgrown by vegetative cells. Microscopic inspection revealed these filaments are much more branched than wild-type filaments (Figure 6A). Like other filamentous fungi, wild-type \( C. neoformans \) hyphae produce branches or secondary filaments that originate from subapical cells in the main

![Figure 3](image1.png)

**Figure 3.** \( C. neoformans \) conjugation tube formation. Wild-type \( \alpha \) and \( a \) cells were preincubated in Alexa Fluor 594 ConA and Alexa Fluor 488 ConA, respectively, to distinguish between \( \alpha \) and \( a \) cells during conjugation. Cells were mixed, incubated on V8 medium for 4 h, and counterstained with filipin to identify sites of conjugation between cells (arrows). Cells were observed with Texas Red (Alexa Fluor 594), fluorescein isothiocyanate (Alexa Fluor 488), and DAPI (filipin) filter sets and the images were merged. Bar, 5 \( \mu \)m.

![Figure 4](image2.png)

**Figure 4.** Fusion and spore production defect in pak1 mutants. (A) Wild-type and pak1 cocultures were incubated for 12 and 24 h on V8 medium. Cells were harvested, fixed, and stained with DAPI to visualize nuclei. DAPI and DIC images were merged to observe nuclear migration. At 12-h, nuclear migration (arrowhead) and mating filaments were detected in the wild-type cocultures. At 24 h, the initial mating filament cells had replicated to form dikaryotic filaments with clamp cells (asterisks), whereas in the pak1 coculture mating filaments were at the nuclear migration (arrowhead) stage of development. Bar, 10 \( \mu \)m. (B) Quantitative mating analysis. Bilateral crosses consisting of wild-type, pak1, and ste20 auxotrophic strains and prototrophic partners were prepared, incubated for 7 d on V8 medium, and serially diluted onto medium to select recombinants that were Ura\(^-\) and either Lys\(^+\) (ste20 cross) or Ade\(^+\) (pak1 cross). Top, progeny from the pak1 bilateral cross (left) and the ste20 bilateral cross (right). Bottom, progeny from the wild-type control cross for pak1 (left) and ste20 (right).
filament (Figure 6A). In addition, branches also can form from the clamp cells that link adjacent filament cells (Kwon-Chung, 1976). To determine the origin of the branches in the ste20 mutant crosses, we stained the septa and cell walls of wild-type and bilateral ste20 filaments with calcofluor (Figures 5A and 6B). Staining revealed that the unusual branches in the ste20 mutant filaments did not originate from the clamp cells but were caused by the growing tip splitting in half (Figure 6B). Clamp cells were produced but were limited to one of the two branches, and nuclear staining revealed that the filament cells contained unequal numbers of nuclei as a result. Whereas some filament cells contained two nuclei, others contained one, three, or zero nuclei (Figure 6B).

Although not previously described in C. neoformans filaments, tip splitting has been described in Aspergillus nidulans polarity-maintenance mutants as dichotomous branching (Momany, 2002). Actin localizes as a cap at the tip of A. nidulans filaments and dichotomous branching occurs when the actin cap is perturbed or displaced, creating an additional axis of polarity. To determine whether actin localization was similarly perturbed in the filaments produced by a ste20α × ste20α bilateral cross, actin in wild-type and ste20 mutant filaments was stained with rhodamine-conjugated phalloidin. In wild-type filaments actin was localized in cortical patches and filaments along the length of the filament cell and was concentrated at the growing tip and in clamp cells, both areas of active polarized growth (Figure 7A). Transient actin rings also were observed at sites of septation between filament cells and at clamp connections (Figure 7B and C). Comparison of wild-type and the ste20 mutant filaments revealed no overall difference in actin polarization (Figure 6C).

By microscopic examination, no normal basidia or basidiospores were identified in the bilateral ste20 cross. Instead, the basidia were deformed and either devoid of basidiospores or contained only a few (4 to 8) basidiospores (Figure 8A). Nuclear staining revealed that these basidiospores contain DNA, suggesting that they may be viable (unpublished data). To determine this, we measured the production of recombinant meiotic progeny in bilateral ste20 mutant crosses. Wild-type and ste20 bilateral mutant crosses were incubated for 7 d on mating medium. Each strain was genetically marked to allow for the selection of recombinant progeny. Whereas recombinant colonies were isolated from the bilateral ste20 cross, there was a fivefold reduction compared with a similarly marked auxotrophic wild-type cross (Figure 4B). Thus, bilateral ste20 crosses produce viable recombinant progeny although at a reduced rate. Together, these results indicate that Ste20 is required to maintain filament polarity and that loss of polarity leads to a reduction in the number of viable basidia and basidiospores.

C. neoformans PAK Kinases Are Not Interchangeable
In S. cerevisiae, Ste20 and Cla4 have overlapping functions and can substitute for each other in certain situations. For example, overexpression of Cla4 can suppress the mating and osmosensitivity defects associated with ste20 mutant strains (Sells et al., 1998; Raitt et al., 2000). A similar situation
has been described in fission yeast *Schizosaccharomyces pombe*; overexpression of the *Cla4* homolog Pak2/Shk2 suppresses the morphology and mating defects associated with deletion of the *Ste20* homolog Pak1/Shk1 (Sells et al., 1998; Yang et al., 1998).

To determine whether overexpression of *C. neoformans* Pak1 can suppress the mating and morphology defects of the *ste20* mutant or vice versa, we performed bilateral mating assays using */H9251* mutant strains overexpressing either the *STE20*/H9251 or the *PAK1* gene. Filamentation equivalent to wild type was restored in control crosses (*/pak1* × */pak1* and *ste20a* × *STE20a* × *ste20a*) (Figure 8A and Table 2). However, overexpression of *PAK1* in the *ste20a* mutant strain did not restore filamentation in a bilateral *ste20* cross, nor did *STE20* restore filamentation in a bilateral *pak1* cross when overexpressed in an */pak1* mutant strain (Figure 8A and Table 2). We also examined the ability of Pak1 to suppress the cytokinesis defects exhibited by *ste20* mutant strains. Whereas overexpression of *STE20a* complemented the cytokinesis defect of the *ste20a* mutant strain, overexpression

---

**Figure 6.** Ste20 is required to maintain filament tip polarity. (A) Wild-type and bilateral *ste20* crosses were incubated on V8 mating medium for 5 d at 24°C and photographed at 200× magnification with DIC optics. Wild-type filaments contain numerous lateral branches, whereas the *ste20* filaments branch at the tip. (B) Glass slide mounts of bilateral *ste20* mating filaments were costained with calcifluor and Sytox Green to visualize septa and nuclei. Each filament cell branches, leading to random segregation of nuclei. Whereas some filament cells maintain a dikaryotic state, other cells are mononucleate or anucleate. (C) Glass slide mounts of bilateral *ste20* mating filaments were costained with rhodamine-conjugated phalloidin and DAPI to visualize actin and nuclei (top) and visualized with DIC optics (bottom). Arrows denote clamp cells containing nuclei. Bar, 10 μm.

**Figure 7.** Localization of actin in *C. neoformans* mating filaments. Glass slide mounts of wild-type mating filaments were costained with rhodamine-conjugated phalloidin and DAPI to visualize actin and nuclear dynamics during filamentous growth. (A) Actin patches are polarized to the tip of the growing dikaryotic filament and to the tip of the clamp cell (arrowheads). (B) Actin rings form at the site of septation during nuclear division. Nuclear migration has already occurred, separating one pair of duplicated nuclei (arrows) on either side of the actin ring, whereas one nucleus of the second duplicated pair prepares to migrate into the clamp cell (asterisk). (C) Actin rings also form at the clamp cell to separate the nucleus in the clamp cell from the terminal filament. Also depicted here is nuclear migration into the basidium (asterisk).
plasmid exhibited a cytokinesis defect, resulting in long chains of connected and elongated cells. Introduction of the 
STE20 complemented the cytokinesis defect, resulting in a wild-type budding yeast cell morphology.

C. B. Nichols et al.

Figure 8. Ste20 and Pak1 are not interchangeable. (A) ste20α ura5 and α pak1 ura5 strains were transformed with plasmids expressing either STE20α or PAK1. Each strain also was transformed with a control plasmid. ste20 (left) and pak1 (right) bilateral crosses were prepared with each transformant and analyzed for filament production. Representative crosses were photographed at 100 and 400× magnification to visualize filamentation and basidiospore production. (B) ste20α transformants were assessed for growth at 37°C. Cells were grown overnight at 30 and 37°C and photographed at 1000× magnification with DIC optics. At 37°C ste20α mutants bearing vector alone or the PAK1 plasmid exhibited a cytokinesis defect, resulting in long chains of connected and elongated cells. Introduction of the STE20α gene complemented the cytokinesis defect, resulting in a wild-type budding yeast cell morphology.

of PAK1 did not (Figure 8B and Table 2). Together, these results indicate that the C. neoformans PAK kinases play functionally specialized roles in mating and morphology.

Ste20 Function Is Mating-Type Independent
In contrast to a ste20 bilateral cross, unilateral ste20α × α and ste20α × α crosses produce abundant filaments with no morphology defects, indicating that one copy of either gene is sufficient. To determine whether there was any mating-type specificity associated with this Ste20 function, we overexpressed STE20α in a ste20α mutant and STE20α in a ste20α mutant. In bilateral ste20 crosses, Ste20α restored normal filament morphology when overexpressed in either a ste20α or a ste20α mutant strain (Table 2). Similarly, Ste20α restored filament morphology when overexpressed in either a ste20α or a ste20α mutant strain (Table 2). In addition, overexpression of Ste20α complemented the cytokinesis defect of the ste20α mutant strain and vice versa. These data indicate that, at least under these experimental conditions, Ste20α and Ste20α perform identical roles during vegetative growth and are redundant during mating. Thus, the functions of Ste20 seem independent of mating type even though the STE20 genes are located in the MAT locus and function in mating.

Ste20 Function Is Serotype Independent
Serotype is correlated with the ability of C. neoformans to cause disease. In the clinic, cryptococcosis caused by serotype A strains predominates over cases involving serotype B, C, or D strains. Recent analyses have shown that certain gene products also have a serotype-dependent impact on C. neoformans virulence. For example, the Ste12 transcription factor is important for virulence in a serotype D strain but not in a serotype A strain (Yue et al., 1999). Conversely, Ste20 is required for virulence in serotype A but not in serotype D. In addition, whereas both serotype A and D ste20 mutants exhibit defects in cytokinesis and mating filament polarity, only serotype A ste20α mutants are temperature sensitive for growth (Wang et al., 2002).

To address the role of Ste20 serotype specificity, we generated a serotype A ste20α mutant by disrupting STE20α in the serotype A MATa strain KN99α (Nielsen et al., 2003). Our previous analysis of Ste20 in serotype A was limited to ste20α due to the unavailability of a serotype A MATa strain. The serotype A ste20α mutant behaved exactly as the serotype A ste20α mutant with respect to cytokinesis and high-temperature growth (unpublished data) (Wang et al., 2002). In addition, the few filaments produced in a bilateral serotype A ste20 cross exhibited defects similar to the filaments produced in a bilateral serotype D ste20 cross (Figure 9). Next, we overexpressed the serotype A STE20α gene in the serotype D ste20α mutant and the serotype D STE20α gene in the serotype A ste20α mutant. When overexpressed, serotype D Ste20α complemented the cytokinesis defect, high-temperature growth defect, and mating filamentation defect of the serotype A ste20α mutant (Table 2). Similarly, serotype A Ste20α overexpression complemented the cytokinesis and mating filamentation defects of the serotype D ste20α mutant (Table 2). Identical results were obtained using serotype A and D STE20α genes (Table 2). Additionally, overexpression of serotype A STE20α complemented the serotype D ste20α mutant (summarized in Table 2). In summary,
Ste20 function is independent of both serotype and mating type.

**Pak1 Function Is Serotype Dependent**

We also examined whether Pak1 could function between the divergent serotypes. To this end, we first attempted to generate a serotype A α pak1 mutant by crossing the serotype A α pak1 mutant with the congenic mating partner KN99a. However, in contrast to serotype D pak1 unilateral crosses, very few filaments were produced (Figure 9). In an alternative approach, we generated a serotype A α pak1 mutant strain by targeted gene disruption. Similar to a serotype A α pak1 unilateral cross, few filaments were produced when the serotype A α pak1 mutant was crossed to a wild-type α strain (Figure 9). No filamentation was observed in the serotype A bilateral pak1 cross (Figure 9). Interestingly, no filamentation was observed in crosses between ste20α and a pak1 mutants or between α pak1 and ste20α mutants (Figure 9).

Overexpression of serotype A PAK1 in either α or a A pak1 mutants restored filamentation in unilateral crosses (Table 2). However, overexpression of the serotype A PAK1 gene in the serotype D pak1 mutant did not restore filamentation to a serotype D pak1 bilateral cross (Table 2). Similar results were obtained with serotype D PAK1; overexpression of serotype D PAK1 complemented the serotype D pak1 mutant but not the serotype A pak1 mutant (Table 2). Thus, in contrast to Ste20, Pak1 function is serotype specific.

**DISCUSSION**

In this study, we find that Ste20 and Pak1 have polarity specific roles in *C. neoformans* mating. Our studies have revealed that the *C. neoformans* PAK kinases have both conserved and unique roles. Whereas Pak1 is required for fusion, a role similar to that performed by *S. cerevisiae* Ste20, *C. neoformans* Ste20 performs a novel role in maintaining polarity in the filament. In addition, the morphogenic roles of each kinase seem to be nonoverlapping and temporally distinct from one another.

**A PAK Kinase for Cell Fusion**

We find that in response to pheromone, *C. neoformans* α and a cells generate mating projections and conjugation tubes and that Pak1 is required for this response. Mating projections and conjugation tubes occur by 4 h, the same time that pheromone transcript levels increase (Shen et al., 2002; Chang et al., 2003). In the basidiomycete *Ustilago maydis*, pheromone gene induction and conjugation tube formation also coincide and are regulated by a pheromone-responsive MAP kinase cascade (Muller et al., 2003). The *C. neoformans* pheromone-responsive MAP kinase pathway, composed of Ste11, Ste7, and Cpk1, is required for pheromone gene induction and cell fusion (Davidson et al., 2000b, 2003). In *S. cerevisiae* the PAK kinase Ste20 activates the pheromone-responsive MAP kinase cascade which in turn induces the processes required for mating, including shmoo formation and cell fusion (Elion, 2000). In *U. maydis*, the Ste20 homolog Sm1 has been recently characterized and smu1 mutant strains exhibit mating defects and decreased pheromone expression (Smith et al., 2004). *C. neoformans* Pak1 is most closely related to *S. cerevisiae* Ste20. However, our data show that pheromone induction is not compromised in pak1 mutants, indicating that the fusion defect is either independent of MAP kinase cascade activation or that Pak1 functions downstream of the MAP kinase cascade, or plays a redundant role with other elements that activate MAP kinase signaling.

Why then do pak1 mutants fail to fuse? In *S. cerevisiae*, Ste20 has additional roles in bud and shmoo morphogenesis. Polarization at the bud tip is maintained by a complex of proteins called the polarisome. This complex consists of Spa1, Pea2, Bud6, and Bni1. Ste20 is thought to activate the polarisome via phosphorylation of Bni1, a formin homology protein and a Cdc42 effector (Goehring et al., 2003). Polarisome components, including Bni1, are required for shmoo formation and cell fusion (Gehrung and Snyder, 1990; Che-nevert et al., 1994; Dorer et al., 1997; Evangelista et al., 1997; Gammie et al., 1998). Our findings are consistent with a model that *C. neoformans* Pak1 induces mating projection formation by activating polarisome components.

**Origin of pak1 Filaments**

The filaments produced in bilateral pak1 crosses seem similar to the conjugation tubes produced by haploid or diploid cells (unpublished data). Typically, fusion occurs only between α and a cells, leading to the production of a dikaryotic filament, basidia, and basidiospores. However, filamentation is thermally repressed and fusion products incubated at 37°C undergo nuclear fusion and become diploid. When grown at 24°C these diploids reenter the sexual cycle and form monokaryotic filaments, basidia, and basidiospores.

---

**Table 2. Overexpression analysis**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene</th>
<th>Restore filamentation? (bilateral cross)*</th>
<th>Suppress cytokinesis defect?</th>
</tr>
</thead>
<tbody>
<tr>
<td>D ste20a</td>
<td>Vector</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>D STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>D STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>D PAK1</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>D ste20a</td>
<td>Vector</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>D STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>D STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>D PAK1</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>A ste20a</td>
<td>Vector</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>D STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>D STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A STE20a</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>D PAK1</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>D α pak1</td>
<td>Vector</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>D STE20a</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>D STE20a</td>
<td>No</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>D PAK1</td>
<td>Yes</td>
<td>Yes</td>
<td>NA</td>
</tr>
<tr>
<td>A α pak1</td>
<td>Vector</td>
<td>No</td>
<td>NA</td>
</tr>
<tr>
<td>A STE20a</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>A STE20a</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>D PAK1</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>A PAK1</td>
<td>Yes</td>
<td>Yes</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not applicable.

* With the exception of serotype A α pak1, which was crossed to wild-type a.
Also, haploid cells can reproduce asexually (haploid fruiting) under nutrient-limiting conditions by forming monokaryotic filaments. Common to all three processes is the ability of haploid cells to form conjugation tubes during nutrient-limiting conditions, suggesting that this may be a default mechanism. A possible explanation for this model is given by the appearance of mononucleate filaments in 
akl mutants. The 
akl mutant cells are unable to respond to pheromone and identify a mating partner, and as a consequence form long conjugation tubes that mature into mononucleate filaments. Our data support this interpretation, but other possibilities also can be considered. For example, 
akl filaments may originate from cell fusion but are unable to maintain both nuclei, either as a diploid or as a dikaryon, thus resulting in mononucleate filaments.

In contrast to mononucleate filaments produced by haploid fruiting or diploid filaments, 
akl mutant filaments do not produce normal basidia or basidiospores; either no basidiospores are formed on the basidia or budding cells are produced instead. Little is known about 
A. neoformans basidia and basidiospore development. In other filamentous fungi, asexual spores form from a specialized structure called a conidiophore. Polarity mutants defective in spore and conidiophore formation have been identified and include a Cla4 homolog from the rice blast fungus 
M. grisea, a septin homolog from 
A. nidulans, a Rac homolog from the human pathogen 
P. marneffei, and a Ras homolog from the alfalfa pathogen 
C. trifolii (Truesdell et al., 1999; Westfall and Momany, 2002; Boyce et al., 2003; Li et al., 2004). Interestingly, both Rac and Ras are signaling components known to function in mediating PAK kinase activation (Daniels and Bokoch, 1999; Fruyne and Bretscher, 2000).

A PAK Kinase for Polarity Maintenance

The tip-branching defect exhibited by 
ste20 mutants during filamentous growth implicates Ste20 in hyphal tip maintenance. PAK kinase homologues from filamentous fungi have been characterized and play roles in filament establishment and hyphal maturation; however, none has been implicated in hyphal tip maintenance (Leberer et al., 1996; Leberer et al., 1997; Ayad-Durieux et al., 2000; Szabo, 2001; Smith et al., 2004). Proteins involved in tip maintenance have been identified in 
P. marneffei, 
A. nidulans, and 
N. crassa. Not surprisingly, some of the genes corresponding to the tip-branching mutants encode cytoskeletal-related proteins, including the Rac homolog CIA (P. marneffei), actin (N. crassa), and a Bni1 homolog SepA (A. nidulans) (Sharpless and Harris, 2002; Seiler and Plamann, 2003; Virag and Griffiths, 2004). Because Bni1 homologues are involved in both tip splitting and shmoo formation, albeit in different organisms, it raises the intriguing possibility that a 
A. neoformans formin protein may be a common target for both Pak1 and Ste20 during morphogenesis. Although no formin proteins have been identified in 
A. neoformans, our BLAST searches of 
A. neoformans sequence databases have revealed that formin domain sequences are present.

In 
A. neoformans, 
A. nidulans, and 
N. crassa filaments contain multiple nuclei per
filament cell (Coppin et al., 1997). Thus, in C. neoformans, tip-splitting has a unique and detrimental impact on nuclear segregation during filamentous growth that affects subsequent basidiospore formation and viability, and Ste20 has a pivotal role in this process.

**Dosage Dependency and Serotype Specificity of PAK Kinases**

During our analysis we found that a single copy of PAK1 was sufficient for function, and this also held true for STE20, where a single copy of either MAT variant would suffice for mating to occur. This redundancy of STE20 function, specifically the lack of discrimination between the mating-type-specific alleles of the gene, was highly unexpected. It is thought that the products of the MAT locus contribute to α- and a-specific signaling pathways controlling reproduction and virulence (Hull and Heitman, 2002; Fraser and Heitman, 2003). The finding that the allelic components of MAT may be interchangeable between mating types without alteration of phenotype implies that despite the large cohort of genes present in this structure, the different MAT alleles may be functionally equivalent with the exception of those components considered the most ancient; the homeodomain protein Sxi1a (Hull et al., 2002) and the pheromones/pheromone receptors (Chang et al., 2003).

Also puzzling is the serotype specificity of Pak1 compared with Ste20. Serotype A and D Ste20 and Pak1 are 94 and 90% identical at the amino acid level, respectively. However, serotype A Pak1 contains several short insertions not shared with serotype D Pak1. Population genetic studies show that C. neoformans A and D serotypes diverged ~18 million years; thus it is likely that the serotype A and D Pak1 homologues have acquired unique functions during mating (Fan et al., 1994; Xu et al., 2000). However, the two different STE20 alleles of each serotype have clearly been diverging for a much longer time due to their presence in the nonrecombinating MAT locus. In contrast to the divergence between the serotype alleles of each gene, at the nucleotide level the coding sequences of the MATa and MATα alleles of STE20 are only 66% identical. The divergence of function of the PAK1 genes between serotype A and D is therefore all the more exceptional; the function has changed dramatically over the past ~20 million years, where the STE20 genes (which are much more divergent) each seem to encode identical functions.

**Cell Polarity and Virulence**

One goal of our study was to gain insight into the connections between mating and virulence in C. neoformans. In U. maydis, the hyphal form is pathogenic and the sexual cycle occurs in the host thus there is a clear connection between mating and virulence. For example, mutations in components of the pheromone responsive MAP kinase cascade or the Ste20 homolog Smu1 exhibit reduced virulence (May- orga and Gold, 1999; Muller et al., 1999, 2003; Smith et al., 2004). In contrast, there is no evidence that mating or filamentation occurs during cryptococcal infections. However, there is a link between mating and virulence and many of the gene products found to impact virulence also are required for mating. In the specific case of Ste20, the link between mating and virulence may result from the temperature-sensitive growth defect exhibited by serotype A ste20 mutants. Serotype D ste20 mutants exhibit a cytokinesis defect, but they are not temperature sensitive for growth and exhibit no virulence defect (Wang et al., 2002).

In contrast, pak1 mutants are not temperature sensitive nor do they exhibit defects in any known virulence factor. Why then are pak1 mutants avirulent in animal models of cryptococcosis? Within the host, C. neoformans cells encounter a combination of stresses, including high temperature, limiting nutrients, and high osmolarity. Our results indicate that Pak1 plays a positive role establishing polarity during mating. During stress, Pak1 also may be required for bud morphogenesis or some other essential function. However, pak1 mutant strains do not seem to be stress sensitive and are slightly more resistant to salt stress than wild-type strains incubated at 37°C (unpublished data). In conclusion, our studies reveal an intriguing association between cell polarity mechanisms and virulence in the human pathogen C. neoformans. Further characterization of Pak1 and Ste20 and the identification of their downstream targets will be necessary to elucidate the connections between mating, cell polarity, and virulence.

**ACKNOWLEDGMENTS**

We thank Andrew Alspaugh, Toshiaki Harashima, and Peter Krauss for critical reading of the manuscript and Marie-Josee Boily for technical assistance. These studies were supported in part by R01 grants AI39115 and AI50113 from the National Institute of Allergy and Infectious Diseases and by P01 grant AI44975 from the National Institute of Allergy and Infectious Diseases to the Duke University Mycology Research Unit. J.H. is an associate investigator of the Howard Hughes Medical Institute and a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology.

**REFERENCES**


Candida albicans


