

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

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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 a 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 morphogenic 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; Dobbelaere *et al.*, 2003; Schmidt *et al.*, 2003; Versele and Thorner, 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 \mathbf{a} 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 \mathbf{a} mating-type, and in serotype D α cells are more virulent than \mathbf{a} 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

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Table 1. Strains and plasmids

Strain	Genotype	Source/Reference
Serotype A		
CSB1	<i>MAT</i> α <i>pak1::URA5 ura5</i>	Wang <i>et al.</i> , 2002
CSB51	<i>MAT</i> α <i>pak1::ura5 ura5</i>	Wang <i>et al.</i> , 2002
F99	<i>MAT</i> α <i>ura5</i>	Wang <i>et al.</i> , 2001
H99	<i>MAT</i> α	Perfect <i>et al.</i> , 1980
JF99	<i>MAT</i> a <i>ura5</i>	This study
JF219	<i>MAT</i> a <i>ste20a::NAT ura5</i>	This study
JF265	<i>MAT</i> a <i>pak1::NAT</i>	This study
JF267	<i>MAT</i> a <i>pak1::NAT ura5</i>	This study
KN99a	<i>MAT</i> a	Nielsen <i>et al.</i> , 2003
PPW91	<i>MAT</i> α <i>ste20α::URA5 ura5</i>	Wang <i>et al.</i> , 2002
PPW96	<i>MAT</i> α <i>ste20α::ura5 ura5</i>	Wang <i>et al.</i> , 2002
Serotype D		
CSB5	<i>MAT</i> a <i>ste20a::ADE2 ade2</i>	Wang <i>et al.</i> , 2002
CSB8	<i>MAT</i> α <i>pak1::URA5 ura5 ade2</i>	Wang <i>et al.</i> , 2002
CSB9	<i>MAT</i> α <i>pak1::URA5 ura5</i>	Wang <i>et al.</i> , 2002
CSB10	<i>MAT</i> a <i>pak1::URA5 ura5</i>	Wang <i>et al.</i> , 2002
CSB11	<i>MAT</i> α <i>ste20α::URA5 ura5 ade2</i>	Wang <i>et al.</i> , 2002
KLB156-1	<i>MAT</i> a <i>ste20a::ADE2 ura5 ade2</i>	Wang <i>et al.</i> , 2002
CSB15	<i>MAT</i> α <i>ste20α::URA5 ura5</i>	Wang <i>et al.</i> , 2002
CSB21	<i>MAT</i> a <i>ste20a::ADE2 ura5 ade2 lys1</i>	Wang <i>et al.</i> , 2002
CSB23	<i>MAT</i> α <i>pak1::ura5 ura5 ade2</i>	Wang <i>et al.</i> , 2002
CSB25	<i>MAT</i> α <i>pak1::ura5 ura5</i>	This study
CSB27	<i>MAT</i> a <i>pak1::URA5 ura5 lys1</i>	This study
CSB48	<i>MAT</i> α <i>ste20α::ura5 ura5</i>	Wang <i>et al.</i> , 2002
RDC5	<i>MAT</i> α <i>cpk1::ADE2 ade2</i>	R. Davidson
JEC20	<i>MAT</i> a	J. Edman
JEC21	<i>MAT</i> α	J. Edman
JEC31	<i>MAT</i> a <i>lys2</i>	J. Edman
JEC34	<i>MAT</i> a <i>ura5</i>	J. Edman
JEC43	<i>MAT</i> α <i>ura5</i>	J. Edman
JEC50	<i>MAT</i> α <i>ade2</i>	J. Edman
JEC53	<i>MAT</i> a <i>ura5 lys1</i>	J. Edman
JEC155	<i>MAT</i> α <i>ura5 ade2</i>	J. Edman
Plasmids		
pRDC83	<i>C. neoformans</i> vector	R. Davidson
pCBN12	serotype A <i>P</i> _{GPD1} - <i>STE20α</i>	This study
pCBN19	serotype D <i>P</i> _{GPD1} - <i>STE20α</i>	This study
pCBN20	serotype D <i>P</i> _{GPD1} - <i>STE20a</i>	This study
pCBN23	serotype D <i>P</i> _{GPD1} - <i>PAK1</i>	This study
pCBN34	serotype A <i>P</i> _{GPD1} - <i>PAK1</i>	This study
pCBN47	serotype A <i>P</i> _{GPD1} - <i>STE20a</i>	This study

(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 Ste20 α and Ste20a are located in the *MAT* locus, *ste20* mutants are unable to mate, and the virulence of a clinical isolate deleted for *STE20 α* 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). Biolistic transformations were performed as described previously (Toffaletti *et al.*, 1993; Davidson *et al.*, 2000a).

Overexpression Analysis

The serotype A *STE20 α* , *STE20a*, and *PAK1* and serotype D *STE20 α* , *STE20a*, and *PAK1* open reading frames were amplified by polymerase chain reaction (PCR) with *Bam*HI sites at each end. The PCR products were subcloned into pCRII-TOPO and sequenced. After digestion with *Bam*HI, each open reading frame was subcloned into *Bam*HI digested *C. neoformans* vector pRDC83 generating plasmids pCBN12 (serotype A *STE20 α*), pCBN47 (serotype A *STE20a*), pCBN34 (serotype A *PAK1*), pCBN19 (serotype D *STE20 α*), pCBN20 (serotype D *STE20a*), and pCBN23 (serotype D *PAK1*). Ura⁻ strains PPW96, JF219, CSB51, CSB48, KLB156-1, and CSB25 were transformed with each plasmid, including pRDC83, 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 *ste20* transformants).

Northern Blot Analysis

Equal numbers (2×10^7 cells) of JEC21, α *cpk1* (RDC5), *ste20a* (CSB5), and α *pak1* (CSB9) cells were grown on separate V8 plates alone or in coculture with equal numbers (2×10^7 cells) of JEC20, *ste20a* (CSB5), or a *pak1* (CSB10) cells for 24 h. Cells were harvested, lyophilized 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 μ g of total RNA per sample. The *Mf α* , *Mf α* , and *ACT1* probes were amplified by PCR, radiolabeled with [α -³²P]dCTP (Amersham Biosciences, Piscataway, NJ) by using a Rediprime II kit (Amersham Biosciences), and used in hybridization reactions as described previously (Church and Gilbert, 1984).

Fusion, Recombinant Spore, and Conjugation Assays

Fusion assays were performed as described previously (Alspaugh *et al.*, 2000) with the following modifications. Bilateral crosses consisting of the wild-type (JEC50 \times JEC34 and JEC50 \times JEC31), *ste20* (CSB11 \times KLB156-1), and *pak1* (CSB8 \times CSB27) mutant strains containing equal numbers of cells were pipetted onto V8 agar medium. Each mating partner contained a complementing auxotrophic mutation (*lys2* and *ura5* or *ade2* and *ura5*) to allow selection of prototrophic fusion products. After 48 h, the mating mixes were excised, resuspended in 1 ml of H₂O, and plated in serial dilutions onto minimal medium. Colonies were counted after 3 d of incubation.

Recombinant spore production was assessed in a quantitative mating assay as described previously (Alspaugh *et al.*, 2000) with the following modifications. Bilateral crosses consisting of the wild-type (JEC21 \times JEC53 and JEC155 \times JEC53), *ste20* (CSB15 \times CSB21), and *pak1* (CSB23 \times CSB10) mutant strains containing equal numbers of cells were pipetted onto V8 agar medium. After 7 d, the mating were excised, resuspended in 1 ml of H₂O, and plated in serial dilutions onto 5-fluoroorotic acid agar medium lacking lysine or adenine. Colonies were counted after 3 d of incubation.

The number of conjugation products present in wild-type and *pak1* cocultures was determined by mixing together equal numbers of wild-type (JEC21 and JEC30) and *pak1* (CSB9 and CSB10) mutant cells on V8 agar medium. After 24 h, the cells were scraped off, resuspended in H₂O, and examined microscopically for the presence of fused cells. Fifty cells were counted for each sample and a total of 300 cells were analyzed from each coculture.

Microscopy

Brightfield, differential interference microscopy (DIC) and fluorescent images were captured with a Zeiss Axioskop 2 Plus fluorescent microscope equipped with an AxioCam MRM digital camera. Additional brightfield images were captured with a Nikon Eclipse E400 microscope equipped with a Nikon CoolPix 990 digital camera.

For sterol staining, cells were harvested from V8 agar medium and resuspended in phosphate-buffered saline (PBS). Cells were incubated with 5 μ g/ml filipin III (Cayman Chemical, Ann Arbor, MI) for 5 min, rinsed in PBS, and observed immediately. For concanavalin A (ConA)-filipin staining, overnight cultures were diluted and incubated for 1 h in either 50 μ g/ml ConA Alexa Fluor 594 (α cells) or ConA Alexa Fluor 488 (a cells) (Molecular Probes, Eugene, OR). Cells were rinsed three times in PBS to remove unbound lectin, mixed, and pipetted onto V8 agar medium. After 4 h cells were harvested, counterstained with filipin, and visualized with the appropriate filters.

For filament staining, slide mounts were prepared as described previously (Fraser *et al.*, 2003) with the following modifications. After incubation, each slide mating was transferred to a fresh petri dish, washed three times with 10 ml of PBS, fixed in 10 ml 10% formaldehyde for 20 min, washed three times with PBS, permeabilized with 1% Triton X-100 in PBS for 10 min, and washed three times with PBS before staining. To visualize septa and DNA, the slide matings were incubated in a 10-ml preparation of calcofluor (fluorescent brightener 28 F-3397; Sigma-Aldrich, St. Louis, MO) at 40 μ g/ml and a 5000 \times dilution of a 5 mM solution of Sytox Green (Molecular Probes) in PBS for 20 min. Agar sections containing filaments were mounted on fresh microscope slides with 5 μ l of antifade (1 mg/ml *p*-phenylenediamine in 50% glycerol). To visualize actin and DNA, similar mating preparations were fixed and incubated in a 10-ml preparation of rhodamine-conjugated phalloidin (P-195; Sigma-Aldrich) at 10 μ g/ml. Agar sections containing filaments were mounted on fresh microscope slides with 5 μ l of antifade mix containing 8 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) (D-21490; Molecular Probes).

Gene Disruption

The *ura5*-serotype A strain JF99 was isolated as a 5-fluoroorotic acid spontaneous mutants of KN99a. The construct for precise replacement of the serotype A *STE20a* open reading frame with the nourseothricin resistance cassette was generated as described previously (Davidson *et al.*, 2002; Fraser *et al.*, 2003) using primers to amplify *STE20a* 5' (JOHE9252 AACGTAAAGCCATGCCAACCA; JOHE9253 AGCTCACATCCTCGACGCGACGATGAAAGCCCTGGTCTAGGT) and *STE20a* 3' (JOHE9254 CCGTGTAATACAGATAAACCCCGCTTATGATGTTATTGTCCG; JOHE9255 CAGTGCCTGGAATAT-

CTTGT), with the overlap construct used to transform JF99 to create JF219 (*MATa ste20a::NAT ura5*). The construct for precise replacement of the serotype A *PAK1* open reading frame with the nourseothricin resistance cassette was generated using primers to amplify *PAK1* 5' (JOHE10447 AGGCATCCATCACCATTCTT; JOHE10448 AGCTCACATCCTCGACGCCCTTTTCTATCGCCGGC-GTCGC) and *PAK1* 3' (JOHE10449 CCGTGTAATACAGATAAACCCATTGGGTAGGAACGAAAACGTGGT; 110450 GCAAACCTGGTCAATCGCATCT), with the overlap construct used to transform KN99a to create JF265 (*MATa pak1::NAT*) 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* \times a *pak1* and *ste20a* \times *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 epistasis 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 obtained 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 *Mf α* 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 *Mf α* pheromone expression in α *pak1* cells during coculture with a *pak1* cells. *Mf α* 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 a *pak1* cells cocultured with a *pak1* cells. Consistent with previous observations, *Mf α* expression was dramatically induced by 24 h when α cells were cocultured with a cells (Shen *et al.*, 2002) (Figure 1B). *Mf α* induction was absent in α *cpk1* cells cocultured with wild-type a cells (Davidson *et al.*, 2003), but

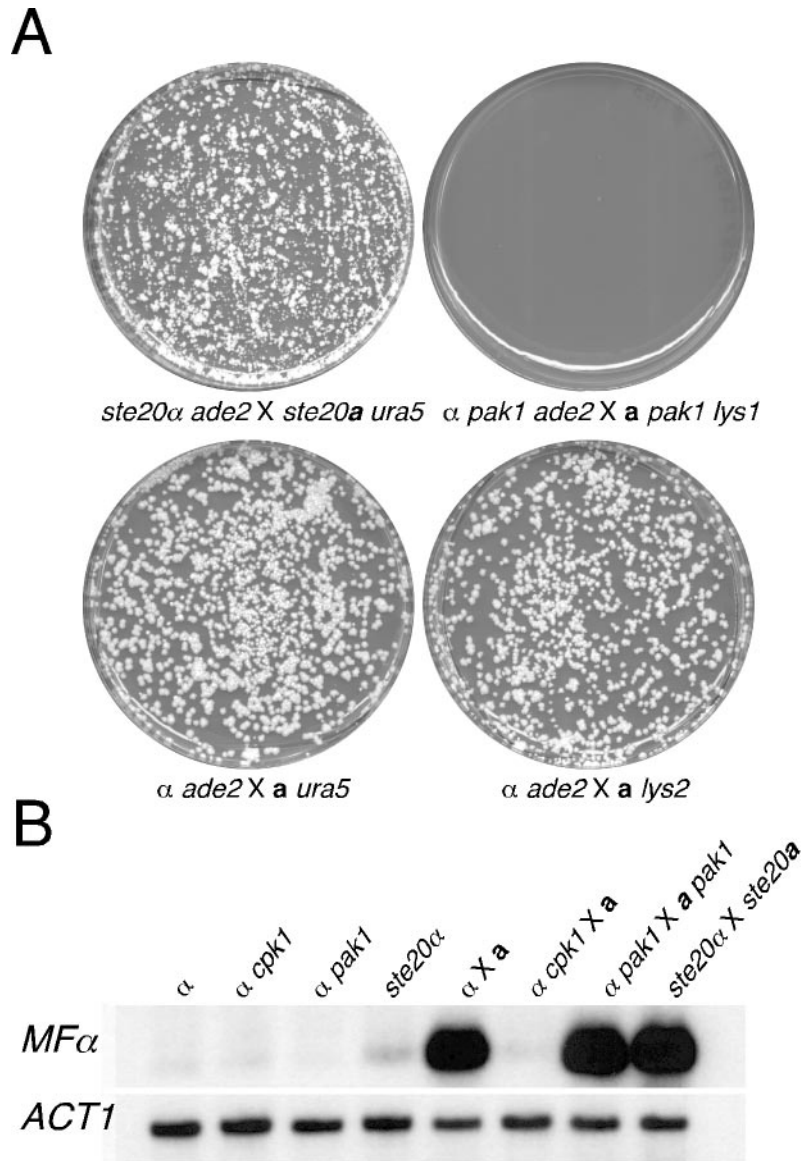


Figure 1. Pak1 is required for fusion during mating. (A) Fusion assay of bilateral *ste20* and *pak1* crosses. Top, media used to select prototrophic fusion products from *ste20α* × *ste20a* (top left) and *α pak1* × *a pak1* (top right) crosses. Bottom, wild-type control crosses corresponding to the marker matched bilateral *ste20* cross (bottom left) and the bilateral *pak1* cross (bottom right). (B) *MFα* pheromone gene expression in wild-type *α* strain and *α cpk1*, *α pak1*, and *ste20α* strains alone, *α* and *α cpk1* strains cocultured with wild-type *a*, *α pak1* cocultured with *a pak1*, and *ste20α* cocultured with *ste20a*. Cells were incubated on V8 medium for 24 h at room temperature, RNA was prepared, and *MFα* gene expression was assessed by Northern blot analysis with an *MFα1* gene probe. *C. neoformans* *ACT1* was used as a loading control.

it was induced when *α pak1* cells were cocultured with *a pak1* cells (Figure 1B). Wild-type levels of *MFα* also were observed in *ste20α* cells cocultured with *ste20a* cells (Figure 1B). In addition, *MFα* pheromone induction occurred normally in the bilateral *pak1* and *ste20* crosses (unpublished data). These results indicate that neither Pak1 nor Ste20 is required for activation of pheromone gene transcription during mating.

pak1 Mutants Fail to Polarize in Response to Pheromone

In *S. cerevisiae*, both *α* 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*, *α* and *a* cells respond differently when confronted with pheromone; *α* 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 *α 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 *α* and *a* cells undergo before and during fusion were monitored using filipin, a fluorescent antibiotic that binds to 3- β -hydroxysterols (Wachtler *et al.*, 2003). Sterols have recently been shown to be enriched in areas of polarized growth and mating projections in *Schizosaccharomyces pombe* and in mating projections in *S. cerevisiae* (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 discernable in wild-type cells (Figure 2, B–E). Whereas budding continued in some cells, the majority of cells (>50%) formed protrusions that stained intensely with filipin (Figure 2B).

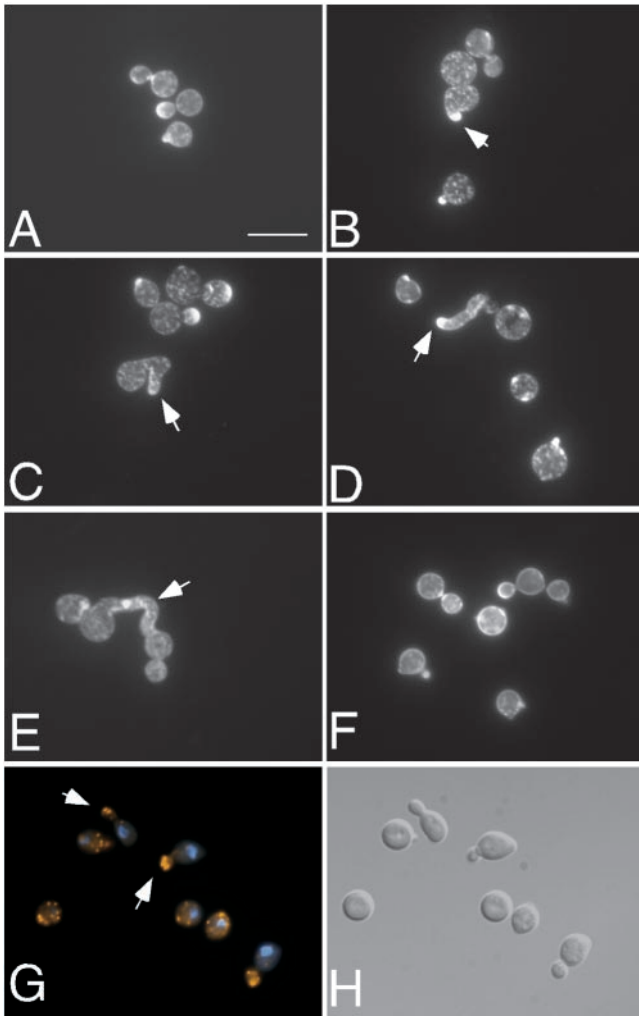


Figure 2. Morphogenic events precede fusion during mating in *C. neoformans*. Sterol distribution was analyzed in wild-type *C. neoformans* cells grown in YPD (A), or wild-type α cells cocultured with wild-type a cells (B–E), and α *pak1* cells cocultured with *a* *pak1* cells (F) on V8 medium for 4 h. Live cells were harvested, incubated for 1 min with filipin and observed immediately. By 4 h, a range of morphogenic changes were observed in the wild-type coculture, including shmoo-like projections (B), conjugation tubes (C and D), and conjugation (E). (G) *C. neoformans* shmoo-like projections contain polarized actin. Cells from the wild-type coculture were fixed and stained with rhodamine-conjugated phalloidin and DAPI to visualize F-actin and nuclei. (H) DIC image corresponding to G. Arrows denote shmoo-like projections and conjugation tubes. Bar, 10 μ m.

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 α *pak1* and a *pak1* mutant cells, but these were less frequent and were delayed compared with those observed in cocultured wild-type cells.

To distinguish between mating types during fusion, wild-type α and a cells were differentially stained with fluorescent conjugates of ConA, a lectin that binds mannose and glucose

residues. In *C. neoformans*, ConA localizes to septa and bud scars (Figure 3; Foster *et al.*, 2004). Overnight cultures of α and a cells were incubated with Alexa 594 ConA (red) and Alexa Fluor 488 ConA (green) conjugates, respectively, for 1 h. Cells were washed to remove unbound lectin, and equal numbers of each mating partner were mixed and plated onto mating medium. After 4 h, the cells were harvested and counterstained with filipin. Fusion products consisting of ConA-labeled α and a cells were identified demonstrating that both mating types can produce conjugation tubes to fuse with a mating partner (Figure 3).

To monitor nuclear migration with respect to cell fusion and postfusion events, we fixed and stained cells from 4-, 12-, and 24-h time points with DAPI. Staining revealed that by 12 h, α and a cells from the wild-type coculture had fused and formed mating filaments into which the α and a nuclei both migrated (Figure 4A); by 24 h, dikaryotic filaments were evident (Figure 4A). In contrast, mating filaments were not detected in the α *pak1* \times a *pak1* coculture until 24 h (Figure 4A). To quantify the defect in *pak1* conjugation, we counted the number of fusion products present in the wild-type and α *pak1* \times a *pak1* mutant cocultures incubated for 24 h. On average, the wild-type coculture samples contained 3.8 ± 0.83 conjugation products per 50 cells, whereas the *pak1* mutant coculture samples contained only 0.3 ± 0.26 conjugation products per 50 cells. Together, these results demonstrate that cells lacking Pak1 fail to polarize toward a mating partner in response to pheromone and, likely as a consequence, exhibit a relative fusion defect compared with wild-type cells.

pak1 Mutants Produce Few Meiotic Progeny

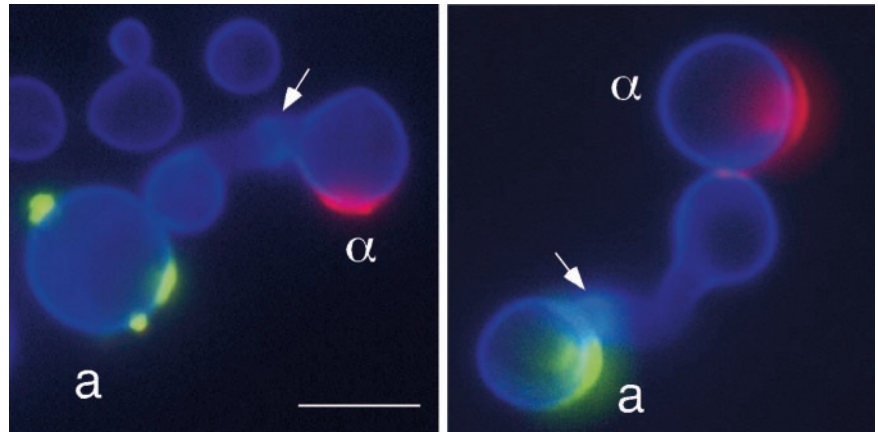
Our results suggest that despite a significant fusion defect, conjugation and filamentation might not be abolished in bilateral *pak1* mutant crosses. To determine whether the *pak1* fusion products produced functional mating filaments and completed the sexual cycle, we measured the number of viable recombinant meiotic progeny produced from wild-type and *pak1* crosses. Cells from wild-type and bilateral *pak1* crosses were genetically marked to permit selection of recombinant haploid progeny, mixed, incubated for 7 d on mating medium, and serially plated onto selective medium. A 200-fold reduction in the production of recombinant progeny was observed in the bilateral *pak1* cross compared with the wild-type control cross (Figure 4B). Thus, α *pak1* and a *pak1* mutant cells exhibit a profound fusion defect and only rarely complete the sexual cycle to produce recombinant haploid meiotic progeny.

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* crosses 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.

Characteristic features of *C. neoformans* dikaryotic hypha include two paired nuclei per cell, nuclear migration into clamp cells, and fused clamp cell connections between filament cells (Figure 5A). However, DNA and cell wall staining of *pak1* filaments revealed that the filament cells were mononucleate for the most part and contained many unfused clamp-cell-like or blastospore-like structures (Figure

Figure 3. *C. neoformans* conjugation tube formation. Wild-type α and a cells were preincubated in Alexa Fluor 594 ConA and Alexa Fluor 488 ConA, respectively, to distinguish between α 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 μ m.



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 unused 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 α and a cells.

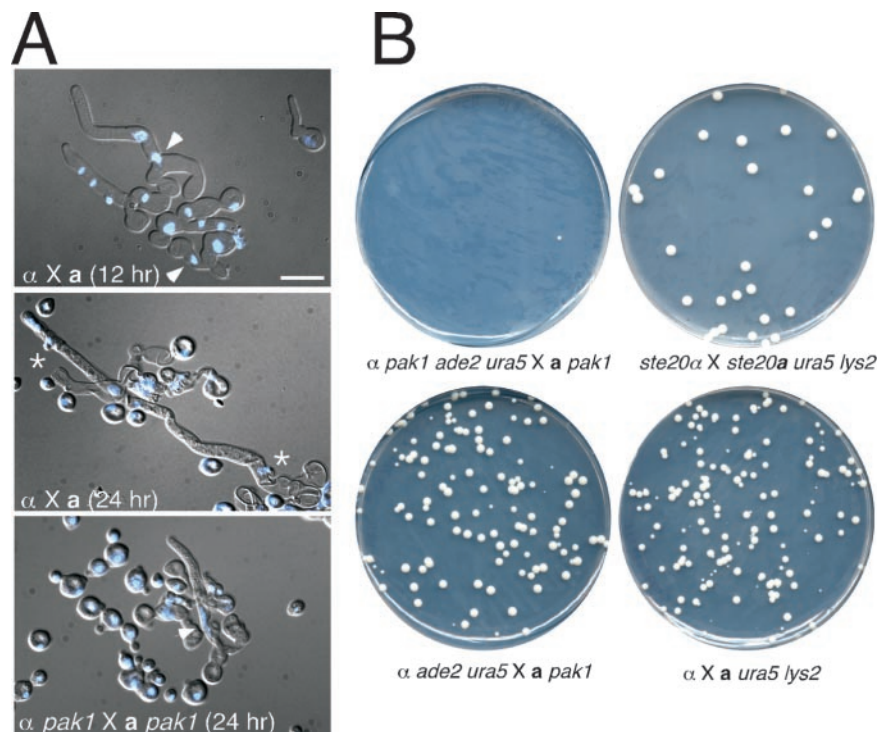
In *C. neoformans*, both dikaryotic and mononucleate (either haploid fruiting or diploid) hyphae produce basidia and

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 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 μ 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).



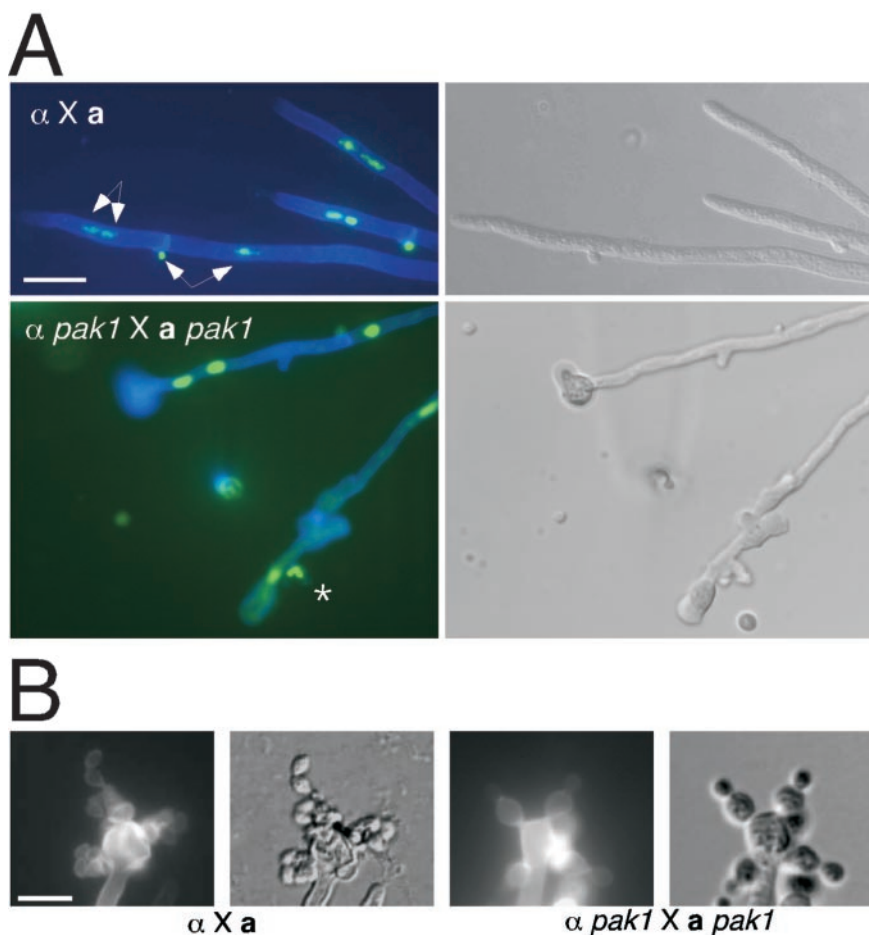


Figure 5. $\alpha pak1 \times a pak1$ crosses produce mononucleate filaments. (A) Bilateral wild-type and *pak1* crosses were prepared on glass slides coated with V8 agar medium and incubated for 5 d. The glass slide mounts were fixed and stained with calcofluor and Sytox Green to visualize septa, including clamp cell junctions, and DNA simultaneously. DIC images depict clamp cells in wild-type filaments and clamp cell-like protrusions in the *pak1* filaments. The wild-type filaments contain two nuclei per filament cell (arrows), whereas the *pak1* filaments contain one nuclei per filament cell with two exceptions: the terminal tip cell in which the single nucleus has divided before filament cell division and also multiple nuclei (asterisk) observed in the clamp cell-like protrusions. (B) Basidiospore formation is defective in *pak1* mutants. Wild-type (top) and *pak1* basidia and basidiospores (bottom) were stained with calcofluor to visualize septa (left). Left panels depict accompanying DIC images. Bar, 10 μ m.

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 costaining 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 *ste20 α \times ste20a* 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

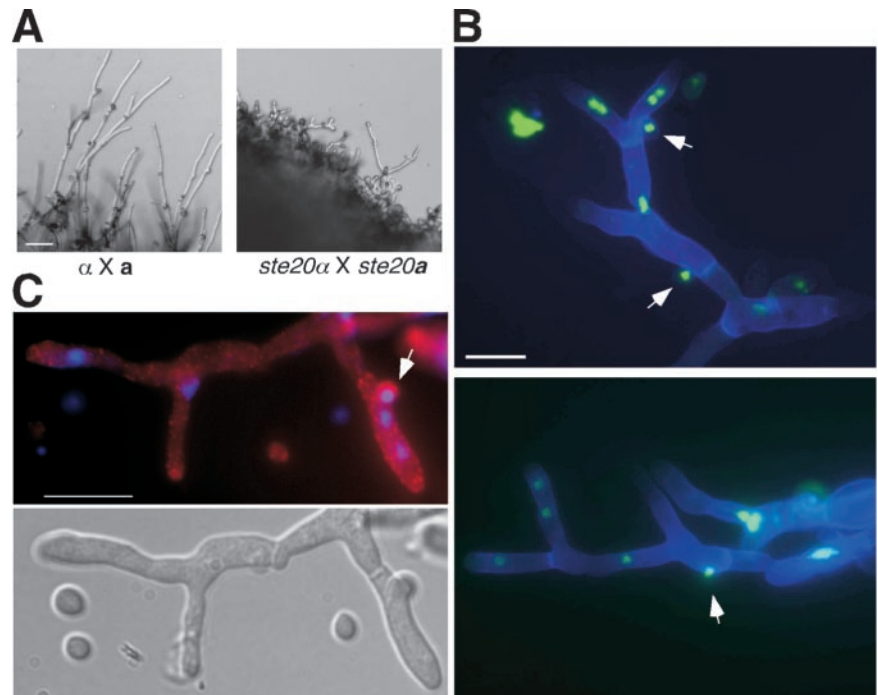
(Figure 7, B and C). Comparison of wild-type and the *ste20* mutant filaments revealed no overall difference in actin polarization (Figure 6C).

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

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 calcofluor 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.

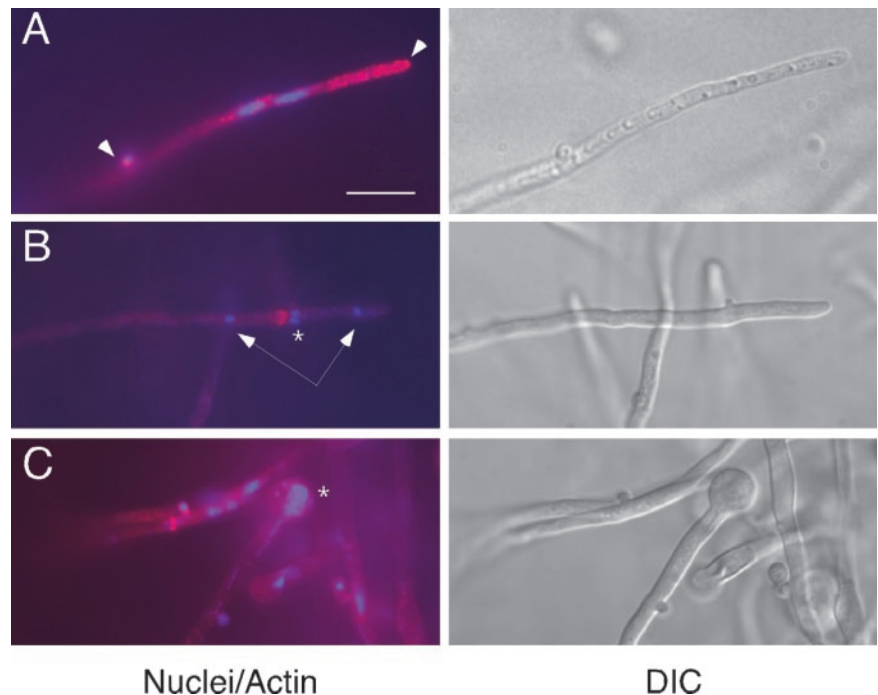


has been described in fission yeast *Schizosaccharomyces pombe*; overexpression of the Cla4 homolog Pak2/Shk2 suppresses the morphology and mating defects associated with deletion of the Ste20 homolog Pak1/Shk1 (Sells *et al.*, 1998; Yang *et al.*, 1998).

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 α mutant strains overexpressing either the *STE20 α* or the *PAK1* gene. Filamentation equivalent to wild

type was restored in control crosses (α *pak1* + *PAK1* × α *pak1* and *ste20 α* + *STE20 α* × *ste20 α*) (Figure 8A and Table 2). However, overexpression of *PAK1* in the *ste20 α* mutant strain did not restore filamentation in a bilateral *ste20* cross, nor did *STE20 α* restore filamentation in a bilateral *pak1* cross when overexpressed in an α *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 *STE20 α* complemented the cytokinesis defect of the *ste20 α* mutant strain, overexpression

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).



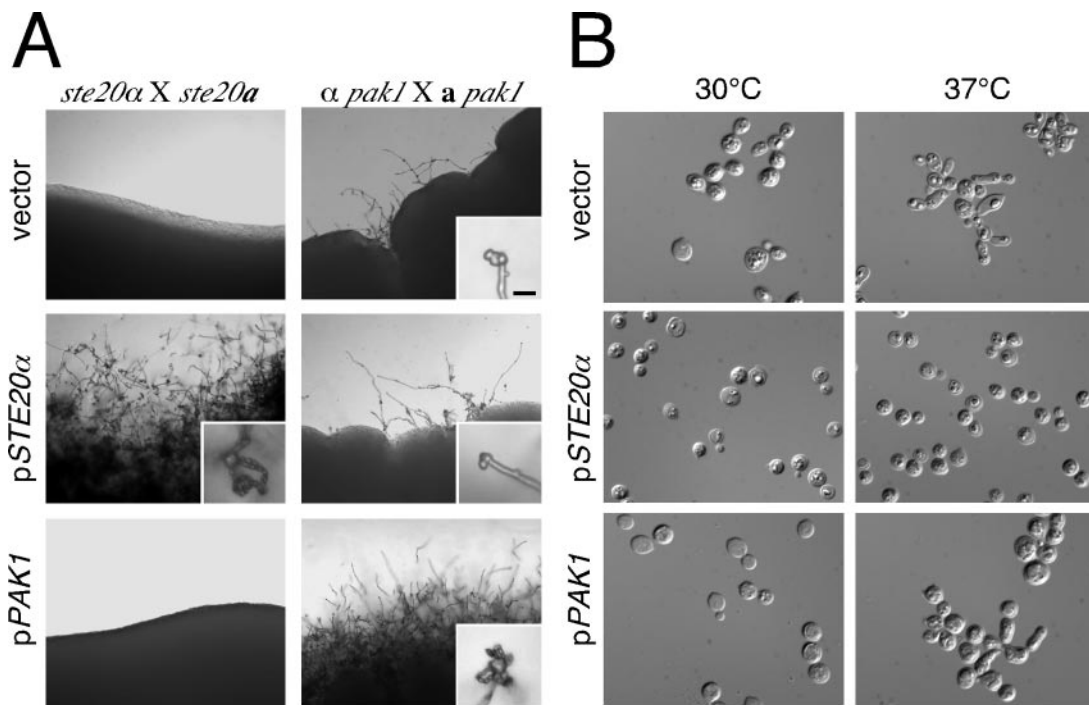


Figure 8. Ste20 and Pak1 are not interchangeable. (A) *ste20α ura5* and α *pak1 ura5* strains were transformed with plasmids expressing either *STE20α* or *PAK1*. Each strain also was transformed with a control plasmid. *ste20* (left) and *pak1* (right) bilateral crosses were prepared with each transformant and analyzed for filament production. Representative crosses were photographed at 100 and 400 \times (insets) 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 \times 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* \times *a* and *ste20a* \times 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 *ste20a* mutant and *STE20a* in a *ste20α* mutant. In bilateral *ste20* crosses, *Ste20α* restored normal filament morphology when overexpressed in either a *ste20α* or a *ste20a* mutant strain (Table 2). Similarly, *Ste20a* restored filament morphology when overexpressed in either a *ste20α* or a *ste20a* mutant strain (Table 2). In addition, overexpression of *Ste20α* complemented the cytokinesis defect of the *ste20a* mutant strain and vice versa. These data indicate that, at least under these experimental conditions, *Ste20α* and *Ste20a* 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 *ste20a* mutant by disrupting *STE20a* in the serotype A *MATa* strain KN99a (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 *ste20a* 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 *STE20a* 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 *Ste20a* 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 *STE20a* genes (Table 2). Additionally, overexpression of serotype A *STE20α* complemented the serotype D *ste20a* mutant (summarized in Table 2). In summary,

Table 2. Overexpression analysis

Mutant	Gene	Restore filamentation? (bilateral cross) ^a	Suppress cytokinesis defect?
D <i>ste20α</i>	Vector	No	No
	D <i>STE20α</i>	Yes	Yes
	D <i>STE20a</i>	Yes	Yes
	A <i>STE20α</i>	Yes	Yes
	A <i>STE20a</i>	Yes	Yes
	D <i>PAK1</i>	No	No
D <i>ste20a</i>	Vector	No	No
	D <i>STE20α</i>	Yes	Yes
	D <i>STE20a</i>	Yes	Yes
	A <i>STE20α</i>	Yes	Yes
	A <i>STE20a</i>	Yes	Yes
	D <i>PAK1</i>	No	No
A <i>ste20α</i>	Vector	No	No
	D <i>STE20α</i>	Yes	Yes
	D <i>STE20a</i>	Yes	Yes
	A <i>STE20α</i>	Yes	Yes
	A <i>STE20a</i>	Yes	Yes
	D <i>PAK1</i>	No	No
A <i>ste20a</i>	Vector	No	No
	D <i>STE20α</i>	Yes	Yes
	D <i>STE20a</i>	Yes	Yes
	A <i>STE20α</i>	Yes	Yes
	A <i>STE20a</i>	Yes	Yes
	D <i>PAK1</i>	No	No
D <i>α pak1</i>	Vector	No	NA
	D <i>STE20α</i>	No	NA
	D <i>STE20a</i>	No	NA
	D <i>PAK1</i>	Yes	NA
	A <i>PAK1</i>	No	NA
A <i>α pak1</i>	Vector	No	NA
	A <i>STE20α</i>	No	NA
	A <i>STE20a</i>	No	NA
	D <i>PAK1</i>	No	NA
	A <i>PAK1</i>	Yes	NA

NA, not applicable.

^a With the exception of serotype A *α pak1*, which was crossed to wild-type **a**.

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 **a** *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 **a** *pak1* mutant strain by targeted gene disruption. Similar to a serotype A *α pak1* unilateral cross, few filaments were produced when the serotype A **a** *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 *ste20a* mutants (Figure 9).

Overexpression of serotype A *PAK1* in either *α* or **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 Smu1 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 (Gehring and Snyder, 1990; Cheverton *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.

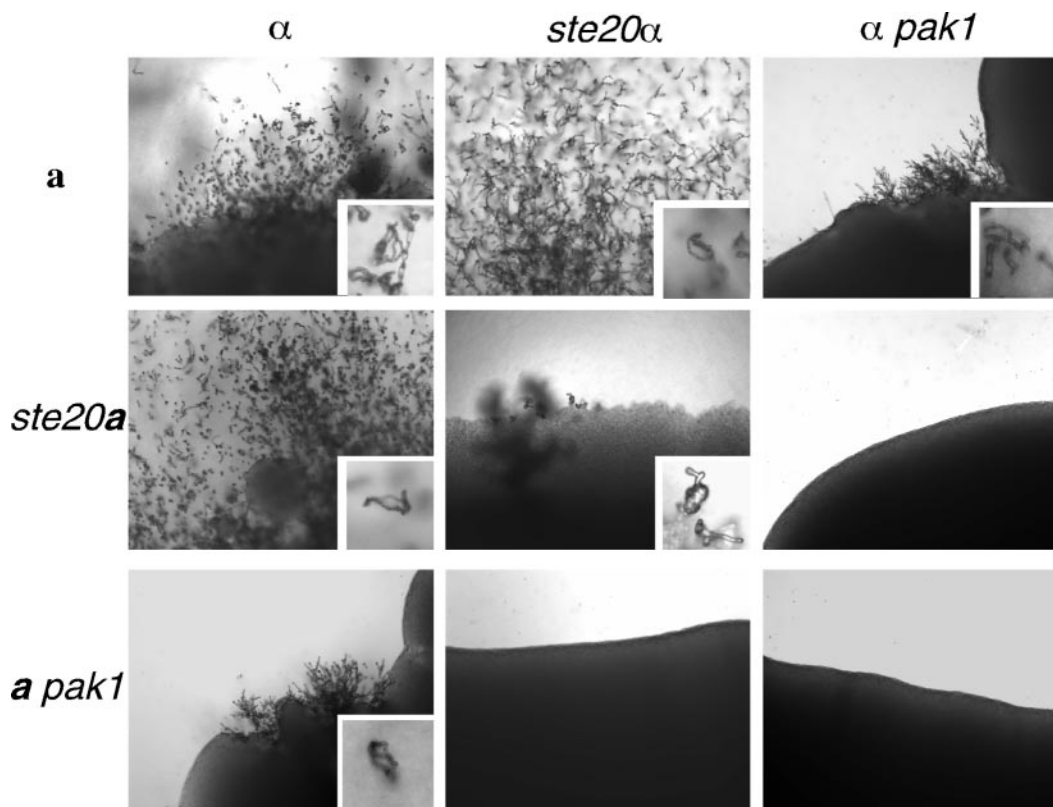


Figure 9. Serotype A bilateral *ste20* and *pak1* crosses exhibit filamentation defects. Serotype A *ste20a* and *a pak1* strains were each crossed to wild-type α , *ste20\alpha*, and α *pak1* strains. Few or no filaments were observed in the mutant bilateral crosses and in crosses between *ste20* and *pak1* mutants. Filamentation also was reduced in the *pak1* unilateral crosses but the few basidia and basidiospores that were produced were normal morphologically (insets). Representative crosses were photographed at 100 and 400 \times (insets) magnification.

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 *pak1* crosses. The *pak1* 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, *pak1* 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, *pak1* 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 *C. 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 *Magnaporthe grisea*, a septin homolog from *Aspergillus nidulans*, a Rac homolog from the human pathogen *Penicillium marneffeii*, and a Ras homolog from the alfalfa pathogen *Colletotrichum 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; Pruyne 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. marneffeii*, *A. nidulans*, and *Neurospora crassa*. Not surprisingly, some of the genes corresponding to the tip-branching mutants encode cytoskeletal-related proteins, including the Rac homolog CflA (*P. marneffeii*), 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 *C. neoformans* formin protein may be a common target for both Pak1 and Ste20 during morphogenesis. Although no formin proteins have been identified in *C. neoformans*, our BLAST searches of *C. neoformans* sequence databases have revealed that formin domain sequences are present.

In *C. neoformans*, α and *a* nuclei must be segregated to maintain the dikaryotic filament. In contrast, *P. marneffeii*, *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 \mathbf{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 Sxi1 α (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 α and Pak1 are 94 and 90% identical at the amino acid level, respectively. However, serotype A Pak1 contains several short insertions not shared by 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 nonrecombining *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 *MAT α* 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 (Mayorga 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.

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