The second *STE12* homologue of *Cryptococcus neoformans* is *MATa*-specific and plays an important role in virulence

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***Cryptococcus neoformans STE12α*, a homologue of *Saccharomyces cerevisiae STE12*, exists only in *MATα* strains. We identified another *STE12* homologue, *STE12a*, which is *MATa* specific. As in the case with Δ*ste12α*, the mating efficiency for Δ*ste12a* was reduced significantly. The Δ*ste12a* strains surprisingly still mated with Δ*ste12α* strains. In *MATα* strains, *STE12a* functionally complemented *STE12α* for mating efficacy, haploid fruiting, and regulation of capsule size in the mouse brain. Furthermore, when *STE12a* was replaced with two copies of *STE12α*, the resulting *MATa* strain produced hyphae on filament agar. *STE12a* regulates mRNA levels of several genes that are important for virulence including *CNLAC1* and *CAP* genes. *STE12a* also modulates enzyme activities of phospholipase and superoxide dismutase. Importantly, deletion of *STE12a* markedly reduced the virulence in mice, as is the case with *STE12α*. Brain smears of mice infected with the Δ*ste12a* strain showed yeast cells with a considerable reduction in capsule size compared with those infected with *STE12a* strains. When the disrupted locus of *ste12a* was replaced with a wild-type *STE12a* gene, both *in vivo* and *in vitro* mutant phenotypes were reversed. These results suggest that *STE12a* and *STE12α* have similar functions, and that the mating type of the cells influences the alleles to exert their biological effects. *C. neoformans*, thus, is the first fungal species that contains a mating-type-specific *STE12* homologue in each mating type. Our results demonstrate that mating-type-specific genes are not only important for saprobic reproduction but also play an important role for survival of the organism in host tissue.**

Cryptococcus neoformans is a pathogenic fungus that primarily infects patients with impaired immune systems, but people with no known underlying immunodeficiencies are affected also (1). *C. neoformans* is a bipolar heterothallic species in which mating is controlled by two alleles: *MATα* and *MATa* (2, 3). In the laboratory, the sexual reproduction cycle begins when two strains of the opposite mating type are crossed under nutrient-deprivation conditions. This cycle is characterized by the formation of dikaryotic hyphae with typical basidiomycetous clamp connections and by the production of basidia. Basidiospores germinate to produce yeast cells that multiply by polar budding without hyphal formation. Previous studies indicate that *MATα* strains are found far more frequently than *MATa* strains among clinical as well as environmental isolates of serotype D (4). For serotype A strains, *MATα* is thus far the only mating type recovered. In a mouse systemic-infection model, *MATα* strains of serotype D are significantly more virulent than *MATa* strains, which suggests an important role of the *MATα* locus for fungal pathogenicity (5). In the absence of *MATa* strains, *MATα* strains also undergo haploid fruiting in response to nitrogen starvation, which results in hyphal production and sporulation (6).

The signal-transduction pathways regulating morphogenesis and pathogenicity of *C. neoformans* have been studied, and homologues of several *Saccharomyces cerevisiae* mitogen-activated protein (MAP) kinase cascade genes involved in the pheromone-response pathway have been identified (7–9). *STE12α*, which encodes a protein similar to the ascomycetous Ste12p, was isolated during molecular analysis of hyphal production in a *MATα* strain of *C. neoformans* (7). The transcriptional factor Ste12p is one of the well conserved elements of the pheromone-response pathway studied in several fungi. In *S. cerevisiae*, *STE12* is an integral part of the conserved MAP kinase signal-transduction pathway involved in mating, pseudohyphal development, and haploid invasive growth (for reviews see refs. 10–13). Unlike the *STE12* genes in other fungi, *STE12α* of *C. neoformans* exists only in *MATα* strains (7). Although *STE12α* influences mating efficiency in *C. neoformans*, the Δ*ste12α* strains of *C. neoformans* are still fertile (14, 15). Deletion of *STE12α* from a wild-type strain of *C. neoformans* had no effect on yeast cell growth but abolished its ability to undergo haploid fruiting on filament agar. In serotype D strains, *STE12α* was found to modulate the expression of several genes associated with virulence. As a consequence, deletion of *STE12α* significantly reduced virulence in a serotype D strain (14). However, disruption of *STE12α* in a serotype A strain showed no effect on virulence (14, 15).

Because *STE12α* is dispensable for mating, it is possible that *C. neoformans* contains another *STE12* homologue that could substitute for the function of Ste12αp during the mating process. To investigate this possibility, we attempted to isolate a putative *STE12* homologue from the cDNA of a culture during mating. Here, we report the identification of another *STE12* homologue, *STE12a*, which only exists in the *MATa* strain of *C. neoformans*. After deleting the *STE12a* gene from a *MATa* strain, we examined the phenotypes and the virulence of the *ste12a* deletants. Deletion of *STE12a* affected the expression of several virulence-associated genes and showed reduced virulence. When the disrupted *ste12a* locus was reconstituted with a wild-type *STE12a*, the resulting strain expressed phenotypes and virulence similar to wild-type levels. These results provide molecular evidence for the important role of the mating type *a*-specific gene, *STE12a*, in the virulence of *C. neoformans MATa* strains.

Materials and Methods

Strains, Media, and General Methods. All strains used in this study were serotype D and are listed in Table 1. YEPD, minimal media, and filament agar have been described (14). The method used for the quantitative assay of mating frequency has been described (14). In brief, two auxotrophic *MATα* strains (JEC31 and JEC33) were used as tester strains to determine the mating frequency of any given *MATa* strain carrying different auxotrophic markers. The relative mating frequency was expressed as a

Abbreviations: MAP, mitogen-activated protein; SOD, superoxide dismutase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF242352).

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Table 1. List of strains relevant to this study

Strain	Genotype/Comment	Source
B4500	*MATα*; congenic strain of B-4476	Ref. 5
B4476	*MATa*; congenic strain of B-4500	Ref. 5
LP1	*MATα ura5 ade2*	Ref. 24
LP2	*MATa ura5 ade2*	Ref. 24
LP8	*MATa ura5 ade2*	Ref. 24
JE31	*MATα lys1*	Gift of J. C. Edmen
JE33	*MATα lys2*	Gift of J. C. Edmen
TYCC245	*MATα ura5 ade2 Δste12α::ADE2*	Ref. 14
TYCC245F1	*MATα Δste12α::ADE2*	Ref. 14
TYCC409AF1	*MATα Δste12α::STE12α*	Ref. 14
TYCC384	*MATa ura5 Δste12a::ADE2*	This study
C429	*MATa Δste12a::ADE2*; F_2 of TYCC384	This study
C437	*MATa ura5 ade2 Δste12a::STE12a*	This study
C446	*MATα ura5 ade2 Δste12α::STE12a*	This study
C448	*MATa ura5 ade2 GAL7(p)::STE12a::ADE2*	This study
C449	*MATa Δste12a::STE12a*; F_2 of C437	This study
C460	*MATα ura5 ade2 GAL7(p)::STE12a::ADE2*	This study
C487	*MATa ura5 ade2 Δste12a::2×STE12α*	This study
C488	*MATa ura5 ade2 Δste12a::STE12α*	This study
C489	*MATα Δste12α::STE12a*; F_1 of C446	This study
C490	*MATa Δste12a::2×STE12α*; F_1 of C487	This study

percentage of the mating frequency of the reference strain (LP2). The data represent the average of results derived from matings with JEC31 and JEC33. The experiments were repeated at least twice to confirm reproducibility. All crosses were performed on V-8 juice agar (16). Phospholipase activity was determined on egg-yolk agar as described (17). Cultures were incubated at 30°C for 72 h, and the diameter of colony plus the zone of precipitate around the colony was measured. The index of phospholipase activity was determined by dividing the colony diameter by the total diameter of the colony plus hue. There is an inverse relationship between the index and enzyme activity; the smaller the index, the higher the enzyme activity of the strain. To determine the superoxide dismutase (SOD) activity, the yeast cells were grown in Sabouraud broth for 20 h, harvested, and resuspended in 50 mM Tris (pH 7.5)/10 mM $MgSO_4$/100 mM KCl/1 mM EDTA. The resuspended cells were disrupted with glass beads in a minibead beater (Biospec Products, Bartlesville, OK). The suspension was centrifuged at 13,000 × *g* for 10 min at 4°C, and the crude cell extract was obtained. SOD activity in the protein extract was detected as described (18). A calibration curve was obtained by using bovine erythrocyte SOD (Sigma 2515) as a standard, and the sample SOD activity was determined from the regression line for standard. The method for virulence studies and the preparations of brain smears have been described (14).

Identification of the *STE12a* Gene. Degenerate primers were synthesized based on the conserved homeodomain regions among the *STE12* gene of *S. cerevisiae*, the *steA* gene of *Aspergillus nidulans*, and the *STE12α* gene of *C. neoformans* (AARAARTTYGARGARGGIRTITT and CCARWARAAIACYTTYGYTTYTT; R = A or G; Y = C or T; W = A or T; I = inosine). Reverse transcription–PCR was performed by using RNAs isolated from 6-h mating cultures of *MATa* and *MATα* strains by 30 cycles of 94°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min. The ≈150-bp PCR product obtained was cloned and sequenced. One of the 46 PCR clones contained a sequence clearly different from the *STE12α* sequence, whereas the rest showed sequences identical to the *STE12α*. To isolate the longer cDNA clone, pYCC360, the rapid amplification of cDNA ends was performed in accordance with the protocol of the Marathon cDNA amplification kit (CLONTECH). The genomic clone containing the entire *STE12a*, pYCC367 (Fig. 2*B*), was obtained by screening the genomic library of B-3502 (a gift from J. C. Edman) with pYCC360 as a probe.

Deletion and Reconstruction of *STE12a*. For the *ste12a* deletion construct, the 1.3-kb *Bam*HI–*Xba*I fragment of *STE12a* was subcloned into the *Bam*HI–*Xba*I site of pCIP3 to yield pYCC382, and the 1.5-kb *Ase*I–*Xba*I fragment of *STE12a* was cloned into the *Bam*HI–*Xba*I site of pYCC123 to yield pYCC383. The 3.3 kb *Xba*I–*Apa*I fragment of pYCC382 was cloned into the *Eco*RI–*Apa*I site of pYCC383 to yield the final deletion construct, pYCC384 (Fig. 2*A*). The electroporation-based positive-negative selection method (19) was used to delete the *STE12a* gene, and a PCR protocol was used to screen the putative deletants as described (14). The deleted *ste12a* locus was reconstituted back to the wild type by using plasmids pYCC367 and pYCC331 (20) by a biolistic-based cotransformation method (14). All putative reconstituted adenine auxotrophs were transferred on YEPD three consecutive times to cure the cotransformed telomere-based plasmid, pYCC331. Uracil and adenine auxotrophs were isolated, and their DNAs were analyzed by Southern blot. To replace the deleted *ste12α* with *STE12a*, plasmid pYCC411 was constructed. Briefly, the coding region of *STE12α* in p18-S1 was replaced with the coding region of *STE12a* from pYCC367 to yield pYCC411. Both pYCC411 and pYCC331 were cotransformed into the *ste12α* deletant, TYCC245. C446 is the resulting strain in which the deleted *ste12α* locus was replaced with *STE12a* and devoid of the cotransformed plasmid pYCC331 (Table 1). Similarly, plasmid pYCC511 was constructed to replace the deleted *ste12a* with *STE12α*. Briefly, the region containing the *STE12a* in pYCC367 was replaced with the region containing the entire *STE12α* from p18-S1 to yield pYCC511. Both pYCC511 and pYCC331 were cotransformed into the *ste12a* deletant, TYCC384. C487 and C488 were the resulting strains devoid of the cotransformed plasmid pYCC331 and contained one or two copies of *STE12α* at the deleted *ste12a* locus, respectively (Table 1).

Overexpression of *STE12a*. An *Nde*I site was generated at the first ATG of *STE12a* by PCR, and the resulting construct containing the entire coding and 3′ flanking region of *STE12a* was subcloned into the *Xba*I–*Nde*I site of pYCC246 to yield pYCC405. The plasmid pYCC405 was linearized with *Apa*I to transform LP1 and LP8. Stable transformants were selected from both mating types by transferring the cultures four times on nonselective media (YEPD). The existence of the intact *GAL7*::*STE12a* construct was confirmed by PCR with primers flanking the construct. C448 and C460 were the resulting strains (Table 1).

Preparation and Analysis of Nucleic Acids. Genomic DNA isolation and analysis were performed as described (19, 21). For low stringency hybridization, the blot was hybridized at 50°C with 6× SSC, 5× Dehnhardt's, and 0.5% SDS and was washed at 50°C with 0.5× SSC/0.1% SDS. To isolate RNA, cells were harvested from 45-h culture as described (14). Total RNA was isolated by using the FastRNA kit (Bio 101), and poly(A)$^+$ RNA was isolated by using the oligotex mRNA kit (Qiagen, Chatsworth, CA). Northern blot analysis was performed as described (22). After hybridization, the blot was exposed to a PhosphorImager Screen and quantified with IMAGEQUANT 1.1 (Molecular Dynamics). Each gene-specific signal was normalized to that of the actin gene. The relative expression levels of each gene were compared between the deletant and its congenic wild-type strain and expressed as a percentage of the wild-type levels.

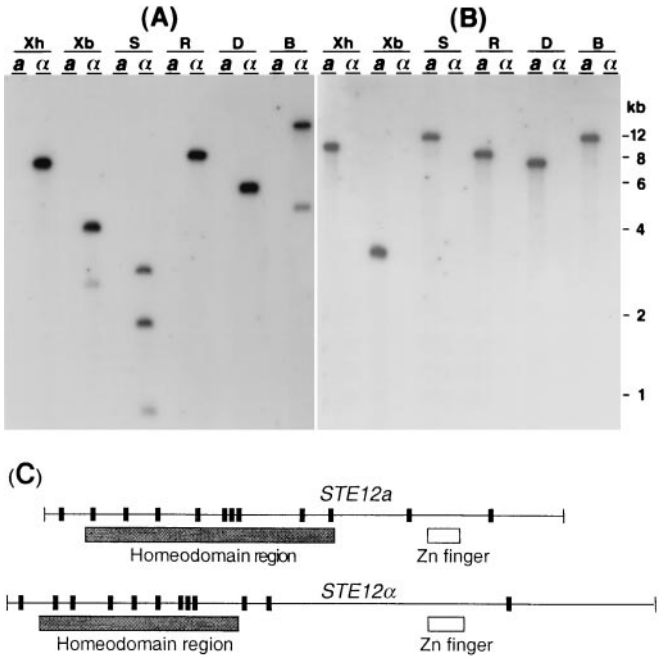

Fig. 1. Detection and structure of *STE12a* and *STE12α*. (*A*) Southern blot analysis of *STE12α*. (*B*) Southern blot analysis of *STE12a*. DNAs of B-4500 (*MATα*) and B-4476 (*MATa*) were digested with different restriction enzymes, fractionated on an agarose gel, and transferred to a nylon membrane. The resulting blot was hybridized with a probe of *STE12α* cDNA (*A*) or *STE12a* cDNA (*B*) and washed at low stringency. B, *Bam*HI; D, *Hin*DIII; R, *Eco*RI; S, *Sal*I; Xb, *Xba*I; Xh, *Xho*I. (*C*) Structure of *STE12a*- and *STE12α*-coding region. Vertical bars represent the intron positions.

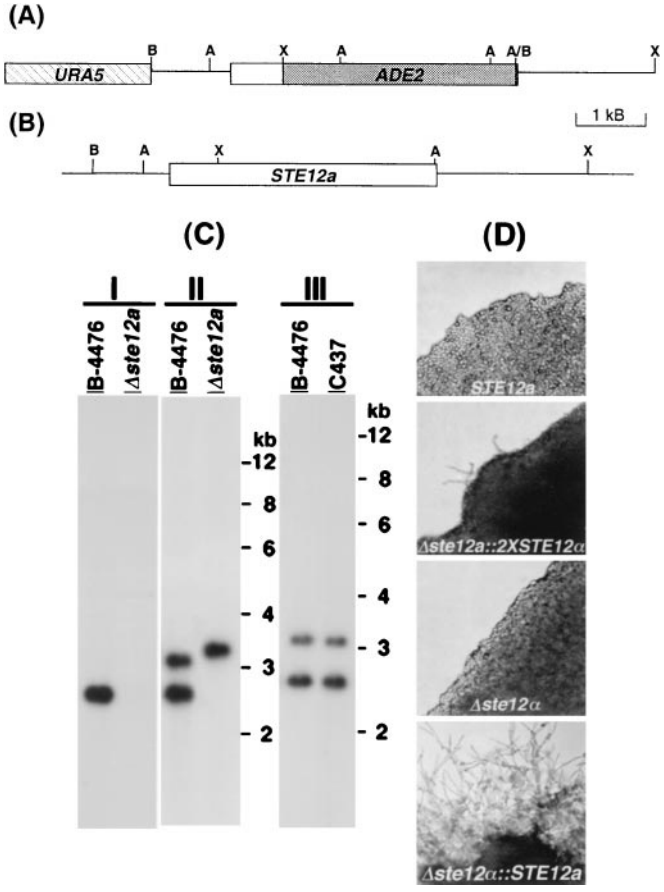

Fig. 2. Deletion and reconstitution of *STE12a*. (*A*) Map of the deletion plasmid construct, pYCC384. (*B*) Map of *STE12a* reconstitution construct, pYCC367. A, *Ase*I; B, *Bam*HI; X, *Xba*I. (*C*) Southern blot analysis of the Δ*ste12a* strain (*I* and *II*) and *STE12a* reconstituted strain (*III*). DNA was digested with *Ase*I, fractionated on an agarose gel, and transferred to a nylon membrane. The resulting blot was hybridized with 2.1-kb *Xba*I–*Ase*I probe of *STE12a* (*I*) or 3.6-kb *Xba*I–*Xba*I probe of *STE12a* (*II* and *III*). Wild type, B-4476; Δ*ste12a*, TYCC384; Δ*ste12*::*STE12a*, C437. (*D*) Hyphae formation on filament agar. Strains B-4476, C490, TYCC245F1, and C489 were inoculated on filament agar. Photographs were taken after 3 days of incubation at room temperature.

Results

Cloning a *STE12* Homologue. Previous studies showed that the Δ*ste12α* strain of *C. neoformans* remains fertile after mating with *MATa* strains albeit with reduced mating efficiency (14, 15). This observation is in contrast to *S. cerevisiae*, in which deletion of the *STE12* gene causes sterility. It is possible that a different *STE12* homologue exists in *C. neoformans*, which may augment the function of *STE12α* in the mating process. Existence of another *STE12* homologue in *MATα* or *MATa* strains, however, was not detectable by Southern blot analysis using a probe of *STE12α* cDNA under low stringency conditions (Fig. 1*A*). Because all the *STE12* homologues identified thus far contain a conserved homeodomain region, it was assumed that the new proposed *STE12* homologue would also contain this conserved homeodomain region. Degenerated PCR primers were designed according to a conserved sequence of the *STE12* homeodomain region. Reverse transcription–PCR was performed then by using RNAs that were isolated from a mating culture containing a mixture of *MATa* and *MATα* cells. A new *STE12* homologue was identified, and then the cDNA and genomic clones containing this *STE12* homologue were isolated. We found that this homologue existed only in the *MATa* strain B-4476 (Fig. 1*B*). This *STE12* homolog is present also in the three other genetically unrelated *MATa* strains reported thus far but was absent in the five randomly chosen genetically unrelated *MATα* strains (data not shown). Because of the sequence similarity and *a* mating-type specificity, we designated this *STE12* homologue as *STE12a*. Sequence analysis suggested that, as in *STE12α*, *STE12a* contains both the homeodomain and the zinc finger regions (7). Further comparison of the sequences between *STE12a* and *STE12α* indicated that the number and distribution pattern of introns in these two genes are similar (Fig. 1*C*), but the overall DNA sequence similarity between *STE12a* and *STE12α* is only 45%. Furthermore, the predicted size of the proteins for Ste12ap and Ste12αp are 69 and 94 kDa, respectively. A higher sequence similarity at the amino acid level was observed between *STE12a* and *STE12α* within the homeodomain (74%) and the zinc finger regions (62%).

Deletion and Reconstitution of the *STE12a* Gene. To study the function of *STE12a*, we deleted *STE12a* from the *MATa* strain, LP2, by using a positive-negative selection method (19). Putative transformants were identified first by PCR and then confirmed by Southern blot analysis. DNA blot was hybridized with a probe of the 2.1-kb *Xba*I–*Ase*I DNA fragment that was deleted in pYCC384 (Fig. 2*C I*). The wild-type strain (B-4476) showed a hybridization signal corresponding to the *STE12a* gene, whereas no signal was detected in the putative deletant. When the 3.6-kb *Xba*I–*Xba*I DNA fragment was used as a probe to hybridize to the same blot, the signals detected in the putative deletant corresponded to fragments of the predicted size (Fig. 2*C II*). These results indicated that we had disrupted the *STE12a* gene. To obtain a relevant control strain for further analysis of *STE12a* function, it was important to complement the deletant. Because complementation by an ectopic copy of a wild-type gene could generate undesirable effects (14), we designed a cotransforma-

tion method that enabled us to reintroduce the wild-type gene into the deletant at the homologous site (14). Adenine auxotrophic transformants, which were derived from a gene-replacement event at the disrupted Δ*ste12a* locus with the wild-type copy of *STE12a*, were isolated and analyzed by Southern blot. Fig. 2*C III* demonstrates that the putative *STE12a* reconstituted strain exhibits the same hybridization pattern as B-4476. This result indicated that we had obtained a *STE12a* reconstituted strain. The auxotrophic markers in the resulting strain were removed subsequently by mating with a wild-type strain.

Phenotype of Δ*ste12a*. Because one of the presumed functions for *STE12a* was its involvement in the mating process, we first examined the influence of the *ste12a* deletion on mating. Like the Δ*ste12α* strain, deletion of *STE12a* affected the mating efficiency, but the Δ*ste12a* strain was still able to mate with *MATα* strains. When the mating frequency was measured quantitatively (14), the Δ*ste12a* strain (TYCC384) and *STE12a* reconstituted strain (C437) had a mating frequency of 18.4 and 106%, respectively, compared with the parent strain, LP2. Although the 5.4-fold reduction in mating efficiency of *ste12a* mutants seems substantial, the degree of reduction is negligible when compared with *S. cerevisiae ste12* mutants (23). Therefore, although *STE12a* is not essential for mating, *STE12a* plays an important role in the regulation of mating proficiency of *C. neoformans*. In addition, when the coding region of *STE12α* was swapped with the coding region of *STE12a* at the disrupted *ste12α* locus, the mating frequency of such a reconstituted strain (C446) was 7.2-fold higher than that of the original Δ*ste12α* strain. This result suggested that *STE12a* could partially substitute the function of *STE12α* in mating. Most surprisingly, however, *C. neoformans* remained fertile even when the *STE12* homologues were removed from both of its mating types. During mating between a Δ*ste12a* and a Δ*ste12α* strain, typical chains of basidiospores were produced, and the spores were viable. Although the mating efficacy of Δ*ste12a* × Δ*ste12α* could not be quantitated by the method described above, we observed an additive effect of the mutation in this cross. The amount of hyphae produced in the Δ*ste12a* × Δ*ste12α* culture was even less than that observed in the cross of Δ*ste12a* × *STE12α* or Δ*ste12α* × *STE12a* (data not shown).

Because *STE12α* is required for haploid fruiting (14, 15), we tested whether *STE12a* could substitute for the function of *STE12α* in this process. Like the wild-type strain B-4500, Δ*ste12α*::*STE12a* strain (C489) produced hyphae on filament agar, whereas the *ste12α* deletant (TYCC245F1) produced only yeast cells (Fig. 2*D*, Δ*ste12α*::*STE12a* vs. Δ*ste12α*). Levels of filamentation in strain C489 were similar to that of the wild-type *MATα* strain B-4500 (data not shown). These data suggested that *STE12a* could substitute for the function of *STE12α* in the production of hyphae on filament agar. It has been shown that *MATa* cells do not produce hyphae on filament agar (6). When the deleted *ste12a* gene was replaced with two copies of the *STE12α* gene, however, the resulting *MATa* strain (C490) produced hyphae sparsely on filament agar (Fig. 2*D*, *STE12a* vs. Δ*ste12a*::2 × *STE12α*). Conversely, when only one copy of the *STE12α* gene was inserted in the *ste12a* locus, the resulting *MATa* strain produced only yeast cells on filament agar (data not shown).

It is known also that when *STE12α* is overexpressed, the *MATα* cells produce hyphal protrusions, and the morphology of *MATα* cells become abnormal (7). To test whether similar morphological changes occur when *STE12a* is overexpressed, we placed *STE12a* under the control of a *GAL7* promoter, and the resulting construct was transformed into both mating-type strains. The resulting stable transformants of both mating types produced protrusions resembling the short hyphal tubes on galactose medium, whereas control strains retained yeast morphology. These structures never extended to form true hyphae, indicating that overexpression of *STE12a* causes abnormal cell morphology regardless of mating type. As in the case of growth on minimal medium with galactose, we observed no clear filamentation on filament agar with galactose when *STE12a* was overexpressed in both mating types (data not shown).

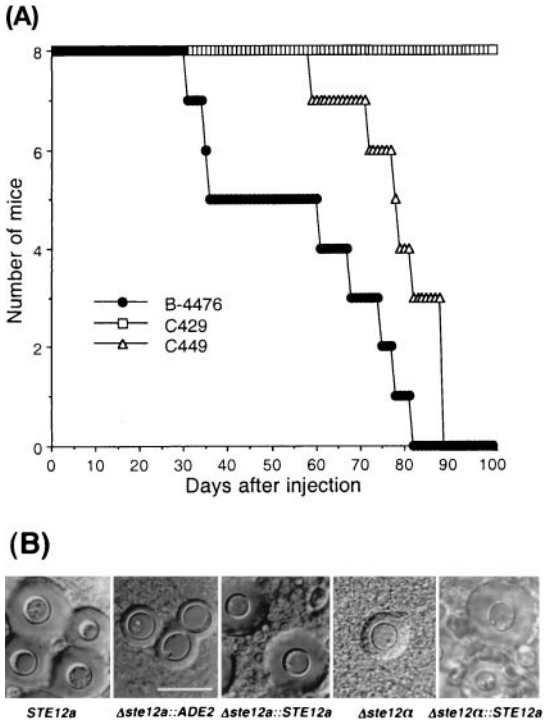

Fig. 3. Effect of *STE12a* on virulence. (*A*) Virulence study. Female BALB/c mice (6–8 weeks old) were injected via the lateral tail vein with 0.2 ml (1×10^6 cells) of a suspension of wild type (B-4476), Δ*ste12a* (C429), and Δ*ste12a*::*STE12a* (C449). Mortality was monitored for 100 days. (*B*) Brain smear of mice challenged with different yeast strains examined under a microscope with a Normalski interference condenser. (Bar = 10 μm.)

***STE12a* and Virulence.** Like *STE12α*, deletion of *STE12a* did not affect the growth rate at 37°C, capsule size or melanin production *in vitro* (data not shown). Because deletion of *STE12α* dramatically affected the virulence of *C. neoformans*, we also tested the importance of *STE12a* in virulence. Groups of mice were infected with F_2 prototrophs of the Δ*ste12a* strain (C429), *STE12a* reconstituted strain (C449), or wild-type congenic strain (B-4476). Although health of the mice infected with these strains was affected, the Δ*ste12a* strain produced no mortality during the 100-day experimental period, whereas all of the mice infected with B-4476 died within 82 days after infection (Fig. 3*A*). In addition, virulence was restored nearly to the wild-type level when the *ste12a* gene was replaced with *STE12a*. Therefore, it was clear that deletion of the *STE12a* gene severely reduced the ability of *C. neoformans* to cause fatal infection. To examine the size of the capsule of yeast cells microscopically, brain smears were prepared from mice infected with each strain of *C. neoformans*. The capsule size of yeast cells in brain smears from mice infected with C429 was considerably smaller (ranging from 1–3 μm) than that seen in mice infected with B-4476 (ranging from 2–7 μm; Fig. 3*B*, Δ*ste12a*::*ADE2* vs. *STE12a*). Furthermore, the capsule size of yeast cells in brain smears from mice infected with the *STE12a* reconstituted strain was the same as that of mice infected with B-4476 (Fig. 3*B*, Δ*ste12a*::*STE12a* vs. *STE12a*).

Because we have demonstrated that *STE12a* could replace

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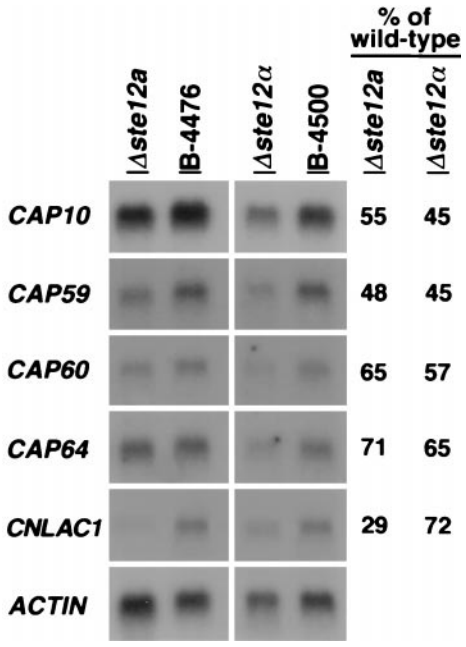

Fig. 4. Northern blot analysis. Poly(A)$^+$ RNA was isolated from yeast cells, fractionated on an agarose gel, and transferred to a nylon membrane. The resulting blot was hybridized with the indicated probe. The numbers in the last two columns represent the percentage of expression levels for each gene in the deletant compared with that of the congenic wild-type strain. Wild type, B-4476; Δ*ste12a*, C429; Δ*ste12α*, TYCC245F1.

several functions of *STE12α*, we also examined the *in vivo* capsule size of the strain in which the *ste12α* gene was substituted with *STE12a*. The yeast cells of brain smear from the mice infected with strain C489 showed a capsule size similar to the wild-type *MATα* strain and was markedly larger than that of the *ste12α* deletant, TYCC245F1 (Fig. 3, Δ*ste12α*::*STE12a* vs. Δ*ste12α*).

***STE12a* and Virulence-Associated Genes.** Because deletion of *STE12a* significantly affected virulence and the capsule size *in vivo*, it was of interest to study the influences of *STE12a* deletion on the expression of virulence-associated genes. Quantitative Northern blot analysis was used to measure the expression levels of four genes required for capsule formation (*CAP10*, *CAP59*, *CAP60*, and *CAP64*) and the laccase gene (*CNLAC1*). The *CNLAC1* gene and the four capsule-associated genes have been shown to be important for the virulence of *C. neoformans* (19, 20, 22, 24, 25). Poly(A)$^+$ RNAs were isolated from stationary phase cells (20) of wild-type *MATa* and *MATα* strains as well as deletants of *ste12a* and *ste12α*. The relative expression levels of these genes were compared between the deletant and its congenic wild-type strain. Fig. 4 demonstrated that the mRNA levels of *CNLAC1* and all four capsule-associated genes were reduced in both Δ*ste12a* and Δ*ste12α* strains compared with their corresponding wild-type strains. The amount of reduction in the expression of these genes was similar between the Δ*ste12a* and Δ*ste12α* strains except that *CNLAC1* mRNA levels were reduced more severely in the Δ*ste12a* than in the Δ*ste12α* strains. These data indicated that both *STE12a* and *STE12α* modulated expression of these virulence-associated genes.

During a subtraction library screening for genes whose expression levels were affected by deletion of *STE12α*, we found that the status of *STE12α* influenced mRNA levels of many genes including phospholipase and SOD (data not shown). Because both phospholipase and SOD are suggested to be putative virulence factors for *C. neoformans*, we measured the enzyme activity for these proteins in the background of Δ*ste12a*, Δ*ste12α*, and wild-type controls. The activity of phospholipase on egg-yolk agar was considerably lower in either Δ*ste12a* or Δ*ste12α* strains compared with that of the corresponding congenic wild-type strains (Table 2). The phospholipase activity was restored to nearly wild-type levels when the deleted gene was reconstituted. In contrast, we found that SOD enzyme activity increased in Δ*ste12a* and Δ*ste12α* strains compared with that of the corresponding congenic wild-type strains (Table 2). Reconstitution of *STE12a* and *STE12α* also restored SOD activity close to wild-type levels. These observations suggested that *STE12a* and *STE12α* modulate the enzyme activity of phospholipase and SOD *in vitro*.

Table 2. Effect of *STE12a* and *STE12α* on the activity of phospholipase and SOD

Strains[†]	Index of phospholipase activity[‡]	SOD activity[§]
STE12a	0.49 ± 0.01**	9.76 ± 0.05**
Δ*ste12a*	0.63 ± 0.03	14.71 ± 0.06
Δ*ste12a*::*STE12a*	0.53 ± 0.03*	8.69 ± 0.04**
STE12α	0.50 ± 0.03**	7.05 ± 0.03**
Δ*ste12α*	0.97 ± 0.03	12.39 ± 0.08
Δ*ste12α*::*STE12α*	0.61 ± 0.03**	7.57 ± 0.03**

[†]*STE12a* (B4476), Δ*ste12a* (C429), Δ*ste12a*::*STE12a* (C449), *STE12α* (B4500), Δ*ste12α* (TYCC245F1), and Δ*ste12α*::*STE12α* (TYCC409AF1).

[‡]There is an inverse relationship between index and enzyme activity, the smaller the index the higher the enzyme activity of the strain (see *Materials and Methods*). Data represent mean ± SD of three samples. *, $P < 0.05$ compared with the congenic deletant. **, $P < 0.01$ compared with the congenic deletant.

[§]Bovine erythrocyte SOD was used as the standard for calibration of *C. neoformans* SOD activity. Data represent mean ± SD of three samples. **, $P < 0.01$ compared with the congenic deletant.

Discussion

C. neoformans is the first reported species containing a functional *STE12* homologue in each of the opposite mating-type strains. Both *STE12* homologues in *C. neoformans*, *STE12a* and *STE12α*, contain a conserved homeodomain and a zinc finger region. These two genes, however, do not cross-hybridize to each other, indicating that they are mating-type-specific genes. Unlike Ste12p of *C. neoformans*, Ste12 proteins of ascomycetous yeasts do not contain a zinc finger region. In this respect, *C. neoformans* Ste12p is similar to steAp of *A. nidulans*. *C. neoformans* Ste12p and steAp share another similarity; they lack a domain conserved among the homologous proteins from *S. cerevisiae*, *Candida albicans*, and *Kluyveromyces lactis*, which is essential for MAP kinase-mediated activation (26). Several transcription factors with two distinct DNA-binding domains have been described (27–33). Depending on the individual protein, homeodomain and zinc finger region can bind to DNA or function as regions of protein–protein interaction. Although Ste12αp binds DNA *in vitro* (unpublished data), the precise molecular function of the homeodomain and zinc finger region in Ste12ap and Ste12αp remains to be elucidated, and it is not clear that Ste12ap and Ste12αp directly interact with the virulence-associated genes analyzed in the present study.

In *C. neoformans*, *MATα* strains predominate among clinical as well as natural isolates (4) contrary to other bipolar heterothallic fungi. The *STE12α* gene was isolated first on the basis of its ability to cause *MATα* cells to form hyphae (7). It has been proposed that the ability to undergo haploid fruiting by *MATα* strains but not by *MATa* strains may be a contributing factor for the predominance of *MATα* strain (6). Gene-disruption studies have shown that *STE12α* is required for haploid fruiting (14, 15). In this study, we found that not only could *STE12a* substitute for

the function of *STE12α* in hyphae formation on filament agar, but also a replacement of *STE12a* with two copies of *STE12α* enabled the *MATa* strain to undergo the same morphogenesis process albeit with lower frequency. Our observations suggest that these two *STE12* alleles of *C. neoformans* can complement each other functionally, conditional to interactions with other mating-type-related factors yet to be identified. The mating-type locus of *C. neoformans* harbors several other pheromone-response MAP-kinase cascade genes (9). It is reasonable, therefore, to assume that *STE12* allele-specific interactions occur between the Ste12p and other proteins encoded by the mating-type locus including components of the MAP-kinase cascade. It is possible also that the *MATa* strains require stronger stimuli than just nitrogen starvation to undergo haploid fruiting, and *STE12α* is a stronger regulator compared with *STE12a*. The mechanism involved in regulating haploid fruiting is not clear.

It is known that *STE12*, a component of the MAP-kinase pathway that signals the mating pheromone response in *S. cerevisiae*, is involved also in filamentous morphogenesis in diploid as well as haploid cells (10–13). Unlike in *C. neoformans*, however, the components of the *S. cerevisiae* pheromone-response MAP-kinase pathway have no association with the mating-type locus. One of the intriguing findings in this study is that, in contrast to the sterile phenotype of *S. cerevisiae ste12* mutants, the Δ*ste12a* strain was not only fertile after mating with *STE12α* strains, but also was able to mate with a *ste12α*-deleted strain. It has been shown in several species of basidiomycetes that interaction between different types of homeodomain-containing transcription factors are required for mating (34). It is possible, therefore, that *C. neoformans* contains other genes that could interact with or substitute for the function of Ste12ap or Ste12αp in the pheromone-response pathway. It is also possible that *C. neoformans STE12α* and *STE12a* belong to different pathways and the effects of both *STE12a* and *STE12α* on mating observed in our study could be an indirect result of cross-talk between the pathways. The involvement of *STE12α* in a different pathway is supported by the observations that *STE12α* is required for the haploid fruiting after nitrogen starvation (14, 15).

Like *STE12α*, *STE12a* seems to be required to express a wild-type level of virulence. Although mice infected with Δ*ste12a* or Δ*ste12α* strains survive significantly longer than those infected with wild-type or reconstituted strains, Δ*ste12a* and Δ*ste12α* cells persist in animal tissue and eventually cause fatal disease (data not shown). Disruption of *STE12a* altered the expression levels of many genes associated with virulence, and consequently the *ste12a* mutant was significantly less virulent in mice compared with the wild type. In fact, the decreased capsule size *in vivo* corroborated the reduced mRNA levels of capsule-associated genes tested *in vitro*. Conversely, when the Δ*ste12a* locus was reconstituted, virulence, capsule size *in vivo*, fertility, and phospholipase activity reverted close to that of wild type.

It is interesting that the enzyme activity of phospholipase was more significantly down-regulated in *ste12α* than in *ste12a*, whereas SOD activity was up-regulated in both Δ*ste12a* and Δ*ste12α* strains. The role of extracellular phospholipase as a potential virulence factor in pathogenic fungi, including *C. albicans*, *C. neoformans*, and *Aspergillus*, has gained acceptance recently (for review see ref. 35). Although SOD is an important housekeeping antioxidant and has been suggested to play a role in virulence, there has not been any experimental evidence supporting such a hypothesis (for review see ref. 36). Our observation with Δ*ste12a* and Δ*ste12α* strains seems to contradict the role of SOD on virulence of *C. neoformans* in mice. The elevated SOD level may benefit the fungus in a stressful environment with high concentration of oxygen radicals such as in animal tissue. The increased SOD activity in Δ*ste12a* or Δ*ste12α* strains, however, may not be enough to offset the negative effects caused by the *STE12a* or *STE12α* deficiency. It is unclear how *STE12a* and *STE12α* render different effects on the activity of phospholipase and SOD. Further studies on the relationship between Ste12p and affected proteins may reveal whether Ste12p directly affects the synthesis of these enzymes. We found additional genes either down-regulated or up-regulated in response to the deletion of *STE12α* (data not shown). These data implicate the multiple roles of *STE12a* and *STE12α* in regulating the expression of other genes and reveal the intricacy of how *STE12a* and *STE12α* modulate virulence.

Previous animal-model studies on virulence of isogenic *MATa* and *MATα* strains suggest that the mating-type locus is the main cause of the higher virulence of *MATα* cells (5). Our present study suggests that not only individual components of the *MATα* locus but also the *MATa* locus play important roles for virulence in the serotype D strains of *C. neoformans*. Therefore, these data support the notion that the fungal genes involved in mating have far more diverse roles than merely for reproduction.

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