Two cyclophilin A homologs with shared and distinct functions important for growth and virulence of *Cryptococcus neoformans*

Ping Wang¹, Maria E. Cardenas¹, Gary M. Cox^{2,3}, John R. Perfect^{2,3} & Joseph Heitman^{1,2,3,4*}

Departments of ¹Genetics, ²Medicine, ³Microbiology and ⁴Pharmacology and Cancer Biology and ⁵Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA

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Cyclophilin A is the target of the immunosuppressant cyclosporin A (CsA) and is encoded by a single unique gene conserved from yeast to humans. In the pathogenic fungus *Cryptococcus neoformans*, two homologous linked genes, *CPA1* and *CPA2*, were found to encode two conserved cyclophilin A proteins. In contrast to *Saccharomyces cerevisiae*, in which cyclophilin A mutations confer CsA resistance but few other phenotypes, cyclophilin A mutations conferred dramatic phenotypes in *C. neoformans*. The *Cpa1* and *Cpa2* cyclophilin A proteins play a shared role in cell growth, mating, virulence and CsA toxicity. The *Cpa1* and *Cpa2* proteins also have divergent functions. *cpa1* mutants are inviable at 39°C and attenuated for virulence, whereas *cpa2* mutants are viable at 39°C and fully virulent. *cpa1 cpa2* double mutants exhibited synthetic defects in growth and virulence. Cyclophilin A active site mutants restored growth of *cpa1 cpa2* mutants at ambient but not at higher temperatures, suggesting that the prolyl isomerase activity of cyclophilin A has an *in vivo* function.

INTRODUCTION

Cyclosporin A (CsA) is a cyclic peptide produced by *Tolypocladium inflatum* that has potent immunosuppressive activity and is widely used to prevent graft rejection in organ transplant recipients. CsA targets a conserved family of proteins, the cyclophilins, which are widely distributed in both uni- and multicellular organisms (Dolinski and Heitman, 1997). Cyclophilins also have peptidyl-prolyl isomerase activity and catalyze protein folding (Fischer and Schmid, 1990). The ubiquitous and

conserved nature of cyclophilins suggests that they have fundamental cellular roles, yet mutants lacking single or multiple cyclophilins have little phenotype in the yeast *Saccharomyces cerevisiae* (Dolinski *et al.*, 1997).

CsA suppresses the immune system by inhibiting signaling cascades required for T-cell activation (Schreiber and Crabtree, 1992; Cardenas *et al.*, 1998). CsA binds to the cyclophilin A protein, and the cyclophilin A–CsA complex targets the Ca²⁺-regulated phosphatase calcineurin in both *S. cerevisiae* and human T cells (Liu *et al.*, 1991; Foor *et al.*, 1992; Breuder *et al.*, 1994). In humans, calcineurin regulates nuclear localization of the NFAT transcription factor, which increases gene expression in response to T-cell activation by antigen presentation to the T-cell receptor (Crabtree, 2001). In yeast, calcineurin controls cell wall biosynthesis and cation homeostasis via Crz1p, a transcription factor regulating the *FKS2*, *PMR2*, *PMC1* and *PMR1* genes (Matheos *et al.*, 1997; Stathopoulos and Cyert, 1997; Stathopoulos-Gerontides *et al.*, 1999).

In addition to immunosuppression, CsA has potent antifungal activity and is toxic to the human pathogen *Cryptococcus neoformans* (Odom *et al.*, 1997). A conserved calcineurin homolog that is required for virulence is the target of CsA action in this fungus (Odom *et al.*, 1997; Cruz *et al.*, 2000b; Fox *et al.*, 2001). CsA analogs with reduced immunosuppressive activity that retain antifungal activity have been identified (Cruz *et al.*, 2000a).

We have cloned, characterized and disrupted the genes encoding two highly related cyclophilin A homologs, *Cpa1* and *Cpa2*, in *C. neoformans*. We show that CsA toxicity is mediated

*Corresponding author at: Department of Genetics, 322 CARL Bldg, Research Drive, Duke University Medical Center, Durham, NC 27710, USA.
Tel: +1 919 684 2824; Fax: +1 919 684 5458; E-mail: heitm001@duke.edu

Fig. 1. *Cryptococcus neoformans* encodes two cyclophilin A homologs. The Cpa1 and Cpa2 proteins from serotype A strains share 93% amino acid sequence identity and those from serotype D strains 97% identity. The Cpa1 and Cpa2 proteins (serotype A) share 66 and 65% amino acid identity with human cyclophilin A (P05092), and 75 and 71% with *S. cerevisiae* cyclophilin A (P14832). Identical amino acid residues are boxed and darkly shaded; similar residues are boxed and lightly shaded.

by CsA in complex with either cyclophilin Cpa1 or Cpa2. Most importantly, we show that Cpa1 has specialized functions in promoting growth at high temperature and virulence, whereas Cpa2 is dispensable under these conditions. This is the first known example in which two cyclophilin A genes have been identified from a single organism and found to encode proteins with both shared and distinct functions. Our studies also reveal that cyclophilin A plays prominent physiological roles in *C. neoformans*, providing a model genetic system to analyze the functions of this ubiquitous but enigmatic family of enzymes.

RESULTS AND DISCUSSION

Cyclophilin A is encoded by two homologous genes in *C. neoformans*

Cyclophilin A is the conserved target of CsA in *S. cerevisiae* and humans. To understand CsA antifungal activity, cyclophilin A homolog(s) were identified from *C. neoformans*. By degenerate PCR, a region of a cyclophilin A homolog was isolated and used as the probe for Southern blots, which revealed two related genes: *CPA1* and *CPA2* (DDBJ/EMBL/GenBank accession Nos AF180336 and AF180337). The *CPA1* and *CPA2* genes are highly conserved, and share 78% nucleotide identity in the coding regions. The *CPA1* and *CPA2* genes encode 162-amino-acid proteins that share 93% protein sequence identity (Figure 1), and 65–75% identity with human and yeast cyclophilin A. The *CPA1* (AF333996) and *CPA2* (AF333997) genes were also present in a divergent serotype D *C. neoformans* strain (Figure 1).

Interestingly, the *CPA1* and *CPA2* genes were found to be linked by Southern blotting, hybridization to common BAC genomic clones and long-range PCR (Figure 2). In chromosome blots, the *CPA1* and *CPA2* genes hybridized to one of two co-migrating ~2.2 Mb chromosomes (Figure 2A). With isolated BAC DNA as template, *CPA1* and *CPA2* gene-specific primers amplified an ~20 kb fragment linking the genes (Figure 2B).

These findings suggest that the two *CPA* genes arose by gene duplication. Duplication of the cyclophilin A gene occurred prior to the divergence of serotype A (var. *grubii*) and serotype D (var. *neoformans*) strains, estimated to have occurred ~20 million years ago (Xu et al., 2000). The *CPA1* and *CPA2* genes are more divergent in serotype A (12 amino acid changes) compared with serotype D (five amino acid changes), suggesting that mutagenesis rates or selective pressures may differ between the two. This is the first known example in *C. neoformans* in which two highly related genes are linked, and could be a common feature in the *C. neoformans* genome. We recently found that the *MFα2* and *MFα3* genes are similarly linked and divergently transcribed. A similar example of gene duplication and divergence is the cyclophilin 40 homologs Cpr6 and Cpr7 in *S. cerevisiae* (Duina et al., 1996a,b; Dolinski et al., 1997). Two pairs of cyclophilins localized to the secretory pathway in *S. cerevisiae*, Cpr2/Cpr5 and Cpr4/Cpr8, may represent additional examples of cyclophilin gene duplication and divergence (Dolinski et al., 1997).

Disruption of the *CPA1* and *CPA2* genes

To determine the functions of cyclophilin A, the *CPA1* and *CPA2* genes were disrupted individually and in combination. The

Fig. 2. The *C. neoformans* *CPA1* and *CPA2* genes are linked. **(A)** The *CPA1* and *CPA2* genes mapped to the same chromosome in both serotype A and D strains. The full-length *CPA1* gene was used as the probe in Southern hybridization. Protoplasts were prepared and chromosomes were separated by CHEF gels. The large chromosome containing the *CPA1* and *CPA2* genes also contains the rDNA gene cluster, and differences in repeat number may account for the difference in chromosome size in the congenic strains. **(B)** The *CPA1* and *CPA2* genes lie 20 kb apart and are divergently transcribed (top diagram). Long-range PCR was used to link the *CPA1* and *CPA2* genes using *CPA1* and *CPA2* gene-specific primers and BAC clone DNA as template. Lanes 1 and 4 are 1 kb DNA ladders. Lane 6 is λ phage DNA cleaved with *Hind*III, also used as marker. Lanes 2 and 3 are PCR products derived from the *CPA1* and *CPA2* genes, respectively, and lane 5 is a 20 kb amplicon linking the *CPA1* and *CPA2* genes. By PCR, the *CPA1* and *CPA2* genes were also present in the serotype A MATa strain 125.91 (data not shown).

CPA1 gene was disrupted with a *cpa1::ADE2* or *cpa1::URA5* allele; the *CPA2* gene was disrupted with a *cpa2::URA5* allele (Figure 3A). The *cpa1::ADE2* allele was transformed into the *ade2* strain M049, and five *cpa1* mutants from 48 Ade⁺ isolates (10%) were identified by PCR and verified by Southern analysis (Figure 3B). Five *cpa1::URA5* and four *cpa2::URA5* mutants were obtained from 49 (10%) and 32 (12.5%) Ura⁺ isolates. Finally, a *cpa1::ADE2* mutant was plated on 5-fluoroorotic acid (5-FOA) medium and the resulting *cpa1::ADE2 ura5* derivative used as a recipient for transformation with the *cpa2::URA5* allele. Seven *cpa1 cpa2* double mutants were obtained from

98 Ura⁺ isolates (7%). The *cpa1*, *cpa2* and *cpa1 cpa2* mutants were reconstituted with the *CPA1*, *CPA2* and *CPA1+CPA2* genes, and verified by PCR and western blotting (Figure 3B and C).

Cpa1 and Cpa2 proteins are both expressed in *C. neoformans*

Proteins were extracted from the wild-type and cyclophilin A mutants, and analyzed by western blotting with polyclonal antiserum against the Cpa1 protein. This antiserum recognized an 18 kDa protein in wild-type cells but not in *cpa1 cpa2* mutants (Figure 3C); 18 kDa proteins were also detected in extracts from the *cpa1* and the *cpa2* single mutants, and represent the Cpa2 and Cpa1 proteins, respectively. Similar 18 kDa protein(s) were detected in the *cpa1 cpa2+CPA1 CPA2* reconstituted strain (Figure 3C). Thus, the antibody raised against Cpa1 also recognizes Cpa2, and the *CPA1* and *CPA2* genes are both expressed and have been disrupted by homologous recombination.

Phenotypic analysis of *cpa1*, *cpa2* and *cpa1 cpa2* mutants

The effects of *cpa1* and *cpa2* mutations on growth, CsA sensitivity and mating of *C. neoformans* were tested. The *cpa1* and *cpa2* single and double mutants exhibited different phenotypes. *cpa1* mutants grew normally at 30 and 37°C, but exhibited a growth defect at 39°C, similar to but less severe than calcineurin mutants (Figure 4A). In contrast, *cpa2* mutants exhibited normal growth at all temperatures (Figure 4A). *cpa1 cpa2* double mutants exhibited a more severe growth defect, and grew poorly at 24, 30 and 37°C and were inviable at 39°C (Figure 4A). We conclude that Cpa1 plays a unique role in growth at 39°C, and that Cpa1 and Cpa2 have partially overlapping functions required for normal cell growth. We note that Cpa1/2 function is specific, as this organism expresses another prolyl isomerase (FKBP12) that is insufficient to protect from loss of cyclophilin A.

Growth of the wild-type and both the *cpa1* and *cpa2* single mutants was sensitive to CsA at 37°C. In contrast, growth of the *cpa1 cpa2* double mutant was completely CsA resistant (Figure 4B). Because mutations in calcineurin (V344K, V344R) known to block cyclophilin A–CsA binding also confer CsA resistance in *C. neoformans* (Cruz *et al.*, 2000b), we conclude that CsA toxicity is mediated by inhibition of calcineurin by cyclosporin in complex with either Cpa1 or Cpa2.

In mating assays, *cpa1* or *cpa2* single mutants were fully fertile and formed abundant filaments, basidia and basidiospores (not shown). In contrast, the *cpa1 cpa2* mutant was sterile and formed no heterokaryotic filaments, basidia or basidiospores, indicating that Cpa1 and Cpa2 are required for mating (Figure 5A).

Reconstitution of the *cpa1* mutant with the wild-type *CPA1* gene restored growth at 39°C. Reconstitution of the *cpa1 cpa2* mutant with the *CPA1* and *CPA2* genes fully restored growth, CsA sensitivity and mating (Figures 4 and 5). Thus, the *cpa1* and *cpa2* mutations confer the observed phenotypes.

The *CPA1* and *CPA2* genes encode proteins that share marked amino acid sequence identity and yet confer distinct phenotypes when mutated. By western blotting, the Cpa1 and Cpa2 proteins are both expressed. The antiserum raised against Cpa1 should cross-react equally or slightly less well with Cpa2. Based on this

Fig. 3. Isolation of *cpa1*, *cpa2* and *cpa1 cpa2* mutant strains of *C. neoformans*. (A) Schematic illustration of the *CPA1* and *CPA2* gene-specific disruptions. The *cpa1::ADE2* allele fragment was transformed into an *ade2* H99 strain, and the *cpa1::URA5* and *cpa2::URA5* alleles were transformed into the *ura5* H99 strain. The *cpa1 cpa2* double mutant strain was constructed by first obtaining a stable 5-FOA-resistant *ura5 cpa1::ADE2* strain and then introducing the *cpa2::URA5* allele. (B) Verification of *cpa1* and *cpa2* gene disruption mutant and reconstituted strains by PCR with *CPA1* (lanes 2–4) and *CPA2* (lanes 5–7) gene-specific primers. The nourseothricin resistance gene marker and biolistic transformation were used to re-introduce the wild-type *CPA1* and *CPA2* genes into the mutant strains. (C) Western blot detection of *C. neoformans* protein extracts indicates that both Cpa1 and Cpa2 cyclophilin A proteins are expressed (~18 kDa). Total proteins (~50 µg per lane) from the wild-type, *cpa1*, *cpa2*, *cpa1 cpa2* and *cpa1 cpa2+CPA1 CPA2* strains were separated by 18% SDS-PAGE, blotted, incubated with anti-Cpa1 antiserum, and detected by ECL (Amersham Pharmacia). (D) Expression of the calcineurin A Cna1 protein was measured in the wild-type, *cpa1*, *cpa2*, *cpa1 cpa2*, *cpa1 cpa2+CPA1 CPA2* and *cna1* strains by overlay blotting with ¹²⁵I-labeled calmodulin.

analysis, the Cpa1 protein is not >2-fold more abundant than Cpa2, and this seems unlikely to explain their differences in function. The promoter regions of the two genes are completely dissimilar, and thus the *CPA1* and *CPA2* genes could be differentially regulated under some conditions. Alternatively, the few subtle differences in amino acid residues between Cpa1 and Cpa2 could endow each with the ability to interact preferentially with different targets.

The phenotypes exhibited by *cpa1 cpa2* mutants are similar to but more severe than calcineurin mutants. Both mutants are inviable at 39°C and avirulent. However, *cpa1 cpa2* cyclophilin mutants are unilaterally sterile and growth impaired at 30°C, whereas calcineurin mutants are bilaterally sterile and grow normally at 30°C. Given that Cpa1 and Cpa2 bind CsA and inhibit calcineurin, Cpa1 and Cpa2 could regulate calcineurin normally. By ¹²⁵I-labeled calmodulin overlay blotting (Cruz *et al.*, 2000b), similar levels of the calcineurin A Cna1 protein were detected in wild-type, *cpa1*, *cpa2* and *cpa1 cpa2* mutants, and reconstituted strains (Figure 3D). No marked difference in calcineurin activity was apparent in crude extracts of wild-type and *cpa1 cpa2* mutant strains; however, this assay is neither highly sensitive nor highly specific (not shown). Calcineurin mutations that block cyclophilin A–CsA binding by impairing

protein–protein interactions (Cna1 V344K, V344R) were fully functional *in vivo* (not shown). Further studies will be required to address whether cyclophilin regulates calcineurin assembly or localization; previous studies have suggested that cyclophilin A may regulate calcineurin in *S. cerevisiae* (Cardenas *et al.*, 1994). Based on phenotypic differences between cyclophilin A and calcineurin mutant strains, cyclophilin A is likely to have targets in addition to calcineurin.

Prolyl isomerase activity is important for Cpa1 function *in vivo*

Two active site mutant alleles of the *CPA1* gene were constructed and expressed to examine the *in vivo* function of cyclophilin A prolyl isomerase activity. Previous studies with mammalian cyclophilin A indicate that replacing the conserved Arg55 or Trp121 residues with alanine results in cyclophilin A proteins with reduced activity (0.1% for R55A and 8.7% for W121A) (Zydowsky *et al.*, 1992). The *CPA1*^{R55A} and the *CPA1*^{W121A} mutants both restored normal growth to *cpa1 cpa2* mutants at 25°C (not shown). At 30°C, the *CPA1*^{R55A} and *CPA1*^{W121A} alleles partially and fully restored growth, respectively (Figure 4C), consistent with their level of activity *in vitro*. Neither

Fig. 4. Cpa1 and Cpa2 cyclophilin A proteins mediate CsA toxicity and their activity is important for growth. (A) The wild-type, *cpa1*, *cpa2* and *cpa1 cpa2* mutant strains, and the *cpa1 cpa2+CPA1 CPA2* reconstituted strain were 10-fold serially diluted in YPD medium, and cell suspension (5 µl) was spotted onto YPD medium and incubated at 30, 37 and 39°C for 60 h. (B) Cpa1 and Cpa2 mediate CsA toxicity. The wild-type, *cpa1*, *cpa2* and *cpa1 cpa2* mutant strains were 10-fold serially diluted, and the cells (5 µl) were spotted onto YPD medium with or without CsA (100 µg/ml) and incubated at 37°C for 72 h. (C) Cells of the wild-type, the *cpa1 cpa2* mutant and the *cpa1 cpa2* mutant strains transformed with vector alone, or the wild-type *CPA1*, *CPA1^{R55A}* or *CPA1^{W121A}* gene, were serially diluted, spotted onto YPD medium, and incubated at 30, 37 or 39°C for 72 h in the absence or presence of CsA (100 µg/ml).

mutant restored wild-type growth at 37 or 39°C, whereas the *CPA1* wild-type gene did (Figure 4C). By western blotting, the cyclophilin A mutants were both expressed under these conditions (not shown). The cyclophilin A active site mutants did not restore CsA sensitivity at 37°C, indicating that these mutants fail to bind CsA or calcineurin (Figure 4C), in accordance with previous studies. Interestingly, either active site mutant restored mating of the *cpa1 cpa2* mutant strain at 24°C (not shown), indicating either that activity is not required for mating or that the residual activity of the mutant enzymes suffices. In summary, the Cpa1 protein and its activity are required for function under certain growth conditions.

Cyclophilin A is required for *C. neoformans* virulence

Because the *C. neoformans* Cpa1 cyclophilin A protein is required for growth at 39°C, and the Cpa1 and Cpa2 proteins are both required for normal growth, Cpa1 and Cpa2 were tested for roles in virulence. In a rabbit model, the wild-type and *cpa2* mutant persisted in the cerebrospinal fluid (CSF) at similar

concentrations ($\sim 10^6$ c.f.u./ml) for the entire 10 days (day 7, $p = 0.13$) (Figure 6A). In contrast, the *cpa1* mutant was reduced by ~ 100 -fold ($\sim 10^4$ c.f.u./ml) compared with the wild type and *cpa2* mutants (day 10, $p = 0.02$) (Figure 6A). In comparison, the *cpa1 cpa2* double strain was reduced $\sim 10\,000$ -fold ($\sim 10^2$ c.f.u./ml) in survival ($p = 0.004$ on day 10) (Figure 6A). Virulence was partially restored in the *cpa1 cpa2+CPA1 CPA2* strain compared with the *cpa1 cpa2* mutant (day 10, $p = 0.03$) (Figure 6A). Similarly to many virulence studies in *Candida albicans* and *C. neoformans*, the reconstituted strain did not reach the full wild-type level, possibly due to other events during transformation or differences in expression levels between the wild-type and ectopic re-integrated genes. Taken together, these findings indicate that Cpa1 is important for virulence of *C. neoformans* and that Cpa2 plays an ancillary role.

Results in a murine model were concordant. The average survival of mice infected with the wild-type and *cpa2* mutant was 17.8 and 20 days, respectively ($p = 0.113$; Figure 6B). Virulence was partially attenuated in the *cpa1* mutant and the average survival was prolonged to 31.4 days (H99 versus *cpa1*,

Fig. 5. *Cryptococcus neoformans* cyclophilin A Cpa1 and Cpa2 proteins are required for mating and melanin biosynthesis. (A) The wild-type, *cpa1 cpa2* mutant and *cpa1 cpa2+CPA1 CPA2* reconstituted strains were co-cultured with the MATa strain JEC20 on V8 agar for 7 days at 25°C and photographed (25×). (B) The wild-type, *cpa1*, *cpa2* and *cpa1 cpa2* mutant, and the *cpa1 cpa2+CPA1 CPA2* reconstituted strains were grown for 48 h at 30°C on Niger seed medium to assay melanin production.

$p < 0.001$). Complete (100%) lethality occurred by day 37 with the *cpa1* mutant compared with day 20 with wild-type and day 27 with the *cpa1+CPA1* reconstituted strain (*cpa1* versus *cpa1+CPA1*, $p < 0.001$) (Figure 6B). The *cpa1 cpa2* mutant was even more significantly attenuated for virulence ($p < 0.0001$), and all 10 mice were still alive on day 50 (Figure 6B). Again, virulence was partially but not completely restored in the *cpa1 cpa2+CPA1 CPA2* reconstituted strain, and the average survival of infected mice was 30.7 days (*cpa1 cpa2* versus *cpa1 cpa2+CPA1 CPA2*, $p < 0.001$) (Figure 6B).

We conclude that Cpa1 is important for virulence, whereas Cpa2 is not required for virulence in a *CPA1* wild-type strain, but does contribute in a *cpa1* mutant background. The cyclophilin A Cpa1 and Cpa2 proteins are likely to contribute to virulence by promoting normal growth and survival under stress conditions. The *cpa1 cpa2* double mutant exhibited defects in both melanin production in response to glucose limitation (Figure 5B) and capsule production in Eagle's medium (not shown). These defects are also likely to contribute to the virulence defect, and reflect the inability of the mutant strain to respond to stress conditions. This is the first report of a role for cyclophilin A in virulence.

METHODS

Strains and media. Serotype A strain H99 and its *ade2* derivative M049 have been described (Toffaletti *et al.*, 1993; Sudarshan *et al.*, 1999). The H99 *ura5* mutant was obtained on 5-FOA

Fig. 6. Cyclophilin A proteins are required for virulence in *C. neoformans*. (A) Rabbits were intrathecally inoculated with 10^6 yeast cells. Three to four rabbits were infected with the wild-type, *cpa1*, *cpa2* and *cpa1 cpa2* mutant, and *cpa1 cpa2+CPA1 CPA2* reconstituted strains. CSF was recovered on days 4, 7 and 10 post-infection, and c.f.u./ml of CSF were plotted with the SEM. (B) Ten female A/Jcr mice per strain were infected by nasal inhalation with $50 \mu\text{l}$ of yeast cells ($10^6/\text{ml}$) of the wild-type, *cpa1*, *cpa2* and *cpa1 cpa2* mutant strains, and the *cpa1+CPA1* and *cpa1 cpa2+CPA1 CPA2* reconstituted strains. Survival was monitored twice daily and the number of surviving mice was plotted against time; p values were calculated by a Kruskal–Wallis analysis.

medium (Kwon-Chung *et al.*, 1992b). All strains used in this study are listed in Supplementary Table 1 (Supplementary data for this paper are available at *EMBO reports* Online). YPD, YNB, V8 and Niger seed agar were as described (Alsbaugh *et al.*, 1997; Wang *et al.*, 2000).

Isolation and characterization of *C. neoformans* cyclophilin A gene homologs. A partial cyclophilin A gene was amplified by PCR from strain H99 and used to probe a genomic Southern blot, which revealed two related cyclophilin A genes. A 3.0 kb *Bam*HI fragment (pPWC1) cloned from a partial library of H99 contains the first *CPA1* gene. A 1.6 kb *Hind*III fragment (pPWC2) contains the second *CPA2* gene. Complete sequences of the *CPA1* and *CPA2* genes were obtained by sequencing both DNA strands, and all introns were determined by sequencing RT–PCR-amplified fragments that overlap the entire coding regions. Primers used to map the *CPA1* and *CPA2* genes to a common 20 kb fragment were 5′-AACGGTACCAGAAGCGGCA (2949) and 5′-ACCCAA-CGAAGCTAGAAGA (2931), and PCR comprised 30 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 15 min. DNA from a

BAC clone containing the *CPA1* and *CPA2* genes was used as template.

Disruption of the *CPA1* and *CPA2* genes, reconstitution of mutants, antibody preparation, and detection of proteins. See Supplementary data Online.

Active site mutagenesis of the *CPA1* gene. Point mutations were introduced in the *CPA1* gene replacing, respectively, triplets CGA encoding Arg55 or TGG encoding Trp121 with GCA encoding alanine by PCR overlap mutagenesis. To construct the *CPA1*^{R55A} allele, the 0.85 kb PCR fragment amplified with primers 5'-TATCGTCTGCGTTGTCTGCT (5267) and 5'-GGA-TCACTGCGTGGAAACC (6093), and also the 0.85 kb fragment with 5'-GGTTTCCACGCGAGTGATCC (6092) and 5'-CGGTAT-GAGAGTCCAAGAGAAG (1335), were pooled and used as the template for a second round of PCR using primers 5267 and 1335. The *CPA1*^{W121A} mutant allele was created using the same approach with primers 5'-GTTACCTCTGCGCTCGACG (6094), 5'-CGTCGAGCGCAGAGGTAAC (6095), 5267 and 1335. A 1.7 kb fragment was cloned, inserted in pGMC200 (pPWC10), verified by sequencing, and transformed into the *cpa1 cpa2* mutant strain. The plasmid with the 1.7 kb wild-type *CPA1* gene (pPWC9) was used as a control.

Animal models of cryptococcal meningitis. New Zealand White rabbits were used, and the maintenance, immunosuppression, sedation, recovery of CSF, and quantitative evaluation of virulence by counting colony-forming units were as described (Wang *et al.*, 2000). In the murine model, 50 µl (total 5 × 10⁴ cells) of yeast cells per strain were used to infect 10, 4- to 6-week-old female A/Jcr mice by nasal inhalation. Mice were monitored twice daily and those that were moribund or in pain were killed by CO₂ inhalation (Cruz *et al.*, 2000b).

A Student's *t*-test was performed to compare mean CSF yeast counts between strains in the rabbit model, and a Kruskal–Wallis analysis was applied to compare survival in mice. Values of *p* < 0.05 were considered statistically significant. Virulence tests in both animal models were repeated twice.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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