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Dynamics of CaCdc10, a septin of *Candida albicans*, in living cells and during infection

Summary. The morphogenetic program in the pathogenic fungus *Candida albicans*, including the dimorphic transition, is an interesting field of study, not only because it is absent in the commonly used model yeast *Saccharomyces cerevisiae*, but because of the close relationship between hyphal development and virulence of *C. albicans*. We studied one of the most important aspects of fungal morphogenesis—the septin ring—in *C. albicans*. By using a fusion construct to green fluorescent protein (GFP), the subcellular localization and dynamics of *C. albicans* Cdc10 in the different morphologies that this fungus is able to adopt was identified. The localization features reached were contrasted and compared with the results obtained from *Candida* cells directly extracted from an animal infection model under environmental conditions as similar as possible to the physiological conditions encountered by *C. albicans* during host infection. [**Int Microbiol** 2004; 7(2):105–112]

Key words: *Candida albicans* · dimorphism · septin Cdc10 · systemic infection

Introduction

Candida albicans is the most prevalent pathogenic fungus that affects humans, producing superficial mucosal or systemic infections in immunocompromised patients [16]. *C. albicans* is able to grow in a broad variety of forms: budding yeast, pseudohyphae (elongated chained cells bearing constrictions between each cell), and hyphae (long homogeneous tubes with no constrictions). It can also switch between these forms in response to external conditions, such as temperature, pH, the presence of serum or macrophages, and certain synthetic culture media [7,41]. The importance of studying hyphal development in *Candida* is supported by the close relationship between this growth form and its virulence [41]. It is clear that the genetic program responsible for the morphologies of *Candida* must be different and unique in each form [37]. This, together with the fact that the most widely employed yeast model for morphogenetic studies, *Saccharomyces cerevisiae*, lacks true hyphal development, make *C. albicans* an appropriate organism for the analysis of such a morphogenetic program. (For a recent review of *Candida* facts see [4].)

In *S. cerevisiae*, one of the key elements in morphogenesis is the septin family, a group of seven proteins. Two of them, Srp3 and Srp28, are produced during the sporulation program of development [12,18]. The other five, encoded by *CDC3*, *10*, *11*, *12* and *SEP7*, are expressed during the mitotic cell cycle and their products assemble in vivo at the mother-daughter neck, forming the so-called 10-nm ring [8,21,25,29]. These proteins are found in many organisms

from yeast to mammals, including flies, worms and mice [17,30,40]. The septin-based ring has been found to be deeply involved in events very important to the cell, including the selection of cell polarity [2,9], chitin deposition at the septum [13], the establishment of the morphogenesis checkpoint [38,39], the spatial localization of the septation machinery at cytokynesis [5,33], and the formation of a barrier separating mother and daughter cells in order to regulate polarity and morphogenesis of the cell [3,48]. (For a recent review on septins see [19].)

In *S. cerevisiae*, septin ring assembly is dependent on START accomplishment in the G1 phase of the cell cycle [10]. It appears as a single ring structure on the mother side of the neck. The ring is kept in that position, with a symmetric neck-spanning morphology, until anaphase, when it splits into a double ring, depending on the state of Clb degradation and CDK inactivation [10]. Recently, the small GTPase Tem1 has been proposed to be essential in this process for the control of septin dynamics during cytokinesis [34].

In *C. albicans*, following the pioneer studies of Byers and Goestch [8], a ring at submembrane level in the mother bud junction was localized by transmission electron microscopy [46]. These authors also detected this structure in the mycelial form of *Candida,* where it was related to the septation process. More recently, the septin ring in this fungus has been detected by employing antibodies that specifically recognize the septin Cdc11 from *S. cerevisiae* [46] and by GFP fusions to CaCdc3 and CaCdc10 [22,50] (hereafter, to avoid confusion, *C. albicans* genes or proteins will be referred to using the prefix "Ca-"). Here we report the localization and dynamics of the septin structures. The physiological significance of the results obtained, both in the "in living cells" approach of this work and in other septin localization reports, has been corroborated here by studying septin localization during host infection.

Materials and methods

Strains, media and culture conditions. The *S. cerevisiae* strains used were VCY1, bearing the thermo-sensitive allele *cdc10–11*, and the respective isogenic wild-type strain 1783. Dr. Víctor J. Cid kindly supplied both. The *C. albicans* strain used was CAI4 (*ura3*::*imm434*/*ura3*::*imm434*) [20]. For general purposes, yeasts were grown in 250-ml flasks containing 50 ml yeast peptone dextrose (YPD) (2% glucose, 1% yeast extract, and 2% peptone, w/v) or SD (1.7 gDifco nitrogen base without amino acids/l; 0.5% ammonium sulfate and 2% glucose, w/v) plus the required amino acids for plasmid maintenance. For the induction of hyphae and pseudohyphae in *C. albicans*, 10 and 5% (v/v) fetal calf serum was added to the culture media, respectively. The Lee's, Spider and SLAD media were as described, respectively, in [24,32,35]. Yeast growth temperatures were 28ºC for general purposes and 37ºC for expression of the phenotype in Ts^- mutant strains or induction of the filamentous morphology. The pseudohyphal morphology was induced at 35ºC.

Mouse strains and infection model. The mice strains employed in the infection model were DBA/2, Balb/C (Charles Breeding Laboratories, Wilmington, MA) and CD1. The animals were infected through the lateral tail vein. In DBA/2, 4×10^6 *C. albicans* cells were injected whereas the more resistant Balb/C and CD1 mouse strains, were injected with 4×10^7 cells. Mice were killed 24–48 h after infection; kidneys and brains were extracted, homogenized and washed with PBS buffer.

DNA manipulations. Except where specified, standard procedures were used for DNA manipulation [43]. *C. albicans* transformations were carried out using a combination of the electroporation and lithium acetate methods [31]. Genomic DNA from CAI4 *C. albicans* strain was obtained as described elsewhere [44]. The oligonucleotides (supplied by Isogen, Maassen, Netherlands) used for sequencing, PCR, or the genomic CaCdc10 fusion to green fluorescent protein (GFP), are listed in Table 1 (the restriction enzyme sequences inserted to facilitate cloning and manipulation are indicated).

The plasmid bearing *GFP3* and optimized for the genetic code of *C. albicans* has been described in [11] and plasmids bearing the cassette for GFP tagging directly to the chromosome have been described in [23]. pIR4 is a plasmid based on the pRM1 plasmid [42], containing *CaURA3* and *CaLEU2* as selectable markers and the ARS2 sequence. pRI4 was constructed by cloning the actin promoter (ACT-p) into the *Bam*HI/*Hin*dIII site of pRM1 plasmid.

Staining procedures and microscopy. Time-lapse microscopy was carried out using a Leica DMRXA fluorescence microscope, to which a thermostatted device (supplied by Linkam, Surrey, UK) had previously been

Table 1. Oligonucleotides used in this study

Primers	Sequences
CDC10upper	GCAGATCTCCCGGGAACATCATGATCGAAGTCC
CDC10lower	GCAGATCTCCCGGGTGGTGGTCTAGCAGCAGCAGTACC
GFP3UP	GCCCCGGGACAAGCTTTATTAAAATGTCTAAAGG
GFP3LW	GCAGGCCTCTGCAGTTATTTGTACAATTC
CaCDC10UP	GCAGATCTCCCGGGAACATCGAGTACTTACCCACTAGATAAGC
CaCDC10RP	GCAGATCTCCCGGGTGGTGGTCTAGCAGCAGCAGTACC
tagCDC10-UP	TTTGAAGAACGCCTCTGGTGTGCCAAATGCTCCTATGTTCCAATCAACTACAGGTACTGCTGCTGCTAGAGGTGGTG GTTCTAAAGGTGAAGAATTATT
tagCDC10-RP	AACACACAAAAGAAGAGGAATACAAAAAAGTAAAATCACATTATATCAATAACAAACATTATTTATCTATTCTAGAAG GACCACCTTTGATTG

fitted in order to maintain the cells at a temperature suitable for the morphological switch. Cells were grown overnight at 28ºC, plated onto a thick layer of adequate medium [26], and subsequently kept at a constant temperature while examined using a thermostatted microscope. The cells of interest were followed, and photographed when interesting morphological changes occurred. The microscope slide was located on the heater device at 28, 35 or 37ºC in order to observe the different morphologies. Nuclei staining was as follows: 4 µl of cells was spread out on a slide, allowed to dry. They were then resuspended in 2.5 µl of DAPI solution (2.5 µg/ml) and mounted for observation.

Cloning of *CaCDC10*. *CaCDC10* had been previously cloned by suppression of a *S. cerevisiae* septin mutant [14]. The sequence was entered into the EMBL database under the accession number Z25870. *CaCDC10* was cloned by PCR, employing the primers CaCDC10UP and CaCDC10RP (see above). The cloned DNA fragment was able to suppress a *cdc10-11 S. cerevisiae* mutant strain (data not shown).

Green fluorescent protein fusions to *CaCDC10*. In all fusions, GFP was placed at the carboxy terminus of CaCdc10. Two prolines were inserted between both proteins in order to preserve their correct folding. The pAG1 plasmid, in which the CaCdc10-GFP fusion was generated, is based on the pIR4 plasmid, which carries the *ACT1* promoter and a *C. albicans* ARS sequence. *GFP3* was previously obtained by PCR, using GFP3UP and GFP3LW as primers and the plasmid pYGFP3 as template (kindly supplied by Dr. Brendan P. Cormack). The PCR product was *Sma*I/*Stu*I-digested and cloned into the *Sma*I site of pIR4. pAG1 was digested again with *Sma*I, and the *CaCDC10* ORF (previously obtained by PCR and confirmed by sequencing) was introduced at this site, affording plasmid pAG2. This plasmid thus bore the *CaCDC10-GFP* fusion under control of the *ACT1* promoter. It was designed to enable genome integration at the *LEU2* locus. In such experiments (data not shown), pAG2 was introduced into the *C. albicans* genome at the *LEU2* locus by digestion of pAG2 with *Kpn*I and linear transformation with the CAI4 strain.

The fusion under the control of the *CaCDC10* self-promoter was developed by PCR amplification of the *CaCDC10* ORF plus 500 bp upstream from the ATG of the gene. The oligonucleotides used were CaCDC10UP and CaCDC10RP (see above). This DNA fragment was introduced into the pGEMT plasmid, digested with *Sma*I and inserted into the *Sma*I site of pAG1, affording the *CaCDC10-GFP* fusion under the control of the *CaCDC10* self-promoter. The chromosome fusion in strain CAI4 was developed following the method described in [23], employing the tagCDC10UP and tagCDC10RP oligonucleotides (see above).

Results

Fusion of *CaCDC10* to GFP. Four different kinds of fusions were developed (see Materials and methods). The localization results obtained employing the different fusions were very similar in all experiments; differences were only detected in the proportion of cells showing the GFP-signal but not in its subcellular distribution. This proportion was almost 100% in the case of integration in the *LEU2* locus and 80% in the case of the self-replicative plasmid. Regarding the promoter employed, the only remarkable difference was a fainter signal when the self-promoter was employed, but identical dynamics and distribution were observed in each case. In order to obtain a better signal, mainly in the time-lapse assays (detailed below), and to avoid bleaching of GFP fluorescence during the prolonged ultraviolet illumination, only the fusion in the self-replicative plasmid under the control of the *ACT1* promoter was followed.

Localization of CaCdc10 in the different morphology patterns of *C. albicans*. Plasmid pAG2 bearing the *CaCDC10-GFP3* fusion under the control of the *ACT1* promoter was introduced into *C. albicans* strain CAI4 [20]. The transformants were grown under conditions that enabled a budding morphology (detailed in Materials and methods). Time-lapse microscopy assays revealed that, before a bud is formed, a ring of CaCdc10 is joined at the area of hottest polarity (Fig. 1A, blunt arrows). The new bud emerges through the septin ring (Fig. 1A, 3rd image from the left). During bud growth, the GFP signal becomes diffuser and broader, extending along the boundary between both cells. Finally, the GFP signal is seen as a double ring (Fig. 1A, arrows), but only when the nuclei are completely separated, indicating that anaphase of the cell cycle has been accomplished, as observed with DAPI and GFP double staining (Fig. 2).

Next, we studied the septin-ring dynamics in hyphal morphology by inducing hyphae production in *C. albicans* strain CAI4 (transformed with pAG2 plasmid). The process was followed by time-lapse microscopy and the results are shown in Fig. 1C, D. In this morphology, the first septin ring was found inside the germ tube but at the mother cell/germ tube junction. This ring followed a trend similar to the one observed in budding cells (see above); first, the appearance of a single ring that becomes diffuse followed by the formation of a double ring (Fig. 1D). At the mother/germ tube junction, as described for CaCdc10 and CaCdc11 [46,50], a septin structure that was not as organized as rings, and that was much fainter, was observed. This "pseudo-ring" was seen just prior to the emergence of the germ tube and disappeared when the first proper septin ring was assembled several micrometers inside the germ tube (Fig. 1C). In order to demonstrate that CaCdc10-septin rings mark the septum within the cylindrical hyphal structure, the septum was co-stained with GFP and calcofluor white (a specific dye for chitin). The two signals colocalized at the same point of the hyphae (data not shown). Furthermore, co-staining hyphae with calcofluor white and DAPI revealed a single nucleus between every two chitin signals (data not shown).

The pseudohyphal pattern of growth was studied next. *C. albicans* cells harboring pAG2 were grown, pseudohyphal development was induced, and the cells were analyzed by time-lapse microscopy. The results are shown in Fig. 1B, in

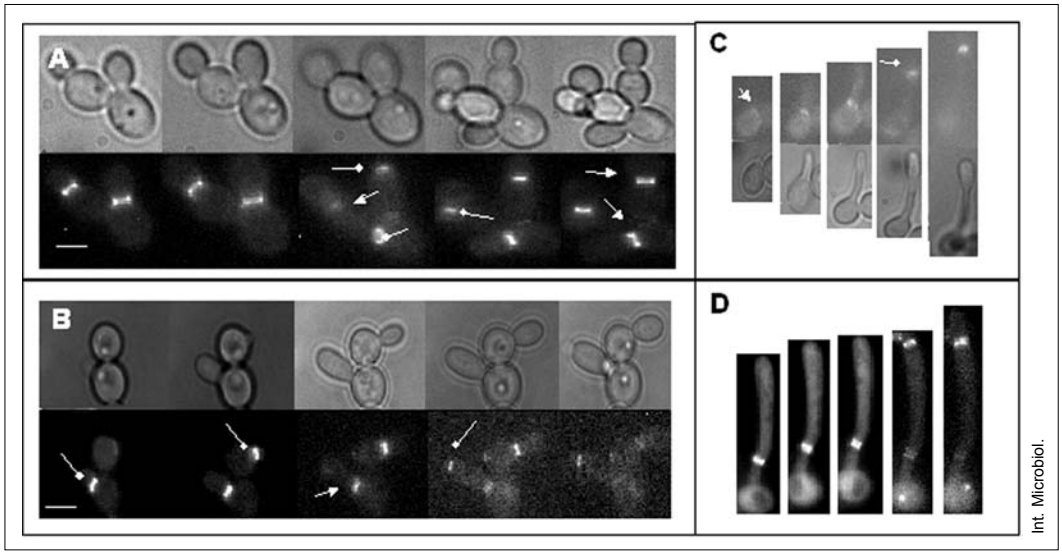

Fig. 1. CaCdc10-GFP localization structure and dynamics. Time-lapse fluorescence microscopy of CAI4 cells transformed with the pAG2 construction containing a GFP3-CaCDC10 carboxy-terminus fusion expressed under the actin ACT1 promoter. Each figure parts consists of a phase-contrast and a fluorescence image (top and bottom, respectively). Arrows indicate the appearance of a double ring, and blunt arrows show the single ring. (A) Cells growing at 28ºC and exhibiting the yeast morphology behavior.(B) The same cells growing on SD agar microlayers containing 5% fetal calf serum and kept at 35ºC to induce pseudohyphal morphology. (C, D) Same cells growing on SD agar microlayers containing 10% fetal calf serum and incubated at 37ºC for hyphal development. In C, the early stages of germ tube emission were followed until the first true ring was established. In this period of time, the existence of the "faint" signal at the base of the germ tube, described in the text, can be observed. In D, a cell displaying the true hyphal morphology at a later stage than in C is shown. No phase-contrast images are shown because of the clearly marked shape of the cell in the fluorescence image.

which the appearance of the septin scaffold just before the emergence of the bud can be appreciated (blunt arrows). These results are similar to those seen in the budding behavior (described above). Subsequently, the septin ring splits into a double ring (Fig. 1B, arrows). Finally, when the cell cycle round is finished, the septin structure is slowly disorganized. However, in our experiments there was a short period of time in which the septin rings from two different cell cycle rounds co-existed.

Localization of CaCdc10 in an animal infection model. With a view to reducing interferences arising from the "in vitro" incubation and culture of *C. albicans* cells, and to better reproduce the environmental conditions encountered by *C. albicans* during host infection, the septin localization assays were repeated in a mouse infection model. To do so, mice were injected through the lateral tail vein with cells of *C. albicans* bearing the *CaCDC10-GFP* fusion, as detailed in Materials and methods. The three different mouse strains employed (Balb/c, DBA/2 and CD1) orchestrate a TH1 protective immune response, a TH2 non-protective immune response, and an intermediate response, respectively [15]. There was no remarkable difference, in terms of virulence, between *Candida* strains with or without the CaCdc10-GFP fusion (data not shown). Also, regarding organ colonization, cell morphology, or hyphae vs. pseudohyphae proportions, no differences were found between the different mouse strains employed (data not shown).

Candida cells obtained directly from the kidneys and brains of the mice were observed directly for GFP staining. In these experiments, the GFP signal was poor, probably due to the exposure of GFP to the high body temperature of the animals for long periods of time. Nonetheless, the GFP signal was localized to the typical single and double ring very similar to the results described above for hyphal morphology (Fig. 3A). However, in kidneys and in brains, the GFP signal was associated with all the septin rings in all the septa of the hyphae. This was a clear difference to the results obtained previously in the "in living cells" experiments, in which the septin signal was only detected in one area of the septum, ie. the area that is active, in terms of polarity, at that time. Similar results were recorded when hyphal development was induced by growing the cells in Lee's medium (Fig. 3B); under these conditions, the CaCdc10 signal remains visible in all the septa of the hyphae but to a lesser extent. This was not the case when cells were grown in Spider or SLAD medium or in other artificial culture media detailed before (data not shown and Fig. 1D).

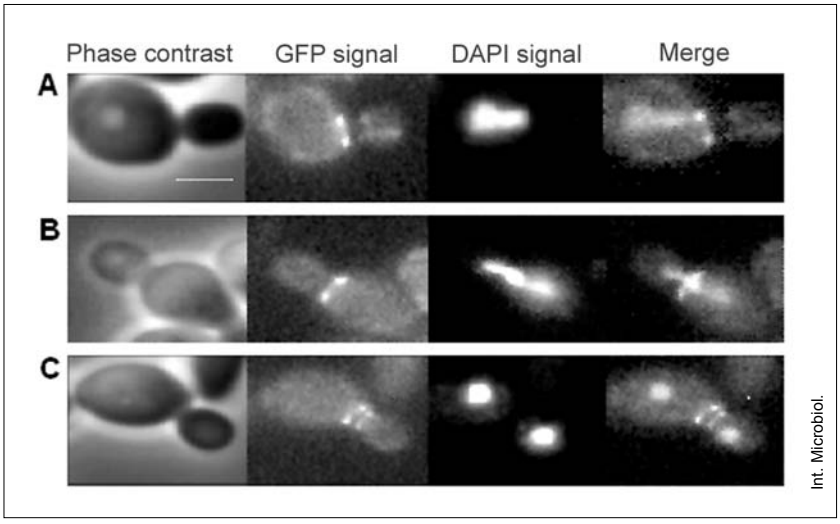

Fig. 2. Cell-cycle dependence of septin dynamics in yeast morphology. CAI4 cells transformed with the pAG2 plasmid were incubated overnight at 28ºC, processed for nucleus staining (using DAPI), and visualized under a fluorescence microscope. (A) G1 cell. (B) Cell in the M phase (metaphase) of the cell cycle. (C) Cell in late-anaphase or telophase of the cell cycle. Nuclei are completely separated and the septin ring appears as a double ring. The merged images are a computer composite of the GFP and DAPI images.

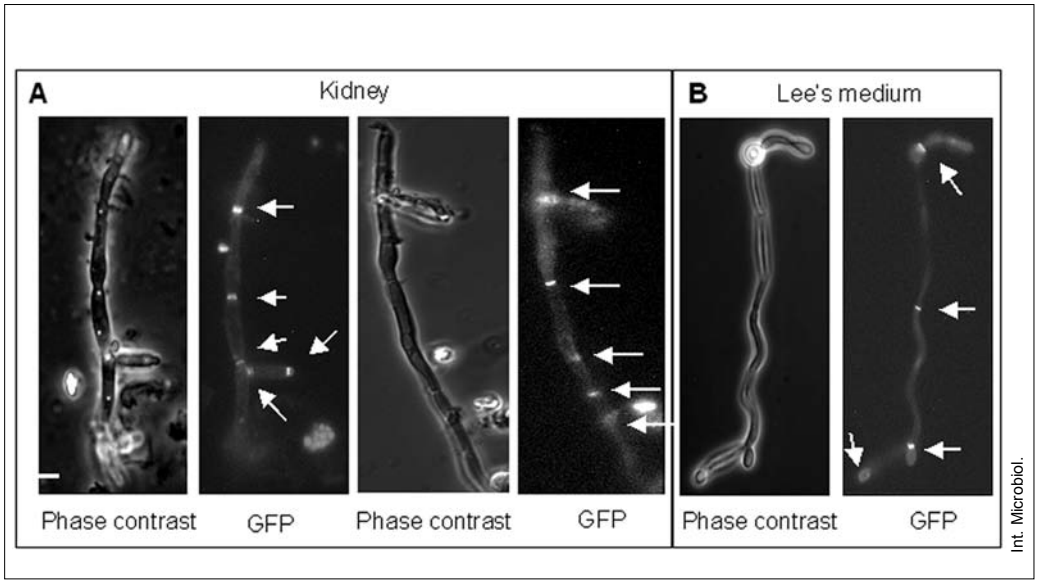

Fig. 3. CaCdc10 localization in C. albicans cells obtained directly from mice in an experimental model of infection. (A) Cells of strain CAI4 bearing the pAG2 plasmid were grown in an overnight culture at 28°C. Subsequently, 4×10^6 and 4×10^7 cells were injected through the lateral tail vein of DBA/2 and Balb/C mice, respectively. Animals were killed 24–48 hours later, and kidneys and brains were extracted, homogenized, washed and mounted for microscope observation. In order to avoid repetitions, only cells extracted from a kidney are shown. (B) Cells of strain CAI4 bearing the pAG2 plasmid were grown overnight at 37ºC in Lee's medium and directly observed under the microscope.

Discussion

Subcellular localization of CaCdc10, a septin from *C. albicans*. Several proteins of the septin structure have been localized in *C. albicans*: CaCdc11, by immunolocalization, employing antibodies generated against *S. cerevisiae* Cdc11 [46]; CaCdc3, by a GFP fusion [22], and, in this work, CaCdc10, by a GFP fusion [50]. However, the results presented here are the only ones in which the dynamics of this protein were studied by time-lapse assays. The localization data obtained by other investigators and by our own group point to some interesting differences, such as the absence of Cdc3 at the base of the germ tube projections, a site where Sudbery, Warenda and Konopka, and our group were able to detect Cdc11 and Cdc10. The structure visualized in that localization differs from the typical single or double ring into which septins are organized; it is more diffuse and relaxed, and is only short-lived (detailed below). All these observations support the possibility that different molecular structures and/or compositions can be assigned to this structure and to the 10-nm ring.

The dynamics of the septin ring structure are the same during the budding cycle in both *S. cerevisiae* [10] and *C. albicans*, as detailed in Results. The data presented here also support the notion of a conserved role for the septin structure in both organisms.

***C. albicans* as a model for the study of hyphal morphogenesis.** In special media or under certain envi-

ronmental conditions, *S. cerevisiae* is able to grow in a pseudohyphal fashion [24] but not with a true hyphal pattern of growth. Interestingly, the hypha is the morphology adopted by *C. albicans* when it infects host tissues [41]. In this context, it is generally accepted that *C. albicans* virulence is closely related to its ability to develop mycelia [15,37]. There are exceptions to this assumption, however, as is the case of *hog1* mutants in the MAP kinase CaHog1 or mutants in the CaTup1 regulator, both of which show a hyper-filamentous phenotype and reduced virulence [1,6]. These features make *C. albicans* an interesting model for the study of morphogenesis during hyphal development. A clue to morphogenesis is the septin-based cytoskeleton. The study of this structure in hyphal development reveals dynamics similar to those previously discussed for budding forms. However, some interesting differences were detected, such as the existence of a "faint septin structure" that appears at the base of the germ tubes, as first described by Sudbery [46]. The existence of this structure supports the idea of the central role played by septins in all polarized patterns of development [36,38].

Based on this hypothesis, it is tempting to speculate that, when a particular pattern of morphogenesis is initiated (germ tube emission), it must be supported by the corresponding septin-based cytoskeleton, such as the "faint signal". In keeping with this idea, the existence of a relationship between the structure in which septins are assembled and the morphology shown by the new cell can be suggested.

Localization of CaCdc10 of *C. albicans* during the infection of a host. The signals and stimuli required by *C. albicans* to switch morphologies are very complex and are not fully understood. Thus, in order to reproduce as much as possible the conditions encountered by *C. albicans* during the infection process, the localization of CaCdc10 was studied in cells extracted directly from a host, without subsequent incubation in culture media. This is the first report of the localization of a GFP-tagged protein in an animal infection model. The results obtained in all host mouse strains and in all the organs analyzed were identical: no differences in septin localization or structure were detected with respect to the different immune responses orchestrated by the different mouse strains employed or by the different accessibility of *C. albicans* to the mouse organs assayed.

The most impressive difference between the "in living cells" and during infection assays was the persistence of septin localization during several rounds of the cell cycle in the hyphae extracted from the host; in other words, the double ring is not disassembled when the next ring is settled. Thus, it is possible to find *C. albicans* hyphae showing several double septin rings. In *S. cerevisiae*, Smt3/SUMO has been described to be involved in the covalent modification of septins [27], and Siz1, an E-3 type enzyme responsible for the conjugation of Smt3 to the septins, has also been described [28,47]. Both proteins, as well as others described in different organisms [45], are involved in the turnover of the old septin structure. It would obviously be of great interest to explore the existence of these proteins in *C. albicans* and, if they were found, why they fail to function when *C. albicans* hyphae are present in the host. Other interesting alternatives are suggested in [49]. These authors described, in *S. cerevisiae*, the Cdc28-kinase-dependent phosphorylation of Cdc3 for correct septin ring degradation during G1. Therefore, understanding the function of CaCdc28 during infection of the host would no doubt shed light on the differences in septin structure described here.

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Dinámica de CaCdc10, una septina de *Candida albicans*, durante el proceso de infección y en células cultivadas in vitro

Resumen. La morfogénesis del hongo patógeno *Candida albicans*, incluyendo el fenómeno de transición dimórfica, es un interesante campo de estudio, no sólo por estar ausente en *Saccharomyces cerevisiae*, que es el modelo habitual de levadura en los estudios morfogenéticos, sino por la correlación existente entre virulencia y filamentación en *C. albicans*. Este trabajo describe el estudio de uno de los aspectos fundamentales de la morfogénesis fúngica, el anillo de septinas, en *C. albicans.* Usando el método de fusión con la proteína verde fluorescente (GFP), se identificó la localización subcelular y la dinámica de la septina Cdc10 de *Candida albicans* en las diferentes formas que puede adoptar este hongo. Los datos obtenidos se compararon y contrastaron con los logrados al extraer las células de *Candida* directamente de ratones previamente infectados con dicho hongo, en condiciones ambientales lo más parecidas posible a las condiciones fisiológicas que *Candida* encuentra al infectar un huésped. [**Int Microbiol** 2004; **7**(2):105–112]

Palabras clave: *Candida albicans* · dimorfismo · septina cdc10 · infección sistémica

Dinâmica de CaCdc10, uma septina de *Candida albicans*, durante o processo de infecção e em células cultivadas in vitro

Resumo. A morfogênesis do fungo patogênico *Candida albicans*, incluindo o fenômeno de transição dimórfica, é um interessante campo de estudo, não só por estar ausente em *Saccharomyces cerevisiae*, que é o modelo habitual de leveduras nos estudos morfogenéticos, como também devido à correlação existente entre a virulência e a formação de hifa em *C. albicans*. O presente trabalho descreve o estudo de um dos aspectos fundamentais da morfogênesis fúngica, o anel de septinas, em *C. albicans*. Usando o método de fusão com a proteína verde fluorescente (GFP), foi observada a localização subcelular e a dinâmica da septina Cdc10 de *Candida albicans* nas diferentes formas que este fungo pode adotar. Os dados obtidos foram comparados e contrastaram com os obtidos ao extrair as células de *Candida* diretamente de modelos de infecção animal com o referido fungo, realizados em condições ambientais o mais semelhante possível às condições fisiológicas que *Candida* encontra ao infectar um hospedeiro. [**Int Microbiol** 2004; 7(2):105–112]

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