Chlamydropse Formation during Hyphal Growth in Cryptococcus neoformans

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Cryptococcus neoformans, a basidiomycetous fungal pathogen, infects hosts through inhalation and can cause fatal meningococcalpahitis in individuals if untreated. This fungus undergoes a dimorphic transition from yeast to filamentous growth during mating and monokaryotic fruiting, which leads to the production of hyphae and airborne infectious basidiospores. Here we characterized a novel morphological feature associated with the filamentous stages of the life cycle of C. neoformans which resembles resting or survival structures known as chlamydospores in other fungi. The C. neoformans chlamydospore-like structure is rich in glycogen, suggesting that it might have a role as an energy store. However, characterization of mutants with decreased or increased levels of glycogen production showed that glycogen levels have little effect on filamentous growth, sporulation, or chlamydospore formation. These results suggest that the formation of chlamydospores is independent of glycogen accumulation level. We also show that chlamydospore formation does not require successful sporulation and that the presence of chlamydospores is not sufficient for sporulation. Although the biological functions of chlamydospores remain to be established for this pathogenic fungus, their formation appears to be an integral part of the filamentation process, suggesting that they could be necessary to support sexual sporulation under adverse conditions and thereby facilitate the production of infectious basidiospores or long-term survival propagules in harsh environments.

Chlamydropse are produced by many fungi and represent enlarged, thick-walled vegetative cells with varied forms and condensed cytoplasm that form within hyphae or at hyphal tips. Despite poor cytological descriptions or documentation of their mode of generation, chlamydospores have been observed in three major clades of the fungal kingdom. For example, the basidiomycete black ink mushroom Coprinus cinereus (28) and Cryptococcus laurentii (30), the ascomycete nematode-trapping fungus Duddingtonia flagrans (20, 41), and zygomycete mucorales, such as Rhizopus schipperae (4, 55), have all been shown to produce chlamydospores. Even the fungus-like oomycete plant pathogens Phytophthora cinnamomi and Phytophthora parasitica produce chlamydospores.

Biological functions ascribed to these chlamydospores differ between species. For example, desiccation-resistant chlamydospores of P. cinnamomi are produced within plant roots during drought and are transported in root fragments or soil, germinating to cause infections when warm, moist conditions are encountered. When chlamydospores of the nematode-trapping fungus D. flagrans are fed to domesticated animals, they can survive passage through the alimentary tract and reduce the number of parasitic nematode larvae that develop from eggs in the feces, thus preventing clinical disease (20, 41). In addition, the chlamydospore developmental phase of Aspergillus parasiticus has been associated with increased aflatoxin production, while chlamydospores of Fusarium species are the principal means of long-term survival during unfavorable peri-

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**C. neoformans** strains can be readily isolated from the environment, particularly from soil contaminated with aged pigeon guano (10). Due to their microscopic nature, structures that promote *C. neoformans* survival in the environment are not known and could be sexual basidiospores, mitotic yeast cells, filaments, or other unknown structures. While many soilborne fungi produce chlamydospores as long-lived survival structures under hostile environmental conditions (15, 44), there has been no previous report of such structures in *C. neoformans*. Here we describe the formation of a morphological structure associated with the filamentous growth of *C. neoformans* that is strikingly similar to chlamydospores produced by other fungi, providing the first documentation of such structures in this organism. These data reveal a novel growth option in the life cycle of *C. neoformans* and provide a robust and genetically tractable model for studying the morphogenesis and molecular basis of the development and function of these cellular structures.

### MATERIALS AND METHODS

**Strains and media.** Strains used in this study are listed in Table 1. YPD medium contained 1% yeast extract, 2% Bacto peptone, and 2% dextrose. YNB medium contained 1% yeast extract, 2% Bacto peptone, and 2% dextrose. YNB-NEO medium contained 1% yeast extract, 2% Bacto peptone, and 2% dextrose. YNB-NEO medium contained 1% yeast extract, 2% Bacto peptone, and 2% dextrose.

**Microscopy.** Cells were grown on V8 medium on the top of slides in the dark for 7 days. Hyphae were fixed in 3.7% formaldehyde in phosphate-buffered saline with 1% Triton X-100. Nuclei and septa were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma) and calcofluor (Sigma) as described previously (53). For FM-64 and Nile Red staining of intracellular organelles and lipid bodies, live cells were used and were processed as described previously (35, 49).

**Transformations.** Dominant selectable markers conferring resistance to nourseothricin (NAT) and G418 (NEO) markers (18) were introduced biolistically using a Bio-Rad model PDS-1000/He biolistic particle delivery system as described previously (50). All the primers used in this study are listed in Table 2.

**Gene disruption and complementation.** To disrupt the *GSY1* gene, an overlap PCR product with the NAT marker amplified from plasmid pAI1 (23) and 5' and 3' flanking sequences of the *TPSI* locus from strain JEC21 (1,092 bp and 1,046 bp, respectively) was generated using primers JOHE117360, JOHE13918, M13 Forward, and M13 Reverse. The PCR product was directly introduced into strain JEC21 (MATa) by biolistic transformation. Homologous replacement mutants were screened by PCR and confirmed by Southern blotting. Isogenic MATa strains with the *GSY1* deletion were obtained by selecting NAT-resistant a progeny from a cross between the mutant a strains and the MATa strain JEC20.

**RESULTS**

Chlamydospore formation during hyphal growth in *Cryptococcus neoformans*. In response to nutrient limitation, desicc-
tion, darkness, and pheromone stimulation, *Cryptococcus neoformans* can switch from a single-celled yeast form to a multicellular filamentous form. During hyphal growth of this fungus, round mitotic blastospores are formed by mitotic budding from the edges of hyphal cells and oval meiotic basidiospores are produced in long chains on the surface of the basidium by basipetal budding (Fig. 1A and B). Close microscopic analysis of the sexual cycle, however, revealed that another type of structure could form either at the intercalary or terminal hyphae, and this structure resembled chlamydospores formed by other fungi (Fig. 1). These structures vary in size, but most are greater than 10 μm in diameter, significantly larger than the almost uniform 2- to 5-μm round blastospores or 1.5- to 3-μm oval basidiospores.

We were quite intrigued by these novel round structures observed during filamentous differentiation and considered three hypotheses. First, they might be water droplets whose role is to survive the desiccating conditions normally required to trigger filamentous growth. Second, they might be enlarged hyphal cells that have responded to pheromone, similar to the dramatically enlarged MATa cells observed during confrontational assays in which MATa cells have undergone isotropic expansion, likely to enhance fusion with conjugation tubes produced by α cells. Third, they might represent a novel cell type, namely, chlamydospores. The structures were not dispersed by micromanipulation, indicating that the structures had a solid surface and thus were not water drops. Since we observed these structures associated with hyphal growth during both mating (produced by a cross between a and α cells) and monokaryotic fruiting (produced by α cells alone) (Fig. 1C and D), this suggests that these structures in *C. neoformans* are not likely to be swollen hyphal cells in response to opposite mating pheromones, in contrast to what occurs with the swollen MATa cells induced by α pheromone prior to cell fusion during mating. Manipulating or isolating these chlamydospores, however, presents a technical challenge due to their large size and physical connection with the hyphae. Unlike blastospores or basidiospores, these structures are not formed by budding; instead, these unusual structures appear to be formed by the conversion of hyphal compartments themselves, consistent with observations of chlamydospore generation in other fungi.

We find it quite remarkable that, despite nearly a century of

FIG. 1. Chlamydospore structures associated with the filamentous growth of *C. neoformans*. (A) Blastospores bud off the hyphae. (B) Basidiospores on the basidium at the top of aerial hyphae. (C) Chlamydospores formed during monokaryotic fruiting. (D) Chlamydospores formed during mating. Cells of JEC21 (monokaryotic fruiting) or a JEC21 and JEC20 (mating) coculture were grown on V8 medium for 2 weeks at 22°C in the dark. Scale bars = 10 μm.
investigation, including an appreciation of the sexual cycle for three decades and of the monokaryotic fruiting cycle for a decade, the chlamydospores of *C. neoformans* have not been previously reported. Yet these structures are readily apparent under a variety of conditions, including during mating, monokaryotic fruiting, and filamentous differentiation of diploid cells. These structures were produced during hyphal growth in both *C. neoformans* var. *neoformans* and var. *grubii* strains, two predominant varieties found in clinical isolates (10). These findings reveal that further detailed morphological analysis of these processes is clearly warranted, as well as studies to understand the formation and function of these unique specialized cells, including their possible roles in energy storage and orchestration and support of the differentiation cascades that are likely involved in the generation of infectious basidiospores.

**Chlamydospores could be independent entities.** Indeed, the development of chlamydospores appears to be temporally and/or spatially regulated, as they first appear in the mycelial zone behind the actively growing hyphae during both mating and monokaryotic fruiting. Chlamydospores are predominantly produced before the production of abundant aerial hyphae (specialized hyphae bearing basidia), and become deflated (devoid of cytoplasm) in the center of an aged mycelium, where large numbers of basidiospores are produced and mature (Fig. 2).

Because in *S. cerevisiae*, G₀ arrest (a stage during which the cell cycle is arrested for an indefinite period) leads to the production of enlarged yeast cells filled with an enlarged vacuole, we hypothesized that the chlamydospore-like structures that we observed during filamentous growth could in fact represent G₀-arrested hyphal compartments filled with an enlarged vacuole. Intracellular organelles can be visualized using FM4-64, a membrane-selective dye that is internalized via endocytosis. As shown in Fig. 3, these chlamydospore-like structures contain numerous small vesicles or internal organelles, suggesting that these structures are not G₀-arrested enlarged hyphal compartments but are, on the contrary, metabolically active cells (Fig. 3A). To establish whether these structures contain genetic material, we stained the hyphae with DAPI and found that chlamydospores indeed contain nuclei (Fig. 3B), raising the possibility that these structures could be independently surviving cells. Interestingly, with prolonged incubation, these intercalary and terminal structures can be released from the hyphae after hyphal autolysis, again supporting the idea that these structures can be independent entities (Fig. 3C) and might survive longer than their producing hyphae. Furthermore, we observed that the chlamydospores could also give rise to yeast cells by budding or generate new hyphal branches, indicating that they are viable and capable of further cell division and reproduction (Fig. 3D). These observations raise the important question of their biological functions in the long-term survival for this pathogenic fungus in harsh environments.

**Chlamydospores are enriched in glycogen and may serve as energy stores.** As described above, chlamydospore formation in *C. neoformans* is first initiated near the periphery of the mycelium prior to the production of basidiospores. The chlamydospores become deflated in the center of the mycelium from which basidiospores have matured. Therefore, we hypothesized that chlamydospores may serve as an energy storage structure for basidiospore production and/or maturation. Nutrient limitation is one of the critical factors triggering the mating and monokaryotic fruiting in *C. neoformans* that lead to filamentous growth and fruiting body formation. Since nutrient transport in the long vegetative mycelium becomes inefficient as the mycelium expands, there may exist some stages of basidiospore formation or maturation that rely on stored carbon sources rather than their transport over longer distances. It is possible that the formation of energy-storing chlamydospores might serve to provide this type of stable energy source for sporulation through regulated translocation and degradation of its stored nutrients.

In order to begin answering these questions, we addressed whether chlamydospores of *C. neoformans* are energy storage structures. Because fungi can store energy in the form of lipids, we stained the mycelium with the lipid body dye Nile Red and...
found that the lipid accumulation level in chlamydospores is comparable with that in other sections of the hyphae (data not shown). We then examined whether these structures are rich in complex carbohydrates by staining the mycelium with iodine, which stains glycogen and starch. We found that the chlamydospores are highly enriched in iodine-stainable substances compared with other regions of the hyphae (Fig. 4A). Since fungi, like animals, store energy primarily in the form of glycogen, whereas plants store energy in the form of starch, this result suggests that the chlamydospore may serve as an energy store by accumulating glycogen.

What is the purpose of this energy storage? One possibility is that the fungus may use this specialized energy storage structure for basidiospore production and/or their maturation, as hypothesized above. Alternatively, energy stored in the chlamydospores could act purely as a carbon reserve for chlamydospores to promote their own long-term survival or reproduction. In many fungi, chlamydospores are long-term survival structures produced in response to harsh environments and a sufficient endogenous energy supply is essential for chlamydospores to conduct their survival functions. Moreover, it is also possible that energy stored in chlamydospores produced by *C. neoformans* during filamentous growth could serve both purposes, and which function they play may depend on the appropriate environmental cues. Characterization of mutants with altered glycogen levels can further elucidate the relationships between energy storage, formation of chlamydospores, and sporulation in *C. neoformans*.

The *gsy1* and *tps1* mutants show altered levels of cellular glycogen. Glycogen metabolism has been shown to be linked to sporulation in some fungi (7, 8, 12, 17, 21, 24, 42, 51). Because both glycogen and trehalose are products of branched glyco- genesis, they share some common precursors (Fig. 5) and there
are possible links between glycogen and trehalose metabolism during growth and differentiation (7, 16, 37, 38, 45, 48, 51, 52). We therefore decided to isolate both glycogen synthase and trehalose synthase mutants to study the effects of altered glycogen levels on the development of chlamydospores in \textit{C. neoformans}.

The glycogen synthase gene \textit{GSY1} and the trehalose synthase gene \textit{TPS1} of \textit{C. neoformans} were identified by BLAST searches with the \textit{S. cerevisiae} orthologous \textit{GSY1} and \textit{TPS1} genes against the \textit{C. neoformans} genome (http://www.tigr.org/tdb/ekl/cna1). Both genes showed high homology with the yeast counterparts (57.3\% identity for Gsy1 and 46.1\% identity for Tps1). The two genes were replaced with a drug resistance marker through biolistic transformation and homologous recombination. Disruption mutants were confirmed by PCR and Southern blot analysis (data not shown). As expected, the \textit{gsy1} mutant produces much less glycogen than the wild type (Fig. 4B). The \textit{tps1} mutant, however, accumulates more glycogen than the wild type. Reintroduction of the wild-type copy of either the \textit{GSY1} or the \textit{TPS1} gene restored each respective mutant to the wild-type phenotype. These mutant phenotypes are similar to those observed in \textit{S. cerevisiae} in which \textit{tps1} mutants hyperaccumulate glycogen and have elevated levels of glycogen synthase activity (9). This could be due to increased substrate availability for glycogen synthesis when the trehalose synthesis pathway is blocked. The \textit{TPS1} gene has also been shown to be essential for virulence in \textit{C. neoformans} var. \textit{grubii} strains (54). Our findings reveal that the \textit{gsy1} and \textit{tps1} mutations have opposing effects on the accumulation of cellular glycogen levels.

The \textit{gsy1} and \textit{tps1} mutants still form chlamydospores during mating yet have differing effects on filamentation and sporulation. Deletions of the \textit{GSY1} and \textit{TPS1} genes conferred opposing phenotypes in many respects. As mentioned above, the \textit{gsy1} mutant accumulates low levels of glycogen, while the \textit{tps1} mutant contains high glycogen levels. Furthermore, the \textit{tps1} mutation blocks monokaryotic fruiting, while the \textit{gsy1} mutation does not (Fig. 6A). Although both mutants do form filaments during bilateral mating (mutant \textit{a} cells crossed with mutant \textit{a} cells), the \textit{tps1} mutant displayed a reduction in filamentation. This is in part due to the fact that cell fusion in the \textit{tps1} mutant is less efficient (10\% of the wild-type level [data not shown]). The \textit{gsy1} mutant mated and sporulated normally, while sporulation in the \textit{tps1} mutant was blocked and development was arrested at the stage of basidium formation (Fig. 6B). Surprisingly, both mutants formed chlamydospores during mating (Fig. 6C), indicating that although glycogen is stored in chlamydospores, altered glycogen levels do not affect the formation of chlamydospores under these conditions; thus, events beyond simple glycogen accumulation must be responsible for the formation of these structures. These data also indicate that chlamydospore formation itself is not sufficient to support sporulation in \textit{C. neoformans} given that the \textit{tps1} mutant forms chlamydospores but not basidiospores.

Chlamydospore formation in \textit{C. neoformans} is species specific. In the dimorphic fungal pathogen \textit{C. albicans}, where some molecular insights into chlamydospore formation are available, the mitogen-activated protein kinase \textit{Hog1p} is required for chlamydospore formation, as are genes involved in morphogenesis (2, 46). Although \textit{Hog1} has also been shown to be a key regulator of stress responses in \textit{C. neoformans} as in other fungi (5), the \textit{hog1} mutation surprisingly did not affect chlamydospore formation in \textit{C. neoformans} (Fig. 7) and neither did mutations in the cyclic AMP signaling pathway (data not shown). In a recent study, a panel of 217 \textit{C. albicans} insertional mutants were screened for defects in chlamydospore formation and several genes were identified (39), suggesting that chlamydospore development in \textit{Candida} is a complex process regulated by multiple genes. In contrast, we screened 2,500 random insertional mutants produced by \textit{Agrobacterium} transformation and did not find any single mutation that blocks chlamydospore formation specifically; that is, all of the insertional mutants that produced monokaryotic hyphae also produced chlamydospores (data not shown). We did, on the other hand, find mutations that block filamentation and, by extension, chlamydospore formation, which supports the hypothesis that chlamydospore formation is intimately associated with filamentous growth. The failure to find genes specifically controlling the production of chlamydospores suggests that their formation is an integral part of filamentous growth in \textit{C. neoformans} or that redundancy in gene function associated with chlamydospore formation may mask developmental defects in strains with only single gene disruptions, as occurs in \textit{Agrobacterium}-mediated mutagenesis. Moreover, there may be far fewer genes that regulate this process in \textit{C. neoformans} and a larger screen may be needed to identify them. Our results therefore suggest that the regulation of chlamydospore development in \textit{C. neoformans} may be fundamentally different from the process in \textit{C. albicans}, necessitating species-specific research into the regulation of their production.

Besides this difference in genetic regulation, the chlamydospores of \textit{C. neoformans} are very different from those of \textit{C. albicans} in that the chlamydospores observed in \textit{C. neoformans} are likely to play a more dynamic role in cell physiology and differentiation. First, chlamydospores of \textit{C. neoformans} are more commonly observed to be intercalary instead of terminal, in contrast to the terminal chlamydospores produced by \textit{C. albicans}. Second, \textit{C. albicans} chlamydospores may have only very transient viability, and the germination of \textit{C. albicans} chlamydospores is very rarely observed (43). By contrast, chlamydospores of \textit{C. neoformans} are viable and capable of further cell divisions (Fig. 3D). These findings suggest that the roles of chlamydospores are likely to be distinct in different fungi and in different pathogenic fungi, and thus their functions and the pathways that give rise to them will require direct identification and analysis in each system.

**DISCUSSION**

A clearer understanding of the reproductive modes and survival structures of fungal pathogens in the environment is of great importance with regard to their ecology and epidemiology. Budding, conidiation, sporulation, fragmentation of hyphae, and conversion of hyphal elements into chlamydospores are common modes of reproduction. In some soilborne fungi, chlamydospores have been documented to have a role as survival structures. This study is the first report of \textit{C. neoformans} chlamydospores, which are produced behind the active hyphal growth zone during filamentous growth, and it elucidates a novel stage of the life cycle for this pathogen. Although
FIG. 6. Effects of the *gsy1* and *tps1* mutations on filamentation, chlamydospore formation, and sporulation. (A) The *tps1* mutant is blocked in filamentation during monokaryotic fruiting. From left to right are wild-type JEC21 (α), XL467 (α *gsy1*), and XL470 (α *tps1*). Cells were cultured on V8 medium for 1 week. (B) The *tps1* mutant is blocked in sporulation during bilateral mating. From left to right are crosses between JEC21 and JEC20, XL467 (α *gsy1*) and XL466 (α *gsy1*), and XL470 (α *tps1*) and XL468 (α *tps1*). Insets are enlarged images of basidia of corresponding mating cultures. Cells were cultured on V8 medium for 2 weeks. (C) Chlamydospore formation in a *gsy1* and *tps1* mutant during bilateral mating is normal. The order is the same as in panel B. Scale bars = 10 μm.
Chlamydospores have been observed in the related fungus *Cryptococcus laurentii* (30), it is, however, dangerous to extrapolate to other *Cryptococcus* species, as in the case in *Candida* species. Among all the *Candida* species, only the two most prevalent pathogens, *Candida albicans* and *Candida dubliniensis*, have been observed to produce chlamydospores, while other *Candida* species, such as *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Candida lusitaniae*, *Candida kefyr*, and *Candida guilliermondii*, do not.

Filaments of *C. neoformans* produce both intercalary and terminal chlamydospores, which are potentially fully functional (independent from the mycelium) and physiologically active, as they are capable of generating new branches and yeast cells. Although we found that chlamydospores in *C. neoformans* are enriched in glycogen, mutants with altered glycogen levels still form chlamydospores, suggesting the possibility for the storage of other materials and regulatory mechanisms independent of glycogen accumulation. Whether the energy stored in the chlamydospores is for their own survival and reproduction or to support proficient basidiospore production and/or matura-
tion is an important unanswered question. Since we were unable to identify any single mutation that specifically blocks chlamydospore formation, the exact nature of the process that leads to chlamydospore production and the biological function of the structures remains to be defined. The inability of our genetic screen to separate the formation of chlamydospores from filamentous growth suggests that chlamydospore production could be an integral part of hyphal growth in *C. neoformans* in response to harsh environments.

Basidiospores are proposed to be the propagules for *C. neoformans* dispersal and infection, and it is likely that basidiospores are also long-term survival structures in nature. However, in many other fungi, chlamydospores serve this role. Our identification of chlamydospores in *C. neoformans* suggests the interesting possibility of an overlooked role for these structures in the popular model of *C. neoformans* survival and propagation. It is possible that these two different reproduction modes of *C. neoformans* coexist in nature and serve independent biological roles, or alternatively, there may be a key connection between the formation of chlamydospores and the production of basidiospores. We have shown that the formation of chlamydospores is apparently independent of the production of basidiospores, given that the *tps1* mutant is blocked in sporulation during mating but can still form chlamydospores, this does not mean that sporulation is independent of chlamydospore formation. The availability of large-scale screens of insertional mutants or of a genome-wide deletion mutation collection in *C. neoformans* may yield insight into the relation-
ship between these two processes and provide a model for chlamydospore production in related fungi.

During our small-scale screen of insertion mutants, we did notice an inverse relationship between blastospore and chlamydospore production by vegetative hyphae, suggesting that there may be a balance between the formation of these two reproductive forms. We also observed that robust blasto-
spore formation is usually associated with suppressed hyphae, poor aerial hyphal production, and sporulation, while chlamydospore formation is associated with better aerial hyphae pro-
duction and sporulation. The different developmental path-
ways to produce blastospores or chlamydospores might reflect the choice that the hyphae make, either to maintain vegetative growth and multiply rapidly or to enter terminal growth, leading to the production of aerial hyphae and basidiospores. The balance between the three reproduction forms (blastospores, chlamydospores, and basidiospores) may be dependent on ge-
etic background, developmental stages, and environmental cues that require further clarification and may yield new clues to the nature and formation of infectious *C. neoformans* propagules.

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