**REVIEW
ARTICLE****Transcription factors in *Candida albicans* – environmental control of morphogenesis**

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Awareness of *Candida albicans* as a major human health threat has risen during recent years. Although infections by *C. albicans* can be relatively mild and superficial, systemic mycoses often occur in immunocompromised patients, or even as a consequence of long-term therapy with broad-spectrum antibiotics or of chemotherapy (reviewed by Odds, 1988). Effective antifungal agents which are free of side-effects are urgently needed. There is hope that recently developed techniques of manipulating *C. albicans* and the sequencing of its genome will lead to a thorough understanding of the virulence and biology of this fungal pathogen, thus offering the possibility of a knowledge-based approach to novel antifungal agents.

Experimental work on *C. albicans* has long been hampered by its asexuality, its diploidy and its non-canonical codon usage (CUG encodes serine) (reviewed by Scherer & Magee, 1990; Santos *et al.*, 1996). Molecular-genetic techniques have overcome these difficulties, and today host strains and transformation vectors are available and an efficient gene-disruption protocol has been developed (Fonzi & Irwin, 1993; Morschhäuser *et al.*, 1999; Wilson *et al.*, 1999). Reporter genes encoding β -galactosidase (Lac4p), luciferase and green fluorescent protein (GFP) are available (Leuker *et al.*, 1992; Srikantha *et al.*, 1996; Cormack *et al.*, 1997). The Stanford genome-sequencing project is expected to complete 10-fold coverage of the *C. albicans* genome in the year 2000 (<http://www-sequence.stanford.edu/group/candida/>).

Which factors determine the virulence of *C. albicans*? In the past possible virulence traits have been suggested, which were characterized on a phenomenological level, but studies could not prove their role in pathogenesis. Modern techniques allow the testing of such hypotheses. It is generally accepted now that dimorphism, i.e. the ability to grow in a yeast and a filamentous growth form, not only is a virulence trait *per se*, but is also co-regulated with other virulence factors, which are associated with cellular morphology (Hube *et al.*, 1996;

Lo *et al.*, 1997). Dimorphism and other striking morphogenetic phenotypes of *C. albicans*, including chlamydospore formation and phenotypic switching, are regulated by environmental conditions. This review focuses on transcription factors that mediate environmental control and thereby constitute key elements of morphogenesis in *C. albicans*.

A variety of developmental programmes

C. albicans is able to develop single spherical cells including typical yeast cells and chlamydospores, as well as elongated cells developing into multicellular true hyphae or pseudohyphae (Fig. 1). Thus, the term dimorphism, which traditionally is reserved for the yeast–true hypha interconversion, in a more general sense designates the main theme of *C. albicans* and possibly fungal morphogenesis in general. Budding-yeast cells can be induced to form true hyphae, which grow by continuous apical extension followed by septation. Pseudohyphae grow differently from true hyphae, by unipolar budding: buds develop into elongated cells, which remain attached to mother cells, stop growth and resume budding. Chlamydospores are thick-walled spherical cells and develop on pseudohyphal support cells (Joshi *et al.*, 1993; Montazeri & Hedrick, 1984). Finally, in some forms of spontaneous phenotypic switching (reviewed by Soll *et al.*, 1993; Soll, 1997), enlarged elongated cells arise, which have a different appearance to yeast and pseudohyphal cells, and which do not form extensive filaments (opaque phenotype) (Fig. 1).

Dimorphic fungi share cell morphologies with both filamentous fungi and yeasts. This raises the question whether in evolution an ancestral yeast-like species has gained the ability to develop hyphae or conversely, whether a primordial filament-forming fungus has acquired the ability to develop a yeast form. The answer to this question is unknown, but some hints favour the latter possibility. Firstly, the position of hypha (germ tube) development on a *C. albicans* yeast mother cell is random with respect to previous sites of budding

Fig. 1. Morphological forms of *Candida albicans*. The yeast and hyphal growth forms are predominant during infection of the human host. Chlamydospores are thick-walled spherical cells arising as terminal cells on pseudohyphae; pathways of chlamydospore germination are unknown. Daughter cells and regions of cell expansion are indicated by dotted lines.

(Chaffin, 1984) and thus is similar to *Saccharomyces cerevisiae* yeast mutants, which lack components for the positioning of polar and axial buds (Chant, 1994). Secondly, several mutations of *C. albicans* discussed below (*efg1*, *tup1*, *rbf1*) favour an elongated cell morphology suggesting that filamentation is under negative control. If this hypothesis is correct, 'add-on' components must have evolved to repress filamentation and to favour spherical cell growth in an ancestral yeast-like organism.

Environmental cues regulate transcription factors

C. albicans morphology is directly related to environmental conditions. Hyphal development depends on two factors: (1) the nature, number and intensity of environmental signals (outside cues) and (2) the activity of signalling pathways including key transcription factors (cellular-response machinery). According to current models, environmental cues trigger separate signal-transduction pathways, which regulate common targets required to initiate hyphal growth (Fig. 2). A threshold level of signalling may be reached either by simultaneous low-level stimulation of several pathways

or by essentially a single pathway, which is fully activated (or hyperactive as in overexpression experiments). The most powerful hypha-inducing protocol involves incubation of *C. albicans* at low cell densities in liquid medium containing serum, GlcNAc or other inducers, at 37 °C (Shepherd *et al.*, 1980). Conceivably, because of strong stimulation of several pathways, minor defects in signalling pathways are not detected under this condition. Liquid induction is usually monitored for a few hours, when initial germ tubes have developed into hyphae that begin to bud off yeast cells. In contrast, on solid media cells develop into colonies over several days of growth, until (pseudo-) hyphal growth emerging from the colonies is recorded. The latter procedure is somewhat problematic, because (1) the molecular environment of a fully developed colony will differ greatly from the initial media composition, not allowing a clear definition of the inducing environment – in fact colonies 'condition' their environment by depletion of nutrients and by secretion of fungal compounds (Ritz & Crawford, 1999) and (2) cells may filament efficiently during initial growth, but may revert quickly to yeast growth and the phenotype of a grown colony will be determined by not only the yeast–hypha transition, but also the reverse process. We found that the use of 1% proline as a single carbon and nitrogen source in solid media strongly induced filaments until microcolonies appeared, but growth later completely reverted to the yeast form, leading to a yeast-only appearance after several days (Ernst, 2000). The 'switch back' to yeast growth is delayed in *cpp1* mutants (Csank *et al.*, 1997) or by overexpression of the *TPK2* gene (Sonneborn *et al.*, 2000), while it occurs more rapidly in *cln1* mutants (Loeb *et al.*, 1999). Compared to liquid medium, induction on solid media appears to represent a weaker hypha-inducing condition, because minor defects in filamentation (as in many mutants, which do not possess the full repertoire of signalling pathways; see below) show a defective phenotype on solid but not in liquid media.

Serum and other inducers

Serum of different sources is still the 'magic potion' to rapidly induce true hyphae in *C. albicans*. The responsible factor is not albumin, since albumin-free serum from a rat mutant was as efficient at promoting hyphae as normal serum (Feng *et al.*, 1999). Two known inducers of hypha formation, *N*-acetylglucosamine (GlcNAc) and proline, may contribute to the serum effect since they are generated by degradation of serum (glyco-) proteins. Thus, several independent signalling pathways are likely to be triggered by serum. Hyphae arise within minutes at 37 °C at 5–20% serum in liquid; on solid media hyphae development is triggered within a few hours. Numerous other compounds induce hyphae *in vitro* (reviewed by Odds, 1988), but their significance for the infection process is unknown.

The Efg1 protein is a strong regulator of morphogenetic processes in *C. albicans*, since it influences not only yeast–hypha interconversions (Stoldt *et al.*, 1997; Lo *et*

Fig. 2. General scheme of hypha induction. Various environmental cues activate separate signalling pathways via sensor proteins. Signalling pathways may converge on separate or identical transcription factors and transcription factors may converge on common target genes to trigger expression of hypha-specific genes.

al., 1997), but also regulates phenotypic switching and chlamyospore formation of this pathogen (Sonneborn *et al.*, 1999a, b) (Fig. 3). The role of Efg1p in hyphal morphogenesis is fascinating, because under standard induction conditions, using serum or GlcNAc as inducers in liquid or on solid media, there is essentially a complete block of hyphal formation in strains lacking Efg1p (Stoldt *et al.*, 1997; Lo *et al.*, 1997). On the other hand, under microaerophilic/embedded conditions hyphal formation is not defective at all in homozygous *efg1* mutants, but rather appears stimulated (Sonneborn *et al.*, 1999b; Riggle *et al.*, 1999; Brown *et al.*, 1999). To make things more complicated, the lack of *EFG1* expression in strains CAI8 and a WO-1 derivative (but not in the common strain CAI4) favours a rod-like, pseudohyphal phenotype (Sonneborn *et al.*, 1999a; Srikantha *et al.*, 2000). These results indicate that, depending on environmental cues (and depending on the genetic background), Efg1p is an activator and repressor of morphogenesis. Efg1p is a member of the APSES protein group, which encompasses putative transcription factors involved in fungal morphogenetic processes leading to (1) a transition from tubular to spherical growth such as in the case of the Asm1 protein of *Neurospora crassa*, which is involved in ascospore maturation (Aramayo *et al.*, 1996) or the StuA protein of *Aspergillus nidulans*, which is required for conidiophore formation (Miller *et al.*, 1992), or (2) regulation of the transition from spherical growth to tubular growth, such as the yeast–filament transition, which is regulated by the Phd1 and Sok2 proteins of *S. cerevisiae* (Gimeno & Fink, 1994; Ward *et al.*, 1995). Efg1p has both types of activities, since it is required for the yeast–hyphal transition, as well as for the pseudohyphal–chlamyospore morphogenesis (Stoldt *et al.*, 1997; Lo *et al.*, 1997; Sonneborn *et al.*, 1999b). APSES proteins share 80–90% identical residues in a domain encompassing about 100 aa. We discovered that the conserved domain of APSES proteins contains a basic helix–loop–helix (bHLH) motif, which is known to be required for dimerization and DNA binding (Stoldt *et al.*, 1997); this theory was supported later by molecular

modelling (Dutton *et al.*, 1997). However, because the Phd1 and Sok2 proteins have opposite functions, as activator and repressor, respectively, the conserved APSES domain is not sufficient to determine the direction of morphogenesis. Auxiliary proteins are likely to determine the specific functions of Efg1p, as in the case of the well-studied Myc protein, whose action as an activator or repressor is determined by interacting proteins (reviewed by Bernards, 1995). The presumed bHLH motif in Efg1p indicates an E box binding site on promoter regions of target genes (CANNTG). However, genes may be regulated by bHLH proteins, even if they do not contain an E box, as shown for the autoregulation of the Myc protein (Facchini *et al.*, 1997). We have recently shown that the *EFG1* gene is also autoregulated (B. Tebarth, M. Weide & J. F. Ernst, unpublished).

Added cAMP has been reported to induce hyphae in *C. albicans* (Niimi *et al.*, 1980; Sabie & Gadd, 1992; Niimi, 1996), suggesting that a cAMP pathway controls hyphal morphogenesis as it controls pseudohyphal development in *S. cerevisiae* (Gimeno *et al.*, 1992; Mösch *et al.*, 1996; Robertson & Fink, 1998; Pan & Heitman, 1999). Recently, *RAS1* has been characterized, which appears to be the only *RAS* gene in *C. albicans* (Feng *et al.*, 1999). Homozygous *ras1* mutants are unable to form true hyphae in the presence of serum, while pseudohyphae are still observed. A dominant-active version of Ras1p stimulates hyphal growth, while a dominant-negative version represses it. Recently, the *TPK2* gene encoding the catalytic subunit of a protein kinase A (PKA) isoform has been characterized (homologue of *S. cerevisiae* *TPK2*) (Sonneborn *et al.*, 2000), which conceptually could function downstream of Ras1p and be regulated by cAMP levels. Hyphal morphogenesis of a homozygous *tpk2* mutant is blocked on solid media containing serum and starvation-type (Spider) medium; interestingly, in liquid a defect is hardly seen with serum as the inducer, while it is apparent in Spider medium. The defects are especially pronounced at 30 °C and are less detectable at 37 °C. Thus, if relatively weak environmental signals (low temperature, solid medium,

Fig. 3. Various roles of the Efg1 morphogenetic regulator. In dimorphism Efg1p is an activator of hypha formation under some conditions (serum, GlcNAc induction), while under other conditions (embedded/microaerophilic) it is not required and possibly acts as a repressor. Chlamydospore formation and the phenotypic switching from the opaque to the white (yeast) phase phenotype requires Efg1p.

Spider medium) encounter a defective response machinery, the result is a defective phenotype, while there is hardly a phenotype under optimal induction conditions, because additional pathways compensate for the defect. The partial effect of the *tpk2* mutation compared to the *ras1* mutation may be due to a second PKA isoform (Tpk1p), which is indicated by the *Candida* genome-sequencing project (<http://www-sequence.stanford.edu/group/candida/>). The *tpk2* morphogenetic phenotype is suppressed by over-expression of *EFG1*, while the reverse is not true indicating that Efg1p is a downstream target of Tpk2p. We recently pointed out that Efg1p contains only a single potential site of phosphorylation by PKA (T206) (Sonneborn *et al.*, 1999b, 2000), at a similar position to other APSES proteins, with the exception of Phd1p (in this respect Sok2p appears more similar to Efg1p than Phd1p). Cells producing an Efg1p mutant version containing the T206A mutation are partially blocked in hyphal formation, while the T206E variant is hyper-filamentous (D. Bockmühl, A. Sonneborn & J. F. Ernst, unpublished). Taken together, these results suggest that Efg1p may be the constituent of a Ras1/cAMP/PKA pathway. Indirect data support the presence of PKA and APSES proteins in common signalling pathways in fungi. A heterologous *C. albicans* chaperonin subunit (Cct8p) blocked pseudohyphal growth in *S. cerevisiae*, if morphogenesis was induced by Ras2p, by Phd1p and the heterologous Efg1 protein, but not if it was triggered by activation of the MAP kinase pathway. In addition, morphogenesis-unrelated Ras2 phenotypes were blocked, suggesting PKA as the target of suppression (Rademacher *et al.*, 1998). We suggested that extraneous Cct8 protein could sequester, but not fold, PKA isoforms, thereby lowering PKA activity levels. These results are consistent with the presence of PKA and APSES proteins in a common pathway (Fig. 4).

Starvation

In *S. cerevisiae* nitrogen limitation stimulates pseudo-hyphal growth (Gimeno *et al.*, 1992). This morphogenesis is not detected in liquid medium, but only on solid nitrogen-limiting (SLAD) medium; here a yeast colony first grows to a considerable size within several days before lateral pseudohyphae appear. *C. albicans* is also triggered on SLAD medium to form filaments (true hyphae) after several days growth (Csank *et al.*, 1998). A similar phenotype occurs on a medium containing mannitol as a source of carbon and a complex source of nitrogen (modified Lee's medium or Spider medium) (Liu *et al.*, 1994). The late development of hyphae on these media suggests that some media component has to be consumed before filamentation is triggered. It has been known for a long time that pregrowth of cells into stationary phase or a period of starvation enhances the efficiency of subsequent hyphal induction by inducers like serum or GlcNAc (Shepherd *et al.*, 1980; Delbrück & Ernst, 1993). The addition of ammonium salts to Spider medium did not block hyphal development, indicating that nitrogen limitation is not responsible for filamentation in this medium (Ernst, 2000).

Nitrogen starvation in *S. cerevisiae* activates a relay of kinases, including Ste20p, Ste7p and the mitogen-activated protein (MAP) kinase Kss1p, to ultimately phosphorylate the transcription factor Ste12p (Liu *et al.*, 1993; Madhani & Fink, 1997). Homologous kinases (Hst11p, Hst7p, Cek1p) exist in *C. albicans*, along with the Ste12p homologue Cph1p; epistasis experiments have ordered most of these components in a linear pathway (Liu *et al.*, 1994; Köhler & Fink, 1996; Leberer *et al.*, 1996; Csank *et al.*, 1998) (Fig. 4). While mutations in the MAP kinase cascade efficiently block pseudo-hyphal development in *S. cerevisiae*, hyphal morphogenesis in *C. albicans* is blocked only on certain solid starvation-type (Spider or SLAD) media (Liu *et al.*, 1994; Köhler & Fink, 1996; Leberer *et al.*, 1996; Csank *et al.*, 1998). On other solid media and generally in liquid media these mutations do not affect hyphal growth. Virulence of *cst20*, *hst7* and *cph1* mutants is partially affected in the mouse model of systemic candidiasis. It is occasionally stated in the literature that both *cph1* and *efg1* mutants are partially defective in hyphal development and that the combination of both mutations blocks hyphal growth. This statement is misleading, because (true) hypha formation in *efg1* mutant is almost completely blocked under all standard induction conditions (Stoldt *et al.*, 1997; Lo *et al.*, 1997; Ernst, 2000) and the presence of a *cph1* mutation does not contribute to the (true) hypha-negative phenotype of a *efg1* mutant (although pseudohypha formation is lower in a *efg1 cph1* double mutant compared to an *efg1* mutant). A minor contribution of the *cph1* mutation to hyphal growth was detected by comparing homozygous *tup1 efg1* and *tup1 efg1 cph1* mutants (Braun & Johnson, 2000). In addition, the *cph1* mutation also contributed to the defective phenotype of *czf1* mutants under microaerophilic/embedded conditions in starvation-type media (see below) (Brown *et al.*, 1999).

Fig. 4. Environmental cues stimulate separate signalling pathways. There is no unitary signalling pathway leading to (true) hyphal formation. Different combinations of pathways, at varying intensities, are triggered in different environments. Transcription factors activated by the respective pathways are boxed. Pathways leading to pseudohypha formation are presumed to be different from the pathways triggering formation of true hyphae (not shown).

Hyperactivation of the MAP kinase pathway leads to filamentation on starvation-type media and has been achieved by overexpression of components of the MAP kinase pathway (Leberer *et al.*, 1996; Csank *et al.*, 1998). The *cph1* mutation also stimulates this pathway, presumably because the Cph1 protein is a member of the VH1 family of dual-specificity phosphatases, whose function could be to dephosphorylate and thereby inactivate the Cek1p MAP kinase (Csank *et al.*, 1997). *In vitro* inactivation of mammalian MAP kinases, its tyrosine-phosphate specificity and the reversal of the *cph1* mutant phenotype by a *cek1* mutation support this function of Cph1p. Hyperactivation of the MAP kinase pathway is also achieved by deletion of the *HOG1* gene (Alonso-Monge *et al.*, 1999) (see below).

Embedded/microaerophilic conditions

Although homozygous *efg1* single mutants have a drastic block in true hyphal formation under most standard induction conditions, considerable filamentation occurs in certain other environments (Brown *et al.*, 1999; Sonneborn *et al.*, 1999b). A limited supply of oxygen, as occurs under a coverslip during induction of chlamydospores, allows wild-type cells to form filaments, which is enhanced in *efg1* mutants (Sonneborn *et al.*, 1999b). Similarly, growth of wild-type colonies embedded in agar stimulates filamentation, which still occurs in homozygous *efg1 cph1* mutants (Riggle *et al.*, 1999; A. Giusani & C. Kumamoto, unpublished). Thus, there appears to exist an Efg1p-independent pathway of filamentation in *C. albicans*, which is operative under microaerophilic/embedded conditions. The filaments produced under microaerophilic conditions have the characteristics of mostly pseudohyphae in *EFG1* wild-type strains and of mostly true hyphae in *efg1* mutants (Sonneborn *et al.*, 1999b); under embedded conditions mostly true hyphae were produced (Brown *et al.*, 1999). Interestingly, the alternative filamentation pathway not only is independent of Efg1p, but it is even repressed by it to some degree. The enhanced filamentation in *efg1*

mutants does not depend on the Cph1 MAP kinase, because a homozygous *efg1 cph1* strain is as hyperfilamentous as the homozygous *efg1* mutant (Sonneborn *et al.*, 1999b). It is possible that agar embedding generates microaerophilic conditions, which activate the same Efg1p-independent pathway of morphogenesis under both conditions (Fig. 4).

The putative transcription factor Czf1 is probably an important element of the alternative pathway of filamentation in *C. albicans* (Brown *et al.*, 1999). The central portion of Czf1p contains four clusters of glutamine residues and the C terminus contains a cysteine-rich region similar to zinc-finger elements. There is no direct homologue of Czf1p in the genome of *S. cerevisiae*. Overexpression of *CZF1* stimulates filamentous growth, but only under embedded conditions and in certain media lacking glucose. Homozygous *czf1* null mutants filament normally under standard induction conditions, but they are defective in hyphal development when embedded in agar. This defective phenotype occurs only during embedding in certain media, such as complex medium containing sucrose or galactose as carbon sources at 25 °C, but not at 37 °C or in media containing strong inducers including serum and GlcNAc. These characteristics suggest that factors other than Czf1p contribute to filamentation under embedded conditions. The defective phenotype of a *czf1* mutant is exacerbated by the presence of a *cph1* mutation, which by itself shows defects in the types of media used for monitoring the *czf1* phenotype. Thus, although the *cph1* mutant phenotype does not appear to be specific for embedded conditions, it worsens filamentation defects caused by the *czf1* mutation.

Hyperfilamentation of *efg1* single and *efg1 cph1* double mutants suggests that Efg1p is a negative modulator of the Czf1p pathway under microaerophilic/embedded conditions (Fig. 4). Thus, these data once again suggest that positive and negative functions are combined in the Efg1 protein. Conceivably, Efg1p and Czf1p collaborate

to allow filamentation in different host environments, as in the blood (serum) and during the passage of tissues or within host cells, at low oxygen partial pressure. It is also possible that Efg1p and Czf1p trigger the formation of different types of hyphae, each of which are equipped with different sets of proteins required for viability and virulence in specific host niches, such as the blood and at limiting oxygen concentrations within cells or within organs.

pH

It is well established that a pH around neutrality (pH ~6.5) favours hyphal development of *C. albicans* *in vitro*, while a low pH (pH <6.5) blocks hyphal formation and stimulates growth of the yeast form (Buffo *et al.*, 1984). The Prr2 transcription factor, which has homologues in other fungi, has a central role in pH-dependent regulation by inducing the expression of alkaline-expressed genes and repressing acid-expressed genes at alkaline pH (Ramon *et al.*, 1999). *prp2* mutants are unable to induce the alkaline-specific gene *PHR1* and to repress the acid-specific gene *PHR2*, which both encode cell-surface proteins required for growth and cell polarization (Fonzi, 1999). Although *prp2* mutants were able to form hyphae in liquid serum media, they were defective on a solid medium containing serum. Under still weaker induction conditions, such as in some liquid media and on solid Spider medium, the *prp2* mutant was clearly defective in hyphal growth. Thus, Prr2p is needed for the full morphogenetic potential of *C. albicans*, while *PRR2* overexpression allows filamentation under non-inducing conditions (A. El Barkani & F. A. Mühlischlegel, unpublished). Prr2p contains a zinc-finger domain that is conserved in the orthologous protein PacC of *A. nidulans* and which is known to recognize the 5'-GCCAAG-3' sequence. Although such sequences occur in the promoter region of the alkaline-induced *PHR1* gene they are not required for transcriptional activation of *PHR1* (Ramon *et al.*, 1999). It is not yet clear if repression of acid-expressed genes is directly or indirectly regulated by Prr2p. Because the expression of *PRR2* depends on the Prr1 protein it was not surprising that *prp1* mutants had similar phenotypes to *prp2* mutants (Porta *et al.*, 1999). Interestingly, forced expression of one downstream target of the Prr1p/Prr2p pathway, *PHR1*, only partially reconstituted the morphogenetic defect of both *prp* mutants (Porta *et al.*, 1999). This result suggests that components other than Phr1p are involved in morphogenetic control by the Prr2 transcription factor.

Repressing environments and factors

Besides low pH, low temperature and high cell density, it is known that high glucose concentrations down-regulate hyphal development of *C. albicans* in liquid media. Glucose repression of morphogenesis is also observed initially during growth on solid media, although glucose consumption permits filamentation after several days of growth. On solid media, high

osmolarity also inhibits hypha formation (Alex *et al.*, 1998). The presence of easily utilizable nitrogen sources, such as ammonium salts, modulates hyphal development negatively to some degree (Ernst, 2000). In the infected host, inhibition of filamentation by γ -interferon occurs at contact of *C. albicans* with lymphocytes (Kalo-Klein & Witkin, 1990; Levitz & North, 1996). Some chemical inhibitors of filamentation are known including diaminobutanone and some antifungals at low concentrations, such as amphotericin and azole antifungals; these compounds inhibit hyphal development by as yet unknown mechanisms (Hawser *et al.*, 1996; Martinez *et al.*, 1990).

The Tup1 transcription factor may be involved in constituting the hypha-repressed state in the presence of glucose and other non-inducing conditions. In *S. cerevisiae*, the Tup1 protein regulates about 60 genes involved in glucose regulation, oxygen stress response and DNA damage. A *C. albicans* homologue of Tup1p was identified that is 67% identical to *S. cerevisiae* Tup1p (Braun & Johnson, 1997). Tup1p contains seven conserved WD40 repeats at the C terminus, which could anchor Tup1p to some of its DNA-binding proteins, and an N-terminal domain that could interact with a homologue of Ssn6p, as in *S. cerevisiae* (Keleher *et al.*, 1992; Komachi & Johnson, 1997). A homozygous *C. albicans* *tup1* mutant grew in filamentous form in all media tested; filaments on most media had the characteristics of pseudohyphae, but in some media had the appearance of true hyphae. Pseudohyphae of a *tup1* mutant (unlike pseudohyphae produced by *EFG1* overexpression; Stoldt *et al.*, 1997) could not be induced to form germ tubes or true hyphae by the addition of serum (Braun & Johnson, 1997). Tup1p had activities besides repression of filamentation, because *tup1* mutants failed to grow at 42 °C, grew faster on glycerol and had misshapen cell walls compared to the wild-type. In epistasis experiments most, but not all, of the filamentation phenotype induced by the *tup1* mutation was abolished by the presence of an *efg1* mutation, while a *cph1* mutation had very little effect; only a comparison of a *tup1 efg1* mutant with a *tup1 efg1 cph1* mutant showed a slight influence of the *cph1* mutation on hyphal morphogenesis (Braun & Johnson, 2000). An analysis of transcript levels of hypha-specific genes including *HYR1*, *ALS1*, *HWP1* and *ECE1* showed no differences between *tup1* and *tup1 cph1* mutants, only the *HWP1* transcript was lowered slightly in the *tup1 efg1 cph1* mutant compared to the *tup1 efg1* mutant. These results indicate that Efg1p is the main and Cph1p a minor contributor to the *tup1* hyphal phenotype. Genes repressed by Tup1p have been identified recently, of which some are expressed in a filament-specific manner (B. Braun & A. Johnson, unpublished).

In *S. cerevisiae*, the Rap1 protein acts as both a transcriptional silencer and a structural protein at telomeres by binding to a sequence designated the RPG box (Drazinic *et al.*, 1996). A *C. albicans* protein was identified, Rbf1p, which is not homologous to Rap1p, but binds to the RPG box of *S. cerevisiae* (Ishii *et al.*,

1997). Rbf1p contains two glutamine-rich regions embedding a region with weak similarity to bHLH domains, which binds to RPG sequences. Homozygous *rbf1* null mutants grew in filamentous form in all media tested; the filaments formed had the characteristics of pseudohyphae rather than true hyphae (Ishii *et al.*, 1997; N. Ishii, M. Watanabe & Y. Aoki, unpublished). Thus, Rbf1p seems to be involved exclusively in pseudohyphal, but not true hyphal growth. In fact, Rbf1p so far seems to be the only component in *C. albicans* that exclusively determines pseudohyphal growth. On an interesting aside, the authors reported that three alleles of *RBF1* were present in the standard disruption strain CAI4. Aneuploidy or triploidy had been previously demonstrated in other *C. albicans* strains, such as strain SGY-243 (Gow *et al.*, 1994; Delbrück *et al.*, 1997), but not in strain CAI4. Besides derepression of filamentous growth, the *rbf1* knockout strain showed significantly slower growth and increased sensitivities to high temperature, high osmolarity and hydrogen peroxide compared to the wild-type strain. Virulence of the *rbf1* mutant in the mouse model of systemic infection was significantly attenuated (N. Ishii, M. Watanabe & Y. Aoki, unpublished). Recently, by screening for sequences that mediate Rbf1p-dependent transcriptional regulation, target genes were identified in the heterologous host *S. cerevisiae*. Among the genes identified as Rbf1p targets was the *WH11* gene, which in phenotypic switching between a white and an opaque phenotype is specifically expressed in the white phase (Soll, 1997); the level of *WH11* transcripts is reduced in homozygous *rbf1* mutants compared to wild-type cells (N. Ishii, M. Watanabe, S. Fukuchi, M. Arisawa & Y. Aoki, unpublished). The Sir2 protein (see below) represses hyphal formation, which is consistent with the role of Sir2p as a repressor in *S. cerevisiae* (although it is unrelated to pseudohyphal growth in this species) (Perez-Martin *et al.*, 1999). Another repressing factor is the Rad6 protein, which besides contributing to UV protection, represses hyphal growth under inducing conditions by an unknown pathway; its deficiency under non-inducing conditions generates a pseudohyphal phenotype (Leng *et al.*, 2000).

Kinases in search of transcriptional pathways

A number of genes encoding conceptual protein kinases of *C. albicans* have been identified whose disruption generates a morphogenesis-defective phenotype, but whose input and output pathways are unknown. The *SLN1*, *COS1* and *HK1* genes encode possible two-component histidine kinases containing sensor and regulator domains. *In vitro* autophosphorylation activity has been shown for the Sln1p and Cos1p proteins (Yamada-Okabe *et al.*, 1999). In *S. cerevisiae*, activation of Sln1p occurs at normal osmolarity and leads to phosphorylation (and thereby inactivation) of the Ssk1 regulator. Although the *C. albicans* Sln1 and Ssk1 proteins are the direct homologues of *S. cerevisiae* Sln1 and Ssk1 proteins, they are not essential in sensing hyperosmolarity. However, hyphal development of *sln1*

and *ssk1* null mutants is blocked on starvation-type media and is severely impaired on serum agar, while filamentation is normal in all liquid media (Nagahashi *et al.*, 1998; Yamada-Okabe *et al.*, 1999; Calera *et al.*, 2000). Interestingly, while growth of the *ssk1* mutant on SLAD medium did not allow formation of hyphae, invasive growth was stimulated significantly compared to the wild-type strain (Calera *et al.*, 2000). A similar phenotype, i.e. a filamentation defect and hyperinvasive growth on solid media, was observed for *C. albicans* *hog1* mutants, which lack a homologue of the *S. cerevisiae* Hog1p MAP kinase, which in this species is a downstream target of Ssk1p (Alonso-Monge *et al.*, 1999). Although to date it has not been resolved if the Sln1p, Ssk1p and Hog1p proteins are in a common pathway it can be speculated that Hog1p downregulates the MAP kinase pathway responsible for filamentation upstream of Cst20p (Ste20p), as occurs in *S. cerevisiae* (O'Rourke & Herskowitz, 1998). Thus, the hyperinvasive characteristics of *ssk1* and *hog1* mutants is possibly related to activation of the Cph1p transcription factor.

The *COS1* (*NIK1*) and *HK1* gene products, which lack transmembrane regions, have no direct homologues in *S. cerevisiae*. The Cos1 protein is a homologue of the *N. crassa* Nik1 histidine kinase, which in this fungus is involved in hyphal growth and protects against osmotic stress. Null mutants lacking *COS1* or *HK1* alleles have no defect in osmoprotection, but they are significantly defective in hyphal formation on solid media (starvation-type or medium containing serum), but not in liquid media (Alex *et al.*, 1998; Yamada-Okabe *et al.*, 1999). In addition, the Hk1 histidine kinase is needed to prevent flocculation of hyphae (Calera & Calderone, 1999). Interestingly, deletions of *SLN1* or *COS1* alleles in a *hk1* mutant restored filamentation and virulence, suggesting that Sln1p and Cos1p act upstream of Hk1p, via a negative regulator (Yamada-Okabe *et al.*, 1999). Possibly histidine kinase pathways including the Sln1p and Cos1p pathways downregulate hyphal development on agar surfaces and within agar.

In *S. cerevisiae*, one function of the protein kinase C (PKC) pathway is to control the expression of genes encoding cell-wall components and presumably the vectorial transport of secretory vesicles (Banuett, 1998); thus, it could be expected that mutation of the gene encoding the *C. albicans* PKC homologue would significantly affect morphogenesis. A homozygous *pkc1* null strain showed a cell-lysis defect, which was osmotically remediable; however, normal hyphal morphogenesis occurred in stabilized liquid serum media (Paravicini *et al.*, 1996). Because high osmolarity inhibited hyphal formation of wild-type cells on solid media, an effect of the *pkc1* mutation on Spider media could not be clarified. A gene encoding a downstream target of PKC, the MAP kinase Mkc1p, was also shown not to be absolutely necessary for morphogenesis (Navarro-García *et al.*, 1995, 1998). Homozygous *mkc1* mutants were less viable and had cell-wall defects, and they were more sensitive to some cell-wall inhibitors. On Spider

media, hyphal development was blocked, but again, in the presence of serum and other inducers, filamentation occurred.

Cyclin-dependent protein kinases (Cdk) regulate cell-cycle progression in eukaryotes. A *C. albicans* gene (*CLN1*) encoding a G1-type cyclin homologue has been isolated and both alleles were disrupted (Loeb *et al.*, 1999). Besides slightly retarded growth the mutants were filamentation-defective on solid Spider and serum media; in liquid media an effect was seen only in Spider medium, not with serum as the inducer. The observed filamentation defects were not apparent immediately after inoculation of solid or liquid media, but *cln1* mutants appeared to revert to yeast growth more rapidly than wild-type cells. Although a gene encoding a homologue of the *S. cerevisiae* Cdc28 protein kinase has been isolated (Sherlock *et al.*, 1994) it is not yet clear if this is the Cdk protein which is activated by Cln1p. Recently, a gene encoding a Cdk homologue *CRK1* has been identified, which has a major effect on hyphal growth (J. Chen, S. Zhou, Q. Wang, X. Chen, T. Pan & H. Liu, unpublished). Hyphal formation under all standard induction conditions, in liquid and on solid media, was severely defective, but not blocked completely. Ectopic expression of *CRK1* promoted hyphal growth under non-inducing conditions. Such relatively drastic effects on morphogenesis were hitherto observed only for mutants lacking the Efg1p, Ras1 and Tpk2 proteins. There is no information yet on regulators or targets of Crk1p.

Targets of transcription factors

Several genes have been identified whose expression is induced during (true) hyphal induction. Such genes include the *HYR1*, *ALS3*, *HWP1* and *ECE1* genes, most of which encode cell-wall proteins (Bailey *et al.*, 1996; Birse *et al.*, 1993; Hoyer *et al.*, 1998; Staab *et al.*, 1999). In addition, the *EFG1* transcript is transiently downregulated during the initial phases of filamentation (Stoldt *et al.*, 1997). To date there is no evidence that any of the above discussed pathways and transcription factors directly regulate these hypha-specific genes. None of the sequence motifs found in their promoter regions, which have been proposed for the above transcription factors, including an E box (Efg1p), a FRE element (Cph1p) and a PacC element (Prr2p), have yet been proven to be essential for gene regulation. Clearly, the absence of expression of a hypha-specific gene in a filamentation-negative mutant, e.g. lacking the transcription factor Efg1p, could be due to direct or indirect effects on gene regulation. In *S. cerevisiae* many signalling pathways converge on the promoter of the *FLO11* gene, whose gene product is similar to Hwp1p (31% identity) and the Als protein family (Rupp *et al.*, 1999); recently, it has been shown that the *FLO11* promoter is regulated by the heterologous Cph1p, Efg1p and Mcm1p proteins (S. Rupp, unpublished). Expression of *HWP1* does not appear to be stringently coupled to hypha formation, since in a *prr1* mutant

HWP1 is induced in some media in the absence of filamentation (Porta *et al.*, 1999).

In phenotypic switching (Soll, 1997) there is a link between the ability to form non-yeast growth forms and switching: (1) smooth colonies are composed only of yeast-form cells, while wrinkled colonies mainly contain branched hyphae and other colony types are a mixture of yeast cells, hyphae and/or pseudohyphal cells; (2) in the white-opaque switching of strain WO-1 and its derivatives, the opaque cells are elongated or rod-like and are unable to form true hyphae, while the white-phase cells have a yeast-cell form and are competent to form hyphae; (3) deletion of the Sir2 protein enhances hypha formation and increases phenotypic switching, while it also increases chromosomal recombination (Soll *et al.*, 1993; Perez-Martín *et al.*, 1999). To date it is unclear whether Sir2p is actually involved in 'natural' switching, or if a general increase in chromosomal recombination is the predominant cause of switch phenotypes (Janbon *et al.*, 1999; Rustchenko *et al.*, 1994). Recent results implicate the Efg1p transcription factor in white-opaque phenotypic switching, because (1) the major *EFG1* transcript is expressed specifically in white-type, not in the opaque-type cells, (2) low *EFG1* expression levels generate an opaque-like cellular phenotype in strain CAI8, which is not known to switch between a white and an opaque form and (3) over-expression of *EFG1* forces opaque-type cells to switch to the white form (Sonneborn *et al.*, 1999a). In support of these data it was recently reported that *efg1* null mutants derived from strain WO-1 have the elongated cell morphology and colony phenotype typical of opaque cells under all conditions and are unable to switch spontaneously to the alternate white phenotype, although other switching phenotypes are Efg1p-independent (Srikantha *et al.*, 2000). As in the case of dimorphism, an Efg1p-target gene in phenotypic switching is not yet known. Likewise, the role of Efg1p in chlamydospore formation needs to be established, for whose morphogenesis it is the only defined factor (Sonneborn *et al.*, 2000).

Saccharomyces as a model of *Candida* morphogenesis?

Although *Sacch. cerevisiae* is an important eukaryotic model organism, homologous components in other fungi, including *Schizosaccharomyces pombe* and *C. albicans*, do not always have identical functions. Furthermore, some morphogenetic phenotypes of *C. albicans* such as true hypha formation, phenotypic switching and chlamydospore formation are not observed in *Sacch. cerevisiae*, which on the other hand is able to develop meiospores. Virulence factors regulating the interaction with host cells or the immune system are relevant for the biology of *C. albicans* as a pathogen, not of *Sacch. cerevisiae*.

On the first look, as detailed above, conserved MAP kinase and cAMP/PKA pathways function in identical morphogenetic processes in *S. cerevisiae* as in *C.*

albicans. The *C. albicans* Cst20p/Hst7p/Cek1p/Cph1p pathway corresponds to the *S. cerevisiae* Ste20p/Ste7/Kss1p/Ste12p pathway with regard to structures and sequential functions of its components (Liu *et al.*, 1993; Madhani & Fink, 1997). Although knowledge of the *C. albicans* cAMP/PKA pathway is still rudimentary, the Ras1/Tpk2/Efg1 components have structurally homologous counterparts in the *S. cerevisiae* Ras2/Tpk2/Phd1 (Sok2) proteins (Gimeno *et al.*, 1992; Gimeno & Fink, 1994; Robertson & Fink, 1998; Ward *et al.*, 1995). However, besides the different morphogenetic outputs, both pathways do not seem equally important in directing morphogenesis in *C. albicans*, as in *S. cerevisiae*. In *S. cerevisiae*, defects in the MAP kinase pathway have a major effect on pseudohypha formation, while corresponding deficiencies in *C. albicans* affect morphogenesis only under special conditions, but not during induction by serum and other inducers. The relative importance of the MAP kinase pathway in *S. cerevisiae* may be related to the fact that some of its components are used in signalling leading to mating, which is a process unknown for *C. albicans* (although recently a mating-type-like locus has been described; Hull & Johnson, 1999). Although the Ras and PKA isoforms appear functionally equivalent in both fungal species, the downstream APSES proteins Efg1p and Phd1p do not seem to represent functional homologues. The function of Phd1p in *S. cerevisiae* is clearly detected only in *phd1 ste12* double mutants, which are more defective than the *ste12* mutant in pseudohyphal growth. In *C. albicans efg1* mutants, the formation of true hyphae is blocked almost completely, while the ability to form pseudohyphae under some conditions remains and is even derepressed (opaque-like phenotype), at least in some genetic backgrounds (Lo *et al.*, 1997; Stoldt *et al.*, 1997). The combination of *efg1* and *cph1* mutations in a single strain reduces pseudohyphal growth (although only partially in our hands), but formation of true hyphae is blocked, as in the *efg1* single mutant. On the other hand, both mutants show florid hypha formation under microaerophilic/embedded conditions (see above). The latter pathway is not known in *S. cerevisiae*, but makes sense in *C. albicans*, which allows it to form filaments in host niches lacking serum or other inducers.

In summary, although *C. albicans* research on dimorphism has profited and will continue to profit from knowledge obtained in *S. cerevisiae*, functions of homologous components cannot be equated. In fact, understanding the differences between the two fungal species will be most important to establish the reasons for the particular virulence of *C. albicans*.

Future directions

The current knowledge on the mechanisms of yeast-hyphal transitions in *C. albicans* can be characterized by the statement 'many components but few connections'. Therefore, one would wish that efforts should be directed more towards an analysis of the functional

linkages instead of an expansion of the list of components affecting morphogenesis. Much of the latter research is driven by the exploration of genes/components with homologous counterparts in *S. cerevisiae*. This is a valid approach, but results should be interpreted with caution and will not necessarily clarify the particular virulence features of *C. albicans*. Most of the about 20 established components involved in dimorphism have no effect in stringent conditions, such as in liquid induction media, but only on solid media after days of growth, in which environmental parameters are changing. In this case the typical 'end-point analysis' should at least be complemented by a temporal analysis of filamentation, taking into account the switch back of (pseudo-) hyphal to yeast growth occurring under this condition. A strong appeal must be made to be absolutely clear about the types of filamentous growths that are monitored. A clear distinction between hyphae and pseudohyphae is not always made in the literature; the designation of both types of filaments as hyphae muddies the water and may deter new researchers from entering this fascinating area of research.

The mechanisms of chlamydospore formation and of spontaneous phenotypic switching are two of the most intriguing areas of research on *C. albicans* biology in the future, which are still in their infancies. As for dimorphism, the presence and the functions of signalling pathways need to be pursued, not only regarding the yeast-hyphae transition, but also regarding the reverse transition; in addition, input and output signals of morphogenetic pathways need to be clarified. Much of the research in the near future will be driven by genomic approaches, which will identify genome-wide expression targets by array techniques. Furthermore, sensors responding to environmental changes, especially in the host during infection, need to be identified, as well as their linkages to downstream signalling pathways.

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