A *Candida albicans* chaperonin subunit (CaCct8p) as a suppressor of morphogenesis and Ras phenotypes in *C. albicans* and *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* and the pathogen *Candida albicans* can be induced to undergo morphogenesis from a yeast to a filamentous form. A *C. albicans* gene (*CaCCT8*) was identified encoding a subunit of the Cct chaperonin complex, whose expression prevents filament formation in both fungi without interfering with growth of the yeast form. In *S. cerevisiae*, pseudohyphal growth induced by Ras2^{Val19}, by overproduction of Phd1p or by expression of the *C. albicans* *EFG1* gene, was blocked by CaCct8p and its N-terminally deleted derivative CaCct8-Δ1p; in contrast, pseudohyphal induction by other components (Cph1p, Cdc42p) could not be suppressed, indicating that morphogenesis *per se* is not inhibited. *CaCCT8* expression also interfered with other Ras2p^{Val19} phenotypes, including heat sensitivity, lack of glycogen accumulation and lack of sporulation. In *C. albicans*, overproduction of CaCct8p effectively blocked hyphal morphogenesis induced by starvation conditions and by serum. The results suggest that the activity of a component in the Ras2p signal transduction pathway is suppressed by excess chaperonin subunits. This component may be a novel folding target for the Cct complex. In agreement with this hypothesis, disruption of one of the two *CaCCT8* alleles in *C. albicans* led to defective hyphal morphogenesis.**

Keywords: *Candida*, morphogenesis, dimorphism, chaperonins, Cct complex

INTRODUCTION

An ability to interconvert between different growth forms is considered an important virulence trait of many pathogenic fungi. The human pathogen *Candida albicans* is able to grow as a budding yeast, but depending on environmental conditions, growth can also occur in a filamentous form. At high temperatures and induced by a variety of compounds, such as serum components, *C. albicans* forms true unconstricted hyphae consisting of mononucleate cells separated by septae (reviewed by Odds, 1988). The molecular mechanisms leading to the establishment of yeast and hyphal growth forms are

poorly understood. An evolutionarily conserved mitogen-activated protein (MAP) kinase cascade that is essential for filamentous growth of the yeast *Saccharomyces cerevisiae* is not absolutely required for morphogenesis and virulence of *C. albicans*. Mutants lacking the MAP kinase components Cst20p, Hst7p and Cph1p show normal hyphal development in liquid media and during serum induction, but reduced hyphal formation on certain solid media; *hst7* mutants are fully virulent in the mouse infection model, while *cst20* and *cph1* mutants only show an attenuated virulence (Köhler & Fink, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1994; Lo *et al.*, 1997). Recently, Efg1p, an essential regulator of *C. albicans* morphogenesis and virulence, has been identified and belongs to a novel class of bHLH proteins that control morphogenetic processes in fungi (Lo *et al.*, 1997; Stoldt *et al.*, 1997).

Although *S. cerevisiae* does not form true septated

Abbreviations: MAP, mitogen-activated protein; PKA, protein kinase A. The GenBank accession number for the sequence reported in this paper is U37371.

hyphae, some diploid strains are able to bud in a unipolar fashion with elongated cells that remain attached, thereby leading to a hypha-like appearance (pseudohypha) (Gimeno *et al.*, 1992). The MAP kinase module containing Ste20p, Ste11p, Ste7p and Kss1p, which activates the transcription factor Ste12p, is required for pseudohyphal morphogenesis (Liu *et al.*, 1993; Madhani *et al.*, 1997). The Tec1 transcription factor acts in concert with Ste12p to regulate genes involved in filamentous growth (Gavrias *et al.*, 1996; Madhani & Fink, 1997). Activated Ras2 protein stimulates morphogenesis via Cdc42p, Ste20p and the MAP kinase pathway, possibly in a PKA (protein kinase A)-independent manner (Mösch *et al.*, 1996). In contrast, recent evidence strongly suggests that a cAMP-dependent pathway is crucial for pseudohyphal formation. Mutations in the MAP kinase pathway can be suppressed by hyperactivation of the cAMP pathway, e.g. by alleles of *GPA2* encoding a constitutively active G-protein α -subunit or by externally added cAMP (Kübler *et al.*, 1997; Lorenz & Heitman, 1997). Similarly, overproduction of Phd1p, a homologue of the *C. albicans* Efg1 protein, is able to suppress mutations in the MAP kinase pathway (Lo *et al.*, 1997).

Filamentous growth in fungi is the consequence of a polarized assembly of components in cells, requiring cytoskeletal proteins, including actin and tubulins (reviewed by Kron & Gow, 1995). It has been shown that in eukaryotes the cytosolic chaperonin complex containing Tcp-1 (Cct1p) has a narrow substrate specificity and is known to fold actin and tubulin monomers (reviewed by Kubota *et al.*, 1995). Cct is a hetero-oligomer complex consisting of eight different Cct subunits, which in yeast are encoded by essential genes (reviewed by Stoldt *et al.*, 1996). Tcp-1 (Cct1p) overproduction is known to partially suppress actin mutations (Ursic *et al.*, 1994) and recently, *CCT6* (*TCP20*) overexpression was shown to suppress mutations in *TOR2* affecting the polarized distribution of actin (Schmidt *et al.*, 1996). Cct can bind newly synthesized actin and tubulin and a restricted set of minor unidentified polypeptides *in vivo* (Sternlicht *et al.*, 1993). Here we present evidence suggesting that a component of the Ras pathway may be one of the unidentified targets for the Cct complex. We show that an extraneous Cct subunit (CaCct8p), while not affecting yeast growth, suppresses filamentous morphogenesis in *C. albicans* and *S. cerevisiae*, as well as several Ras phenotypes. Thus, excess chaperonin subunits appear to act as dominant-negative suppressors of the cAMP branch of signal transduction pathways leading to fungal morphogenesis.

METHODS

Strains and growth conditions. *C. albicans* strain CAI4 (Fonzi & Irwin, 1993) was used as host for recombinant plasmids and gene disruption. *S. cerevisiae* strain MB331-17A, previously referred to as B76 (Stoldt *et al.*, 1997), had been obtained from a cross of MB211-3B (Σ 1278b background) and DBY785 (Σ 288C background) (Brandriss, 1983); the isogenic diploid of

MB331-17A, designated B76/4, developed pseudohyphae in low nitrogen conditions (Stoldt *et al.*, 1997). *S. cerevisiae* strain L5976 (*MATa/MAT α ura3-52/ura3-52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG*), which is congenic to the Σ 1278b background (Gimeno *et al.*, 1992), was provided by H.-U. Mösch (Universität Göttingen, Germany). *S. cerevisiae* strain B76leu2::URA3 was constructed by transformation of B76 with a *leu2::URA3* disruption fragment, followed by diploidization using a *HO*-containing plasmid, as described by Stoldt *et al.* (1997). Fungal transformations were carried out by the lithium acetate method or, for *C. albicans*, by the spheroplast method (Sherman *et al.*, 1986). Yeast cells were grown in YPD, YP (YPD without glucose), YPGal (YP containing 2% galactose) or on supplemented SD minimal medium (Sherman *et al.*, 1986). Pseudohyphal growth of *S. cerevisiae* cells was induced on SLAHD plates (Gimeno *et al.*, 1992) containing 50 μ M ammonium sulfate and amino acids to allow growth of auxotrophic strains, or on SLAHGal plates containing 2% galactose and 0.1% glucose to induce expression by the *GAL1/10* promoter. Pseudohyphal growth was inspected microscopically after 2 d at 30 °C using a Zeiss Axioscop. To induce hyphae, *C. albicans* cells were pregrown at 30 °C in YPD or in 1% Casamino acids (for pBI1-CCT8) to exponential growth phase, starved for 1 h at 30 °C in 0.45% NaCl/0.335% yeast nitrogen base without amino acids (pH 5.5)/10 mM proline and then diluted (OD_{600} 0.1) into the same medium containing 1 mM N-acetylglucosamine at pH 6.5 and 37 °C. Alternatively, cells were diluted into YP medium containing 5% horse serum at 37 °C (Delbrück & Ernst, 1993). Hyphal formation on solid media was allowed on 'Spider' medium (Liu *et al.*, 1994) or on 2% agar containing 1% horse serum, at 37 °C. The phenotypes generated by the dominant *RAS2*^{Val19} mutation were examined essentially as described by Toda *et al.* (1985), except that during the tests to determine heat sensitivity or glycogen accumulation, transformants were grown in YPD or in YPGal, which induces the *GAL1/10* promoter of *GAL-CCT* fusion genes. Because of poor sporulation of B76/4 transformants, strain VW1D (*MATa/MAT α trp1/trp1 ura3-52/ura3-52 leu2-3,112/leu2-3,112 his3/his3*; obtained from M. Ciriacy, Universität Düsseldorf, Germany) was used as a host to test the effect of *CCT* genes on sporulation. Sporulation was allowed to proceed in liquid 1% potassium acetate for 5–7 d.

CCT alleles and expression vectors. *S. cerevisiae* B76/4 was transformed with plasmid p606/1 encoding the *C. albicans* Phd1p homologue Efg1p (Stoldt *et al.*, 1997). The transformant, which showed enhanced pseudohyphal growth on SLAHD plates, was retransformed with a genomic bank of *C. albicans* ATCC 10231 in plasmid pLG89-B1 (Losberger & Ernst, 1989; Leuker & Ernst, 1994). From one transformant, in which morphogenesis was suppressed, plasmid pU1, containing a 2.95 kb *Sau3A* genomic fragment, was isolated. A shortened 2.8 kb *EcoRI* subfragment, in pRS426 (Christianson *et al.*, 1992) (pRS426U1), was shown to be sufficient for suppression; its sequence has the potential to encode a deleted CaCct8p protein (*Cacct8- Δ 1*) commencing with methionine residue 96 (Stoldt *et al.*, 1996) (5' GAATTCGAAATG; *EcoRI* site underlined, Met codon in bold). The missing 5' end of CaCCT8 was isolated from a genomic bank of *C. albicans* ATCC 10231 (Losberger & Ernst, 1989) and sequenced. A *BamHI* site was constructed upstream of the reconstituted CaCCT8 coding region by PCR mutagenesis (primer PRFGes: 5' TTAGGATCCAATGTCGTTGAAG; *BamHI* site underlined, start codon in bold); this generated a 3 kb *BamHI* fragment carrying CaCCT8.

CCT expression plasmids include insertions of the 2.8 kb

EcoRI fragment of pU1 (*Cacct8-Δ1*) into the *EcoRI* sites of plasmids pRS426 or pRS424 (Christianson *et al.*, 1992) (pRS426U1 or pRS424U1) or YCplac33 (Gietz & Sugino, 1988) (YCplac33-RFG1); a fortuitous plasmid promoter drives transcription in these plasmids. The 2.8 kb *EcoRI* fragment was also placed into the *EcoRI* site downstream of the *GAL10* promoter (Johnston & Davis, 1984); basic vectors YEplac195 (Gietz & Sugino, 1988) and pRS424 contained this fusion as *BamHI-EcoRI* inserts, resulting in vectors YEplac195Gal10-RFG1 and pRS424-Gal10-RFG1, respectively. YEplacGal1/10 contains only the *GAL1/10* promoter inserted in YEplac195. Full-length *CaCCT8*, as a 3.1 kb *BamHI* fragment, was inserted into the *BamHI* site downstream of the *GAL1* promoter in YEplac195 (YEplacGal1CCT8) or pRS424 (pRS424-Gal1-CCT8).

A *C. albicans* expression plasmid carrying *CaCCT8* under transcriptional control of the *ACT1* promoter, pCARS-Act-RFG1, was constructed by joining the *C. albicans ACT1* promoter as a 1 kb *BamHI-EcoRI* fragment (Delbrück & Ernst, 1993) to the 2.8 kb *EcoRI-BamHI* fragment of plasmid pRS426U1 and inserting the fusion into the *BamHI* site of plasmid pCARS (Kelly *et al.*, 1988). pBI-CCT8, in which *CaCCT8* is regulated by the *CaPCK1* promoter, was constructed by first inserting the promoter, as a 1.4 kb *BamHI-StuI* fragment of plasmid pCA01 (Leuker *et al.*, 1997) between the *BamHI/SmaI* sites of pRC2312 (Cannon *et al.*, 1992) to generate pBI-1; the 3.1 kb *BamHI* fragment of YEplacGal1CCT8 was then transferred into the *BglII* site of pBI-1 to generate pBI1-CCT8.

Other plasmids. Plasmids pHY2 (*STE12-CPH1*) (Singh *et al.*, 1994) and pCG38 (*PHD1*) (Gimeno & Fink, 1994) have been described. YCplac22RAS2^{Val19} was constructed by inserting the 2.9 kb *EcoRI-HindIII* fragment of YCpR2V (Gimeno *et al.*, 1992) into YCplac22. pILC5-C, which contains the *TEC1* gene on a *URA3* and 2μ marked plasmid, was obtained from E. Dubois (Université Libre de Bruxelles, Belgium; Laloux *et al.*, 1994). Plasmid pGAL1-cdc42^{Val12} carrying the dominant CDC42^{Val12} allele (Ziman *et al.*, 1991) was a gift from D. Johnson (University of Vermont, USA). Plasmid pFG-(TYA)::lacZ-URA encoding the FG(TyA)::lacZ reporter fusion (Mösch *et al.*, 1996) was kindly supplied by H.-U. Mösch.

Gene disruption. To place a *URA3*-containing disruption cassette into *CaCCT8*, PCR was performed using YEplacGal1CCT8 as template and two primers (5' TTAAGATCTG-AAATCAAGCATTTTC and 5' TTAAGATCTCATTTGGTT-TCAACAC; *BglII* sites underlined) designed to amplify *CaCCT8* termini encoding residues 1–264 and the 3' untranslated region along with plasmid sequences. The PCR product was cut with *BglII* and ligated; into the *BglII* site of the resulting vector we inserted a 3.5 kb *BamHI* fragment of plasmid pCAC (obtained from P. Magee, University of Minnesota, USA), which contains the *CaURA3* gene flanked by direct repeats of the *Escherichia coli cat* gene. From the resulting plasmid, pBLAST-CCT8, the 4.5 kb *Sall* fragment carrying the disrupted *CaCCT8* was excised and used to transform *C. albicans* CA14 to prototrophy. DNA of transformants was cut with *EcoRI* and probed, in a Southern blot, with a 0.5 kb *Sall* fragment of pRS424U1 containing the 3' untranslated region of *CaCCT8*. The wild-type strain yielded the expected fragment of 2.8 kb, while the heterozygous disruptants (FR1-1, 2, 3) contained the 2.8 kb fragment and a 1.9 kb fragment of the disrupted allele.

RESULTS

CaCCT8 suppresses pseudohyphal morphogenesis in *S. cerevisiae*

We recently identified a *C. albicans* gene, *EFG1*, encoding a close homologue of Phd1p, which enhances *S. cerevisiae* pseudohyphal morphogenesis (Stoldt *et al.*, 1997). To identify suppressors of morphogenesis, we retransformed an *S. cerevisiae* transformant expressing *EFG1* with a *C. albicans* genomic library and looked for transformant colonies that did not show pseudohyphal induction. From one of these transformants we recovered a library plasmid (pU1) and analysed its genomic insert by sequencing. The insert was found to contain a deleted version of the *C. albicans CaCCT8* gene (Stoldt *et al.*, 1996) encoding a subunit of the cytosolic chaperonin complex on a 2.95 kb *Sau3A* fragment (see Methods). The deleted version of *CaCCT8*, designated *Cacct8-Δ1*, has the potential to encode a *CaCct8* protein commencing at residue 96; a fortuitous promoter located within plasmid sequences appeared to drive transcription of the truncated allele.

The missing 5' end of the *CaCCT8* gene was identified in a partial genomic *C. albicans* bank using the *Cacct8-Δ1* allele as probe. The 5' fragment thus isolated was used to reconstitute the full-length *CaCCT8* sequence. To allow conditional expression of *CaCCT8*, a convenient *BamHI* site was placed immediately upstream of the ATG translational start sequence. This *CaCCT8* allele was placed under transcriptional control of the *S. cerevisiae GAL1* promoter (Johnston & Davis, 1984).

Transformants of *S. cerevisiae* strain B76/4 (Stoldt *et al.*, 1997) expressing *CaCCT8* on a replicating vector grew in the yeast form at identical rates and with identical cytological appearance compared to control strains carrying an empty vector in all conditions. This result demonstrated that *CaCCT8* expression in *S. cerevisiae* did not affect growth in general. In contrast, pseudohyphal morphogenesis induced by *EFG1*-carrying plasmids was completely blocked by the simultaneous presence of plasmids containing *CaCCT8* or its deleted derivative *Cacct8-Δ1* (Fig. 1). Effective suppression of morphogenesis was obtained with *Cacct8-Δ1* contained in either high-copy-number vectors (pRS426-U1, YEplac195Gal10-RFG1) or in a low-copy-number centromeric vector (YCplac33-RFG1).

These results raised the question whether *CaCCT8* alleles mediated specific suppression of morphogenesis induced by the *C. albicans EFG1* gene or affected morphogenesis in general. Therefore, we tested if pseudohyphal growth induced by a variety of genes, including genes encoding Phd1p (Gimeno & Fink, 1994), Ras2p^{Val19} (Gimeno *et al.*, 1992), Cph1p (Singh *et al.*, 1994), Cdc42p (Mösch *et al.*, 1996) and Tec1p (Gavrias *et al.*, 1996), could be suppressed. Morphogenesis induced by the *EFG1* homologue *PHD1* was effectively blocked by *CaCCT8* or *Cacct8-Δ1* (Fig. 1). Similarly, the strong pseudohyphal induction by RAS2^{Val19} was effec-

Fig. 1. Colony phenotypes of *S. cerevisiae* transformants. Transformants contained two types of plasmid, one inducing pseudohyphal growth and a second that was tested for suppression of morphogenesis. Colonies of transformants were grown for 2–3 d on SLAHD medium or (if required for induction of the GAL promoter) in SLAHGal low-nitrogen medium. The following combinations of genes (plasmids) were tested, using B76/4 as the host strain: *EFG1* (p606/1) and *RAS2*^{Val19} (YCplac22*RAS2*^{Val19}) in combination with control vector YEplac195, or with *CaCCT8* (YEplacGal1*CCT8*), or with *Cacct8-Δ1* (YCplac33-*RFG1*); *PHD1* (pCG38) and a *STE12-CPH1* fusion (PHY2) in combination with control vector YCplac22, or with *CaCCT8* (pRS424-Gal1-*CCT8*), or with *Cacct8-Δ1* (pRS424-Gal10-*RFG1*). In addition a B76/4 transformant containing two control vectors (YEplac195 and YCplac22) was tested.

Table 1. Regulation of *FG(TyA)::lacZ* expression

Transformants of both strains carried pFG(TYA)::*lacZ*-URA encoding the *FG(TyA)::lacZ* reporter fusion, as well as a plasmid containing the indicated gene. Strains were grown on solid SD (high nitrogen) or SLAHD medium containing 50 μM NH₄SO₄ (low nitrogen) and β-galactosidase levels in cellular extracts were determined as described by Mösch *et al.* (1996). The activity of at least two transformants, each tested in duplicate, was determined. Activities are expressed relative to the mean value of the control strain B76/4(pRS424) ± SD.

Strain	Added gene (plasmid)		Relative β-galactosidase activity	
			High nitrogen	Low nitrogen
B76/4	–	(pRS424)	1.00 ± 0.19	3.53 ± 1.86
	<i>Cacct8-Δ1</i>	(pRS424U1)	0.99 ± 0.53	2.79 ± 1.1
	–	(YEplac112)	0.56 ± 0.06	2.88 ± 0.71
	<i>RAS2</i> ^{Val19}	(YCplac22 <i>RAS2</i> ^{Val19})	0.83 ± 0.35	14.33 ± 6.93
L5976	–	(pRS424)	1.02 ± 0.14	6.90 ± 1.0
	<i>Cacct8-Δ1</i>	(pRS424U1)	0.90 ± 0.42	6.17 ± 0.75

tively inhibited (Fig. 1), indicating that suppression by *CaCCT8* alleles was not specific for the *Efg1p* inducer. Suppression of morphogenesis by *CaCCT8* alleles was

also not specific for the host strain used (B76/4) because it was also observed in the Σ1298b derivative L5976 (data not shown).

Ras2p is known to trigger filamentous growth either via Cdc42p and an MAP kinase cascade activating transcription factor Ste12p or via a signalling pathway mediated by cAMP (Kübler *et al.*, 1997; Lorenz & Heitman, 1997). The *C. albicans* CPH1 gene encodes a homologue of transcription factor Ste12p which is functional and is activated by the MAP kinase cascade in *S. cerevisiae* (Singh *et al.*, 1994). Transformants expressing an STE12-CPH1 fusion showed vigorous pseudohyphal growth, which could not be suppressed by CaCCT8 or *Cacct8-Δ1* (Fig. 1). Similarly, transformants of strain B76leu2::URA3 expressing the dominant active CDC42^{Val12} allele (Ziman *et al.*, 1991; on plasmid pGAL1-cdc42^{Val12}) showed enhanced morphogenesis (Mösch *et al.*, 1996), but this phenotype could not be suppressed by simultaneous expression of *Cacct8-Δ1* (on plasmid pRS424-Gal10-RFG1; data not shown). TEC1 encodes a transcription factor acting in concert with Ste12p to regulate genes required for pseudohyphal growth (Gavrias *et al.*, 1996; Madhani & Fink, 1997). Overexpression of TEC1 (on plasmid pILC5-C) in strain B76/4 stimulated pseudohyphal development as expected, but morphogenesis could not be suppressed by simultaneous expression of *Cacct8-Δ1* (on plasmid pRS424-Gal10-RFG1; data not shown).

These results indicated that CaCCT8 alleles did not affect morphogenesis in general and suggested that the putative target of suppression was not a part of the MAP kinase pathway, but was possibly located in the cAMP pathway stimulated by Ras2p. We confirmed that the suppression of morphogenesis was not due to a block of the MAP kinase cascade by examining the activity of the FG(TyA) promoter, which depends on this pathway (Lorenz & Heitman, 1997; Mösch *et al.*, 1996). The expression of the FG(TyA)::lacZ construct was increased four- to sevenfold in strains B76/4 and L5976 by growth in low nitrogen conditions, as expected, but the simultaneous expression of *Cacct8-Δ1* did not significantly influence expression levels (Table 1).

CaCCT8 alleles suppress morphogenesis-unrelated phenotypes of Ras2p^{Val19}

Activation of the Ras pathway in *S. cerevisiae* not only stimulates pseudohyphal morphogenesis, but also leads to heat sensitivity, lack of glycogen/trehalose accumulation and lack of sporulation (Toda *et al.*, 1985). To explore if these additional phenotypes were also affected by CaCCT8 expression, we used double transformants of strain B76/4 carrying a RAS2^{Val19}-containing plasmid as well as a plasmid expressing the CaCCT8 or *Cacct8-Δ1* alleles. As controls, transformants carrying an empty vector along with the RAS2^{Val19}-containing plasmid were analysed.

We tested heat sensitivity of transformants by heat shock of stationary cells at 50 °C, followed by plating of cells on SD minimal medium. As expected, the presence of the RAS2^{Val19} allele rendered transformants heat-sensitive. This sensitivity was completely reversed by coexpression of CaCCT8 and *Cacct8-Δ1* (Fig. 2a).

Fig. 2. Suppression of RAS2^{Val19} phenotypes by chaperonin subunits. (a) Suppression of heat sensitivity. *S. cerevisiae* strain B76/4 was transformed by YCplac22RAS2^{Val19} and with a second plasmid containing CaCCT8 (YEplacGal1CCT8), *Cacct8-Δ1* (YEplac195Gal10-RFG1) or no CCT gene (Control; YEplacGal1/10). Transformants were grown in SGal medium to stationary phase, heat-shocked at 50 °C for 30 min (or not heat-shocked) and plated on SD. (b) Restoration of glycogen accumulation. Strains were grown in liquid YPGal, spotted on solid YPGal and grown at 30 °C for 1 d. The accumulation of glycogen in colonies was visualized by iodine staining. (1) Glycogen accumulation (dark coloration) of a control transformant not expressing RAS2^{Val19} (YEplacGal1/10; YCplac22); (2) lack of glycogen accumulation in a transformant carrying RAS2^{Val19} (YEplacGal1/10; YCplac22RAS2^{Val19}). Restoration of glycogen accumulation occurred in RAS2^{Val19} transformants (YCplac22RAS2^{Val19}) expressing (3) *Cacct8-Δ1* (YEplac195Gal10-RFG1) or (4) CaCCT8 (YEplacGal1CCT8).

To examine chaperonin effects on sporulation we used the diploid strain VW1D as the host for the RAS2^{Val19}-encoding plasmid (YCplac22RAS2^{Val19}), because strain B76/4 sporulates poorly. In this experiment we used a plasmid containing the *Cacct8-Δ1* allele under control of the galactose-inducible GAL10 promoter (YEplac195-Gal10-RFG1). RAS2^{Val19}-expressing control strains (carrying plasmids YCplac22RAS2^{Val19} and YEplac195) did not sporulate, as expected, in either glucose- or galactose-containing sporulation media (0% asci after 5–7 d). On the other hand, a transformant containing YCplac22RAS2^{Val19} and YEplac195Gal10-RFG1 sporulated significantly only if the expression of *Cacct8-Δ1* was induced by pregrowth in galactose (17% asci); after pregrowth on glucose medium sporulation was reduced due to the low levels of *Cacct8-Δ1* expression (3% asci).

We tested the accumulation of storage carbohydrates in a RAS2^{Val19}-carrying strain using iodine staining of

Fig. 3. Chaperonins suppress hyphal morphogenesis of *C. albicans*. Strain CAI4 was transformed with plasmid pBI1-CCT8 expressing CaCCT8 or with a control plasmid (pBI-1). Hyphae were induced by (a) *N*-acetylglucosamine in liquid medium, (b) on agar containing 1% serum or (c) on Spider plates. (a, $\square$), (b, i) and (c, i) are the results of the control strain CAI4(pBI-1); (a, $\triangle$), (b, ii) and (c, ii) are the results of transformant CAI4(pBI1-CCT8). The cellular phenotype on serum plates (b) is shown after 3 h growth; the colony phenotype on Spider plates (c) was determined after 5 d growth at 37 °C. The phenotype of the heterozygous strain FR1 (*CaCCT8/Cacct8*) on Spider plates is shown in (c, iii).

colonies (Fig. 2b). Dark coloration of colonies, indicating glycogen accumulation within cells, was observed for a control strain but not for a transformant expressing *RAS2*^{Val19}. The simultaneous expression of *CaCCT8* or *Cacct8-Δ1* was able to restore glycogen accumulation to wild-type levels, even in the presence of *RAS2*^{Val19}.

The suppression of all *RAS2*^{Val19} phenotypes, related or unrelated to morphogenesis, suggested that the *C. albicans* chaperonin subunits produced in the heterologous host act as dominant-negative inhibitors of a component of the Ras2 signal transduction pathway.

CaCCT8 suppresses hyphal development in *C. albicans*

True septated hyphae in *C. albicans* arise because of at least two signalling pathways operative either on certain solid media (Köhler & Fink, 1996; Liu *et al.*, 1994) or in liquid media in the presence of certain positive factors, including serum or GlcNAc (Lo *et al.*, 1997; Stoldt *et al.*, 1997). To test if any hyphal induction pathway is affected by *CaCCT8* overexpression, we constructed pBI1-CCT8, in which *CaCCT8* is under transcriptional control of the glucose-regulated *PCK1* promoter (Leuker *et al.*, 1997). Transformants of *C. albicans* strain CAI4 carrying either pBI1-CCT8 or the 'empty' control vector

pBI-1 were tested for hyphal morphogenesis after pregrowth in 1% Casamino acids (promoter induction) or SD medium (promoter repression).

Transformants carrying pBI-1 did not show differences in growth rates compared to the control strain in any medium and irrespective of the induction of the *PCK1* promoter. However, pBI1-CCT8 transformants grown in Casamino acids and shifted to liquid medium containing 1 mM GlcNAc showed a reproducible delay in the formation of hyphae compared to control transformants carrying pBI-1; a typical result obtained with several independent transformants is shown in Fig. 3(a). Under these conditions hyphal morphogenesis was not abolished completely and after about 4 h induction both the pBI1-CCT8 and pBI-1 transformants had formed hyphae equally. No differences in the rate of hyphal formation were observed if the transformants were pregrown in SD medium, when the *PCK1* promoter is repressed, suggesting that *CaCCT8* overexpression is the cause for the delay of morphogenesis. A suppression of hyphal growth, but not a complete block, by *CaCCT8* overexpression was also observed on solid plates containing 1% serum (Fig. 3b). Transformants were also tested on Spider medium (Liu *et al.*, 1994) for hyphal induction. After 5 d growth, control cells had formed visible lateral hyphae emerging from colonies (Fig. 3c, i),

Fig. 4. Disruption of *CCT8* alleles. The genomic fragment carrying *CaCCT8* (grey arrow) is shown on top, along with the insertion site of the *cat-URA3-cat* cassette. B, *Bam*HI; R, *Eco*RI; S, *Sal*I. The 4.5 kb *Sal*I disruption fragment was transformed into strain CAI4. DNA of transformants (lanes 1–3) and of CAI4 (lane 4) was analysed after cutting with *Eco*RI and Southern blotting using a *CaCCT8* probe fragment (asterisks). Transformants FR1–1, 2, 3 (lanes 1–3, respectively) contain one intact allele (*CaCCT8*; 2.8 kb) and one disrupted allele (*Cacct8Δ*; 1.9 kb).

while colonies formed by CAI4(pBI1-CCT8) cells did not show any hyphae (Fig. 3c, ii). Overexpression of the *Cacct8-Δ1* allele in *C. albicans* under transcriptional control of the constitutive *ACT1* promoter (pCARS-Act-RFG1) led to an identical partial suppression of hyphal growth compared to the *PCK1p-CaCCT8* transformants described above. Furthermore, transformants carrying pCARS-Act-RFG1 showed a slowed hyphal formation in liquid medium containing 1% GlcNAc and on solid plates containing 1% serum (data not shown).

These results demonstrate that *CaCCT8* overexpression partially suppresses hyphal formation induced by the serum/GlcNAc pathway and completely blocks morphogenesis induced on Spider medium in *C. albicans*.

Lowered *CaCCT8* gene dosage in a heterozygous strain blocks *C. albicans* morphogenesis

The above results suggested that excess levels of chaperonin subunits can act as dominant-negative suppressors of morphogenesis and Ras phenotypes by inhibiting a component of the Ras2p signalling pathway. As a mechanism for this effect we hypothesized that overproduced chaperonins are not assembled into a functional Cct complex, which presumably requires all

individual subunits Cct1p–Cct8p. Instead, orphan subunits would still be able to bind to their folding target proteins and, therefore, a lowered level of active target protein within cells. Conversely, lowering chaperonin levels within cells would also be predicted to reduce the activity of the target protein. To test if morphogenesis is blocked at reduced chaperonin levels, as in the case of chaperonin overproduction, we attempted to disrupt the *C. albicans CaCCT8* gene.

Using the 'URA3 blaster' method (Fonzi & Irwin, 1993) we were able to disrupt one *CaCCT8* allele in strain CAI4 without difficulty by using a *URA3*-containing disruption fragment. In Southern blots of genomic DNA a 2.8 kb *Eco*RI fragment, representing the wild-type allele, and a 1.9 kb fragment, corresponding to the disrupted allele, were found in three independent mutants (Fig. 4). Next we isolated Ura[–] mutant derivatives of the disruptant strains, which had arisen because of recombination between the *cat* repeats flanking the *URA3* selection marker; Ura[–] derivatives were used in a second round of transformation with the disruption fragment in an attempt to mutate the second *CaCCT8* allele. Among 54 transformants no double-deleted mutant could be obtained (as determined by Southern blotting), suggesting that the deletion of both *CaCCT8* alleles in *C. albicans* is lethal, as has been observed for *S. cerevisiae* mutants lacking individual Cct subunits (reviewed by Stoldt *et al.*, 1996). Standard protocols were used to test the ability of the *CaCCT8/Cacct8* heterozygous strain to form filaments. Remarkably, a clear phenotypic difference of the heterozygous disruptant compared to the parental strain was observed on solid Spider medium. Under these conditions only the parental strain, but not the *CaCCT8/Cacct8* heterozygous strain, was able to form hyphae (Fig. 3c, iii). On the other hand, hyphae were formed at wild-type rates in the presence of 5% serum in liquid or on solid media.

These results suggest that both excess and lowered levels of chaperonins can abolish morphogenesis of *C. albicans*, which may occur in both cases because of lowered activity of a protein in the Ras2 pathway.

DISCUSSION

In a search for suppressors of *C. albicans* morphogenesis we discovered that high levels of an individual subunit of the eukaryotic Cct chaperonin complex lead to a specific block of fungal morphogenesis. We found that overexpression of *CaCCT8*, encoding subunit 8 of the Cct complex, inhibits hyphal morphogenesis in *C. albicans* and that its expression in the heterologous host *S. cerevisiae* blocks pseudohyphal growth. No effects of *CaCCT8* overexpression on growth and on cytological appearance of the yeast growth forms of both fungi were observed. The analyses revealed that, rather than morphogenesis *per se*, it is very likely that a component of the Ras signal transduction pathway is inhibited by extraneous *CaCct8p* levels. These results suggest a yet undefined component of fungal morphogenesis path-

ways, as well as a novel cytosolic folding target protein of the Cct complex.

A component of the Ras pathway as the target of suppression by CaCct8p

Recent evidence indicates that Ras2p activates two signal transduction pathways as a response of *S. cerevisiae* diploids to nitrogen starvation. One pathway, whose activity can be monitored by the *FG(TyA)* promoter (Mösch *et al.*, 1996) leads to the activation of a conserved MAP kinase (Kss1p) and phosphorylation of the transcription factor Ste12p (Liu *et al.*, 1993; Madhani & Fink, 1997; Madhani *et al.*, 1997). The second pathway includes cAMP, whose levels are up-regulated by both Ras2p and Gpa2p, a G-protein α -subunit (Kübler *et al.*, 1997; Lorenz & Heitman, 1997). It is still not clear, if cAMP exerts its effects via PKA (*TPK1-3* gene products; Toda *et al.*, 1987), or is independent of PKA. A function of PKA in yeast morphogenesis can be deduced from the suppression pattern of the heterologous CaCct8p chaperonin subunit in *S. cerevisiae* described here. CaCct8p did not block pseudohyphal growth induced by Cdc42p or Cph1p (Ste12p), indicating that the 'chaperonin effect' is not due to a block of the 'MAP kinase branch' of signal transduction pathways leading to morphogenesis. This result could be confirmed by measurements of the *FG(TyA)* promoter, which was unaffected by *CaCCT8* expression. Nevertheless, morphogenesis stimulated by *RAS2*^{Val19} could be effectively suppressed, suggesting that the target of suppression is located in the 'cAMP branch' of the morphogenetic signalling pathways. Either PKA itself or a functionally related protein could be the target of suppression, because all additional known *RAS2*^{Val19} phenotypes mediated by PKA are inhibited: heat sensitivity, lack of sporulation and lack of glycogen/trehalose accumulation phenotypes (Durnez *et al.*, 1994; Ruis & Schüller, 1995; Toda *et al.*, 1985) were all restored to wild-type phenotypes by simultaneous expression of *CaCCT8*. It has been reported that overexpression of *TPK1*, encoding PKA, did not stimulate filamentation in a Σ 1298b derivative (Mösch *et al.*, 1996), while we found this to be the case in strains of different genetic backgrounds (F. Rademacher & J. F. Ernst, unpublished results). Thus, it is possible that specific PKA isoforms (possibly to different extents depending on the genetic background of strains) are required for morphogenesis via the Ras2/cAMP pathway.

Morphogenetic pathways in *C. albicans* are less defined than in *S. cerevisiae*. Mutations in the *CST20*, *HST7* and *CPH1* genes (homologues of *STE20*, *STE7* and *STE12* genes of *S. cerevisiae*, respectively) block hyphal development on solid Spider medium, but have no effect on hyphal growth induced by positive signals, such as serum components (Köhler & Fink, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1993). Most likely, the 'starvation pathway' on Spider medium seems to represent the functional equivalent of the *S. cerevisiae* MAP kinase pathway. The observed suppression of both the star-

vation and the positive signals pathway by CaCct8p suggests that one or more common elements occur in both pathways. In *C. albicans*, exogenous cAMP stimulates hyphal growth, indicating a role for cAMP and suggesting a role for PKA in hyphal morphogenesis (Niimi *et al.*, 1980; Niimi, 1996). Stimulation of fungal morphogenesis via cAMP and PKA has also been reported for *Ustilago maydis* (Gold *et al.*, 1994) and other fungi (Kronstad, 1997).

Our finding that morphogenesis induced by *PHD1* or *EFG1* can be effectively suppressed by *CaCCT8* expression suggests that their gene products are components of the cAMP morphogenetic pathway. Phd1p contains a region that is highly homologous to the Sok2 protein, which appears to act as a suppressor of pseudohyphal growth (Gimeno & Fink, 1994; Ward *et al.*, 1996) and to bHLH proteins regulating morphogenesis in other fungal species, including Efg1p of *C. albicans* and StuA of *Aspergillus nidulans* (Miller *et al.*, 1992; Stoldt *et al.*, 1997). Genetic evidence suggests that the homologous Sok2 protein acts downstream of PKA to regulate the expression of genes important in growth and development (Ward *et al.*, 1996). Possibly, Phd1p and Efg1p may assume similar functions in the cAMP pathway, conceivably as direct or indirect phosphorylation targets of PKA.

Mechanism of morphogenesis suppression by CaCct8p

It has been established that the Cct complex has a narrow substrate specificity and mainly folds newly synthesized actin and tubulin, but also binds to small amounts of unidentified proteins *in vivo* (Sternlicht *et al.*, 1993; Ursic *et al.*, 1994; Yaffe *et al.*, 1992). Our results suggest that a protein of the Ras2/cAMP signalling pathways is one of the unidentified folding targets of the Cct complex. We propose that excess CaCct8 proteins within cells that cannot be assembled into a functional Cct complex [which appears to consist of equimolar amounts of eight specific subunits (Lin *et al.*, 1997)] still bind to their target proteins, but are unable to fold them. Sequestration of target proteins (e.g. PKA) may decrease their cellular activity levels and thereby lead to a dominant-negative phenotype (e.g. prevention of hyphal morphogenesis). Conceivably, chaperonin target proteins existing at low levels within cells are affected more significantly in their cellular activity levels than abundant target proteins, such as actin or tubulin, whose inhibition could cause a general growth defect. We do not consider it likely that our results are due to a positive function of excess chaperonins, as has been suggested from *in vitro* experiments (Zahn *et al.*, 1996), because even a deleted chaperonin (CaCct8- Δ 1p) lacking the ATP-binding region that is required for protein folding (Kubota *et al.*, 1995) was as active a suppressor as the full-length chaperonin. Possibly, the 'antimorphic' nature of some alleles of the mouse *T* locus is due to dominant-negative effects of full-length or deleted versions of chaperonin Tcp-1/Cct1, which is encoded by this locus (MacMurray &

Shin, 1988). Recently, overexpression of *ScCCT6* was reported to restore growth and polarized distribution of the actin cytoskeleton in a *tor2* mutant (Schmidt *et al.*, 1996). This result may be due to a dominant-negative effect, e.g. on *Sac7p*, since *sac7* mutations restore the growth of *tor2* strains (Schmidt *et al.*, 1997).

Both *CaCCT8* alleles are required for *C. albicans* morphogenesis

A dominant-negative function of excess chaperonins in morphogenesis implies that the respective target protein is an essential component of the suppressed pathway. We predicted therefore, that a reduced dosage of a single chaperonin subunit would diminish the concentration of functional Cct complexes and therefore abolish or delay hyphal morphogenesis. Although we were not able to delete both *CaCCT8* genes in *C. albicans*, in agreement with the lethality of single *cct* mutants of *S. cerevisiae* (reviewed by Stoldt *et al.*, 1996), we found that in a *CaCCT8/Cacct8* heterozygous strain hyphae no longer could be induced on Spider medium. Hyphal induction by serum was still possible, suggesting that the 'starvation pathway' is more affected by reduced activity of the target protein than the 'serum pathway', in agreement with our finding that excess chaperonins completely abolish morphogenesis on Spider medium, while only partially affecting the response to serum.

In summary, our findings (1) that overproduction and lowering of *CaCct8p* levels lead to comparable phenotypes, (2) that pseudohyphal morphogenesis induced by high activity of the Ras pathway, but not the MAP kinase pathway, is suppressed by *CaCct8p* and (3) that morphogenesis-unrelated phenotypes of *Ras2^{Val19}* are suppressed by *CaCct8p* strongly support our hypothesis that excess chaperonins inhibit a specific target protein in the Ras pathway.

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