5 CO_2 Sensing and Virulence of *Candida albicans*

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I. Introduction

The genus *Candida* includes more than 150 species, among which six are most frequently isolated from candidiasis-suffering patients. Although *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. lusitaniae* are clinically prevalent species, *C. albicans* is the main causative agent of invasive candidiasis, affecting 50% of patients worldwide. In addition, it is the most virulent of all species (Ben-Abraham et al. 2004; Tortorano et al. 2004; Almirante et al. 2005; Avila-Aguero et al. 2005; Martin et al. 2005; Fridkin et al. 2006).

A. *Candida albicans* and Candidiasis

Candida albicans is the main fungal pathogen of humans. This commensal yeast belongs to the normal flora of skin as well as gastro-intestinal and genital tracts of healthy individuals. *Candida* infections are frequently superficial and initiated when the epithelial barrier functions are impaired. However, in immunocompromised patients (premature newborns, elderly individuals, chemotherapy-treated patients, HIV patients, transplant recipients), *C. albicans* can enter the bloodstream and infect almost all internal organs, causing life-threatening systemic infections (Odds 1988; Calderone 2002). Disseminated candidiasis can reach mortality rates up to 40% (Rangel-Frausto et al. 1999; MacPhail et al. 2002; Kibbler et al. 2003). The population of patients with immune dysfunction is currently increasing and recent reports have shown that *Candida* is simultaneously developing resistance to azoles and polyene antibiotics, the most common treatments administered against fungal infections (Sanglard and Odds 2002; Tortorano et al. 2004; Almirante et al. 2005; Richter et al. 2005). Therefore, there is a need for identifying new drug targets and improving diagnostic procedures to combat this devastating situation. To achieve these objectives, the scientific and the medical communities require an improved understanding of the biology of *C. albicans* and in particular how this pathogen responds and adapts to host environmental cues.

B. Virulence Determinants in *C. albicans*

Virulence of *C. albicans* is triggered by several factors, including the secretion of adhesins required for host recognition, mannoproteins and integrin-like

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proteins necessary for adherence of the pathogen to epithelial cells, in addition to proteolytic and lipolytic enzymes which promote tissue invasiveness (Calderone and Fonzi 2001). One key characteristic of *C. albicans* is its ability to switch between yeast and filamentous (hyphae, pseudohyphae) forms (Odds 1988). This reversible morphogenetic transition also enables *C. albicans* to escape the immune system, and hence it is considered as a major virulence attribute (Lo et al. 1997; Leberer et al. 2001; Saville et al. 2003).

C. Environmental Sensing in *C. albicans*

As a commensal yeast, *C. albicans* is able to survive in various anatomic niches of its mammalian host. During its life cycle, it is exposed to a multitude of environmental cues that fluctuate dramaticallydepending on the host niches essentially temperature, pH, serum, amino acids, sugar availability and carbon dioxide. *C. albicans* is able to sense these cues and, upon exposure to physiological levels of such factors, can respond by switching from yeast to hyphal growth forms. This morphological change is caused by the expression of target genes regulated by various signal transduction pathways (Eckert et al. 2007).

This chapter focuses on the filamentation of *C. albicans* in response to the key signalling molecule carbon dioxide (CO_2). The CO_2 concentration in mammals (5%) is above 150-fold higher than in atmospheric air (0.036%). Levels of CO_2 on the mammalian host's skin are low as a result from equilibration with the atmosphere (Frame et al. 1972). Consequently, *C. albicans* must adapt to the dramatic variations in CO_2 concentrations encountered during skin infection and systemic infection. It is of crucial relevance for the pathogen to sense and respond to such variations. Therefore, this chapter aims to give the latest insights gained about the mechanism of CO_2 sensing in *C. albicans* at a molecular level and reveals the importance of this process for the virulence of the pathogen.

II. CO_2 Sensing and Signalling

Carbon dioxide plays a vital role in most ecosystems. Micro-organisms and mammals produce CO_2 as the final product of fermentation and/or cellular respiration. Moreover, photoautotrophic organisms fix atmospheric CO_2 to produce glucose by means of photosynthesis, which creates a cycle of CO_2.

CO_2 has also been identified as a key signalling molecule in various organisms. For instance, female mosquitos locate their host by detecting the level of released CO_2 (Dekker et al. 2005), and avoidance behaviour in *Drosophila* results from the activation of sensory neurons by CO_2 via a G protein-coupled receptor (Suh et al. 2004).

Importantly, CO_2 also plays a role in bacterial virulence. High concentrations of CO_2 induce the synthesis of a tripartite exotoxin (encoded by the genes *pagA*, *lef* and *cya*) and of an anti-phagocytic polysaccharide capsule (encoded by the polycistronic operon *capBCAD*) in *Bacillus anthracis*. Transcription of the toxin and capsule encoding genes is activated by the two *trans*acting regulatory proteins AtxA and AcpA, encoded by genes located on two virulence plasmids (Guignot et al. 1997; Uchida et al 1997). Drysdale et al. (2005) have shown that 5% CO_2 enhances the transcription of *atxA*. Deletion of this gene interferes with the virulence of *B. anthracis* in a mouse model of anthrax and reduces the immunological response to toxins in infected mice (Dai et al. 1995). Furthermore, it has been reported in group A *Streptococci* that elevated levels of CO_2 enhance the synthesis of the regulatory protein Mga which activates the transcription of two virulence genes, *emm* and *scpA*, encoding the antiphagocytic M protein and the C5 a endopeptidase Scp, respectively (Okada et al. 1993; McIver et al. 1995). However, no CO_2 sensor proteins have been so far identified in this model.

It has recently been shown that environmental CO_2 also strongly influences growth and morphogenesis of the pathogenic yeast *Cryptococcus neoformans* (Bahn et al. 2005; Klengel et al. 2005; Mogensen et al. 2006; Bahn and Mühlschlegel 2006). *C. neoformans* is a leading cause of central nervous system infections affecting immunosuppressed patients (Perfect and Casadevall 2002). The ubiquitous fungus is exposed to atmospheric CO_2 levels during growth in its natural habitat. Upon inhalation and subsequent lung and brain infection of its host, *C. neoformans* responds to elevated concentrations of CO_2 by producing a polysaccharide capsule surrounding its cell wall. This virulence factor interferes with phagocytosis and clearance by the immune system (Bose et al. 2003).

A. Role of CO_2 as a Signalling Molecule in *C. albicans*

CO_2 has been proven to be a powerful signal affecting dimorphism in *C. albicans* (Sims 1986). Sheth et al. (2005) recently extended this observation by investigating the response of different *Candida* species to CO_2. The authors showed that, out of 13 species tested, only *C. albicans* filaments (Fig. 5.1) when exposed to physiological concentrations of CO_2, demonstrating that filamentation in response to CO_2 is specific to *C. albicans*.

The next objectives were to determine at a molecular level how CO_2 is metabolised in the cell, what sensors detect fluctuations of CO_2 concentrations in the environment, and how the signal is transduced in *C. albicans*.

B. Metabolism of CO_2 in *C. albicans*

1. Carbonic Anhydrase

After diffusing from the surrounding environment into the cell cytoplasm, carbon dioxide is spontaneously hydrated to bicarbonate, but this reaction is accelerated by carbonic anhydrase (see Eq. 5.1). This zinc metalloenzyme is ubiquitous and has been classified in three main evolutionarily independent classes (Tripp et al. 2001). All mammalian carbonic anhydrases, as well as a few bacterial isozymes, belong to the α-class. The β-class is found in bacteria, archaea, algae and plants and has recently been identified in the basidiomycete *Cryptococcus neoformans* (Bahn et al. 2005; Mogensen et al. 2006). To date, the γ-class only consists of the archeon *Methanosarcina thermophila* enzyme (Smith and Ferry 2000).

Despite their unrelated origins, all three classes share a similar two-step enzymatic mechanism. The first step consists in the nucleophilic attack of CO_2 by a zinc-bound hydroxide ion (see Eq. 5.2). Zinc is ligated by one histidine and two cystein residues. During the second step, the active site is restored by ionisation of a zinc-bound water molecule and elimination of a proton from the active site (see Eq. 5.3; Lindskog 1997). Although this process is conserved among all characterised enzymes, the prokaryote *M. thermophila* has been shown to contain a γ-class carbonic anhydrase which is activated by iron instead of zinc (Tripp et al. 2004).

$$CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^- \quad (5.1)$$

$$E\text{–}Zn_2^+\text{–}OH^- + CO_2 + H_2O \Leftrightarrow E\text{–}Zn_2^+\text{–}HCO_3^- + H_2O \quad (5.2)$$

$$E - Zn_2^+ - HCO_3^- + H_2O \Leftrightarrow E - Zn_2^+ - H_2O + HCO_3^- \Leftrightarrow E - Zn_2^+ - OH^- + H^+ \quad (5.3)$$

Multiple copies of carbonic anhydrase-encoding genes are commonly found in many organisms, including fungal species such as *Neurospora crassa and Magnaporthe grisea*. Most fungal carbonic anhydrases belong to the β-class but within each organism the enzymes encountered may belong

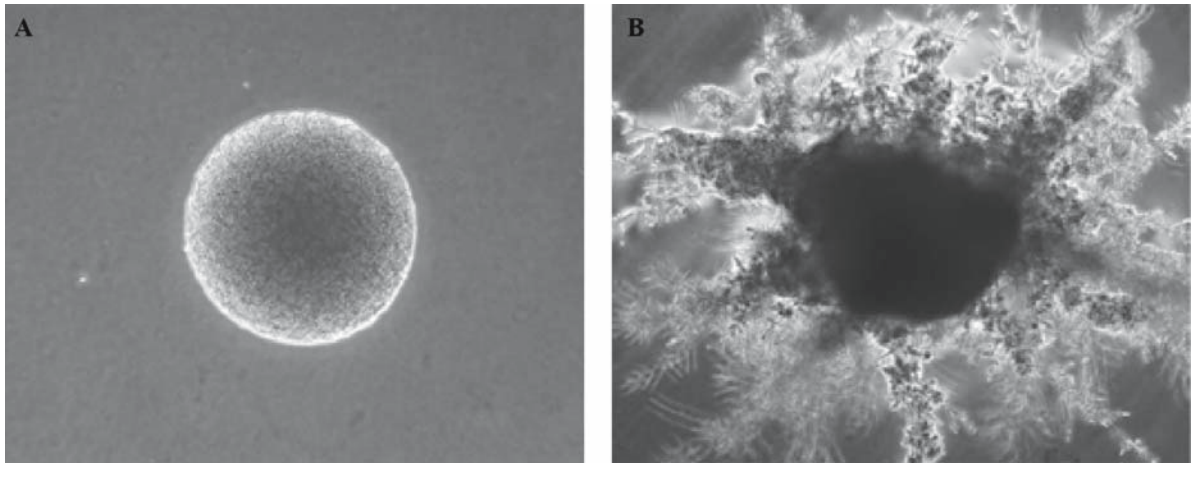

Fig. 5.1. CO_2 mediated filamentation in *Candida albicans* observed on DMEM medium at pH 7. When exposed to atmospheric air (0.033% CO_2) *C. albicans* is found as a yeast form (**A**), but physiological concentrations of CO_2 trigger filamentation (**B**)

to different families (Hewett-Emmett 2000). For instance, *Aspergillus fumigatus*, a major airborne fungal pathogen causing pulmonary infections, contains three β-CA homologues. However, it is still unknown which enzyme is required for virulence of this pathogen (Bahn and Mühlschlegel 2006).

2. Pathological Growth of *C. albicans* Under Low Levels of CO_2

The carbonic anhydrase encoding-gene *NCE103* has been cloned and characterised in *Candida albicans* (Klengel et al. 2005). It encodes a β-class enzyme, similar to those of the two yeasts *Saccharomyces cerevisiae* and *Cryptococcus neoformans* (Bahn et al. 2005; Mogensen et al. 2006). Carbonic anhydrase double deletion mutants are unable to grow in atmospheric concentrations of CO_2. Exposure to 5% CO_2 totally restores their growth defect. More importantly, carbonic anhydrase mutants of *Candida albicans* fail to invade human reconstituted epithelium in air concentrations of CO_2 but recover a phenotype similar to that of wild-type and revertant strains under physiological levels of CO_2. Moreover, *nce103* null mutants are as virulent as the revertants in a murine model of systemic infection. Interestingly, the *C. albicans* Nce103 is constitutively expressed (Klengel et al. 2005), whereas the expression of the *S. cerevisiae* ortholog is induced by low CO_2 levels (Amoroso et al. 2005). Taken together, these data demonstrate that carbonic anhydrase functions as a CO_2 scavenger and is essential for the survival of *C. albicans* in host niches where the CO_2 is limiting such as on the skin, but dispensable during systemic infection where physiological concentrations of CO_2 are encountered (Klengel et al. 2005). As CO_2 is present as concentration gradients within various niches of the host, Nce103 may be differentially expressed during colonisation and/or subsequent invasion. Consistent with these results, Allen and King (1978) have shown that *Candida* infections were enhanced when CO_2 concentrations increased on the skin surface of patients.

III. Chemosensing of CO_2/Bicarbonate

A. Adenylyl Cyclase

The second messenger cyclic adenosine 3′ -5′ -monophosphate (cAMP) is synthesised by adenylyl cyclase. Mammalian cells contain two classes of adenylyl cyclases which harbour a conserved catalytic domain but differ in their subcellular localisations and their regulation mechanism (Kamenetsky et al. 2006). Transmembrane cyclases are encoded by nine distinct genes and are activated by heterotrimeric G proteins (Hanoune and Defer 2001). The more recently discovered soluble adenylyl cyclase is present in various intracellular compartments, including mitochondria and the nucleus. Its activity is directly regulated by bicarbonate (HCO_3^-) and calcium (Chen et al. 2000; Jaiswal and Conti 2003; Litvin et al. 2003). The catalytic domains of the soluble cyclase have been shown to be more closely related to those of cyanobacteria than to those of other eukaryotes, revealing a link between prokaryotic and eukaryotic signal transduction processes (Litvin et al. 2003).

The adenylyl cyclase-encoding-gene *CYR1* (formerly named *CDC35*) was identified and cloned by Rocha and collaborators (2001). The authors showed that *cyr1* null mutants were unable to filament when incubated in various inducing media (serum, low ammonium, Spider or Lee's medium). Such mutants also presented a defect in hyphal formation when co-cultured with macrophages, preventing them from escaping from these cells. Moreover, the authors reported that *cyr1* deletion strains lost their capability to cause infection in a mouse model. These results demonstrate the essential role of adenylyl cyclase in hyphal development and virulence in *C. albicans*.

B. Role of Adenylyl Cyclase in CO_2-Dependant Filamentation

The molecular sensor and the signal transduction pathways activated by CO_2 have only recently been described in *C. albicans*. Klengel et al. (2005) demonstrated that *ras1* null mutants were able to filament and to invade agar medium upon exposure to 5% CO_2, whereas *cyr1* null mutants were not. These findings reveal that CO_2 signalling in *C. albicans* requires Cyr1 and bypasses Ras1. The adenylylcyclase encoding gene *CYR1*, expressed under the control of its own promoter or the constitutive strong *TEF2* promoter, was serially truncated and reintroduced into a *cyr1Δ/cyr1Δ* strain of *C. albicans*. The transformants were phenotypically screened for filamentation in response to physiological concentrations of CO_2. The minimal functional fragment

enabling CO_2-induced filamentation was identified as containing 120 amino acids only, showing that the core catalytic domain of Cyr1 is sufficient for CO_2/HCO_3^- activation (Eckert et al. 2007).

C. Direct Activation of Adenylyl Cyclase by Bicarbonate

Bacterial and mammalian cells contain soluble adenylyl cyclases which are directly activated by physiological concentrations of bicarbonate (Chen et al. 2000; Wuttke et al. 2001; Zippin et al. 2001). The catalytic domain of the *C. albicans* adenylyl cyclase was purified and assayed in the presence of a range of concentrations of sodium bicarbonate. Cyclase activity was stimulated more than 20-fold, demonstrating that the cellular effect of CO_2 can be mediated by its hydrated form, bicarbonate, and that bicarbonate directly activates adenylyl cyclase (Klengel et al. 2005). At physiological levels of CO_2, the intracellular bicarbonate concentration is equilibrated at 25 mM in the absence of carbonic anhydrase activity. At this concentration, the *C. albicans* cyclase Cyr1 has reached its maximal activity to induce filamentation. Therefore, this result is in agreement with the finding that carbonic anhydrase is dispensable for hyphal growth of *C. albicans* under physiological concentrations of CO_2.

Interestingly, the activity of the purified adenylyl cyclase from the closely related pathogen *Cryptococcus neoformans* increased more than six-fold in the presence of bicarbonate, hence presenting a similar pattern of direct enzymatic activation (Klengel et al. 2005; Mogensen et al. 2006). Moreover, soluble-like adenylyl cyclases have been shown to present a similar mode of activation by bicarbonate in cyanobacteria and eubacteria (Litvin et al. 2003; Zippin et al. 2004), mycobacteria (Cann et al. 2003) and the malaria-causing parasite *Plasmodium falciparum* (Levin and Buck, personal communication). These findings reveal an evolutionary link between cAMP signalling and CO_2/HCO_3^- sensing which is conserved across kingdoms.

Recent structural studies proved that bicarbonate stimulates bacterial and mammalian soluble-like adenylyl cyclases by inducing a conformational change which facilitates catalysis. Binding of bicarbonate provokes a shift of the α1 helix and of the β7–β8 loop in the same direction, causing a closure of the active site. These shifts force the β- and γ-phosphates of the ATP analog out of its binding site, which facilitate the release of the reaction product pyrophosphate from the cAMP (Steegborn et al. 2005). Nevertheless, although a number of point mutations have been introduced in the *Candida albicans* Cyr1, it is still unknown what key residues are required for the activation mechanism of the adenylyl cyclase by CO_2/HCO_3^- (Cann et al. 2003; Steegborn et al. 2005). Moreover, Hammer et al. (2006) have identified two prokaryotic cyclases isolated from *Synechocystis* and *Anabaena* which are directly activated by molecular carbon dioxide. Therefore, it remains to be elucidated which inorganic carbon species (CO_2 or/and HCO_3^-) directly activates the *C. albicans* adenylyl cyclase.

IV. Signalling Pathways Involving Adenylyl Cyclase

The dimorphic transition in *C. albicans* is regulated by two signal transduction pathways. This dual control includes the mitogen-activated protein kinases (MAPK) pathway and the cyclic AMP/protein kinase A (cAMP/PKA) pathway that are interconnected by the GTP-binding protein Ras1 (Leberer et al. 2001; Fig. 5.2).

A. Mitogen-Activated Protein Kinases Pathway

The first signalling cascade that was characterised in *C. albicans* is the MAPK pathway. In this pathway, hyphae-specific genes are activated by the transcription factor Cph1, phosphorylation of which is sequentially regulated by the kinases Cst20, Ste11, Hst7 and Cek1 (for reviews, see Brown and Gow 1999; Lengeler et al. 2000; Monge et al. 2006). Null mutations in any of the genes encoding these regulatory proteins (except *ste11*) conferred filamentation defects on synthetic low ammonium dextrose medium, Spider and solid Lee's media; however, they did not affect hyphal development in medium containing serum (liquid and solid) as well as in liquid Lee's medium (Kohler and Fink 1996; Leberer et al. 1996; Csank et al. 1998). Interestingly, *cek1Δ/cek1Δ* and *cst20Δ/cst20Δ* deletion strains of *C. albicans* present a reduced virulence compared to the wild-type strain (Leberer et al. 1996; Csank et al. 1998; Guhad et al. 1998). However, *hst7* and *cph1* null mutants are able to cause systemic candidiasis in

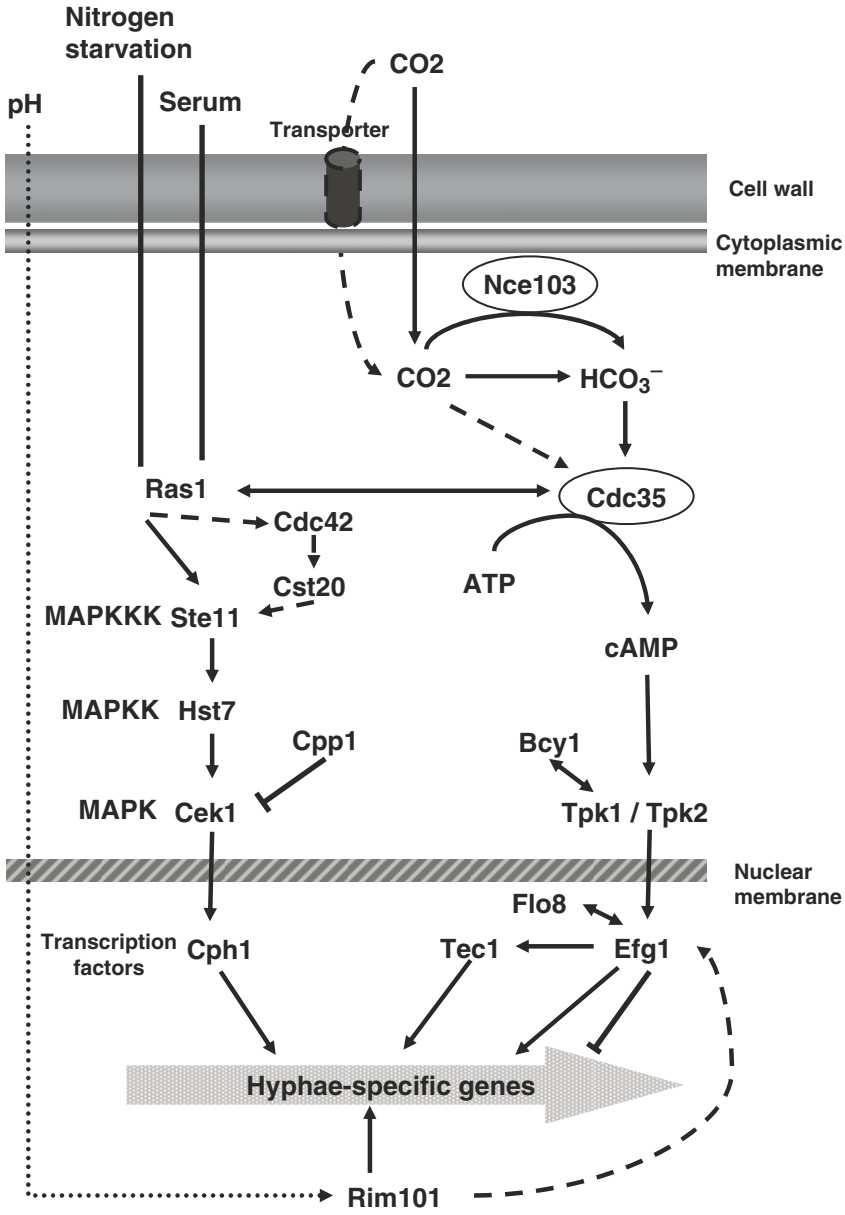

Fig. 5.2. Schematic representation of the CO_2 signal transduction pathway activating filamentation in *Candida albicans* and its connection to both the MAPK and Rim pathways. Physiological concentrations of CO_2 activate the cAMP/PKA pathway via adenylyl cyclase, leading to the expression of hyphae-specific genes. In addition, Cyr1 is activated by Ras1 which also activates the MAPK signalling cascade. The link between the Rim101 pathway and the cAMP/PKA pathway still remains hypothetical. *Dashed lines* indicate hypothesised pathways and effectors. *Dotted lines* show indirect activations. *Lines with double arrows* indicate protein–protein interactions

a mouse model (Leberer et al. 1996; Lo et al. 1997). An additional component, the MAPK phosphatase Cpp1, has been shown to inhibit Cek1. Disruption of both *cpp1* alleles reduces virulence (Csank et al. 1997; Guhad et al. 1998). In addition, the Rho-type GTP-binding protein Cdc42 and its exchange factor Cdc24 are required for hyphal development in *C. albicans* (Ushinsky et al. 2002; Bassilana et al. 2003; Vandenberg et al. 2004). Leberer et al. (2001) hypothesised that Cdc42 acts downstream Ras1 and activates the kinase Cst20.

In a study performed to determine the role of filamentation in *C. albicans* virulence, Lo et al. (1997) have shown that a single mutation in either the *cph1*

or the *efg1* gene did not affect the virulence of *C. albicans*, whereas *cph1 efg1*double mutants were shown to be avirulent. This result suggests that *cph1* and *efg1* are involved in virulence related to filamentation via two different signalling pathways (Section IVB).

B. Cyclic AMP/Protein Kinase A Pathway

Another pathway controlling development in *C. albicans* includes cAMP and the protein kinase A. The cascade consists of Ras1, adenylyl cyclase, the two isoforms Tpk1 and Tpk2 of the protein kinase A (PKA), the transcription factors Efg1 (which belongs to an important group of helix–loop–helix transcription factors controlling development in fungi and vertebrates; Stoldt et al. 1997), and Tec1 (Fig. 5.2).

While the MAPK pathway is activated mainly by starvation conditions, the cAMP/PKA cascade is stimulated by serum, N-acetylglucosamine and CO_2/HCO_3^- in *C. albicans*.

The CO_2/HCO_3^- activation of Cyr1 has been shown to bypass Ras1 (see Section IIIB). However, Feng et al. (1999) showed that *ras1* double deletion mutants were unable to form hyphae in response to serum, demonstrating that Ras1 is required for serum-induced filamentation of *C. albicans*. The interaction between Ras1 and Cyr1 has been proved to be critical for increasing the levels of cAMP, hence for filamentation (Fang and Wang 2006). Using yeast two-hybrid and binding assays, the authors showed that this GTP-dependant interaction is direct and that Ras1 binds to Cyr1 at a unique Ras association domain. In the latter, at least two conserved residues (one lysine, one leucine) are critical for the interaction of Ras1 with Cyr1.

C. albicans strains deleted for both *tpk1* alleles presented a defect in hyphal formation on solid media but their morphology was only slightly affected in liquid media. In contrast, partial filamentation of *tpk2* deletion mutants was observed on solid media whereas their hyphal morphology was totally inhibited in liquid cultures. Only *tpk2* mutants lost their capability to invade solid media (starvation and serum containing-media; Sonneborn et al. 2000). Moreover, homozygous *tpk1Δ/tpk1Δ tpk2Δ/tpk2Δ* mutants grew as much as the wild-type, but a conditional *tpk2Δ/tpk2Δ* strain containing an allele of *tpk1* under the expression of a regulatable promoter grew very slowly and was impaired in hyphal differentiation (Bockmühl et al. 2001). These results suggest that both catalytic subunits of the protein kinase A share growth function but have distinctive roles in filamentation. Tpk1 seems to induce hyphal development on solid media but is not essential for agar invasion, whereas Tpk2 is required for both filamentation and invasiveness. Construction of hybrid genes was used to demonstrate that the catalytic domains of the isoforms are responsible for morphogenesis while invasion is mediated by the N-terminal domain of Tpk2 (Bockmühl et al. 2001).

Moreover, *tpk1* deletion mutants present a delay in germ-tube formation compared to *tpk2Δ* and wild-type strains in various inducing media, which suggests that *tpk1* plays a role in early filamentation response (Souto et al. 2006). More importantly, Sonneborn et al. (2000) proved that *tpk2* deletion mutants presented a reduction in virulence. Consistent with these results, Park et al. (2005) demonstrated that homozygous *tpk2* deletion mutants present a reduced ability to invade and damage reconstituted oral epithelium compared to *tpk1* double deletion strains and to the wild-type strain. These findings suggest that Tpk2 is essential for virulence of *C. albicans*. Souto et al. (2006) have recently shown that *C. albicans* produces two *TPK2* transcripts, a major one of 1.8 kb and a minor one of 1.4 kb. They observed that transcript levels of *TPK1* are lower than those of *TPK2* at all time during vegetative growth of the pathogen. The mRNA levels of either gene were similar in *tpk1* and *tpk2* mutants compared to the wild-type strain, suggesting that each PKA isoform does not compensate the loss of the other one. Furthermore, Cassola et al. (2004) cloned Byc1, the regulatory subunit of PKA. They determined that Tpk1 was localised mostly to the nucleus in both the wild-type strain and the *tpk2* null mutant, whereas it was disseminated throughout the cell in a *bcy1 tpk2* double mutant. Moreover, they proved that Bcy1 interacts with Tpk1, suggesting that Bcy1 triggers the nucleic localisation of Tpk1.

The invasion defect observed with *tpk2* deletion mutants was reversed by overexpressing *EFG1* or *CEK1*, whereas the *efg1* phenotype was not suppressed by overexpressing *TPK2*, suggesting that Efg1 is a downstream target of Tpk2. Interestingly, Efg1 contains a potential site of phosphorylation by PKA (Sonneborn et al. 2000).

Lo et al. (1997) have reported that the response of *efg1* mutants to serum was greatly attenuated. A *C. albicans EFG1/efg1Δ* heterozygous strain develops hyphae but is less virulent than the wild-type strain, suggesting that the cAMP/PKA pathway

regulates not only morphogenesis but also the expression of other virulence factors. In addition, the cAMP/PKA cascade is also involved in programmed cell death of *C. albicans* (Phillips et al. 2006). Moreover, *hst7* and *cph1* deletion mutants present mating and hyphal development defects, demonstrating that the MAPK pathway controls mating and sporulation in addition to invasiveness (Csank et al. 1998; Chen et al. 2002; Magee et al. 2002). The finding that the MAPK and the cAMP/PKA pathways have a pleiotropic effect elucidates why both pathways are essential for filamentation in *C. albicans*. Moreover, these two pathways are complementary as they respond to different environmental signals. For instance, serum and CO_2/HCO_3^- activates specifically the cAMP/PKA pathway.

Phan et al. (2000) showed that *cph1* and *efg1* deletion mutants present a defect in invading and damage endothelial cells, demonstrating that the two genes trigger filamentation and virulence. Moreover, *cph1Δ/cph1Δ efg1Δ/efg1Δ* mutants did not stimulate leukocyte production, whereas single mutants of either gene did. Therefore, both Cph1 and Efg1 are required to induce a proinflammatory response in endothelial cells. It is of importance to mention that the transcription factor Flo8 plays an crucial role in invasive and filamentous growth of *S. cerevisiae*. Interestingly, Cao et al. (2006) have recently cloned the *C. albicans* homolog and demonstrated that *fl08* deletion mutants failed to express hyphae-specific genes. These mutants were shown to be avirulent in infected mice. It was suggested that Flo8, via its interaction with Efg1, functions downstream the cAMP/PKA pathway and regulates filamentation and virulence in *C. albicans*.

C. Regulation of Morphogenesis Mediated by pH

The pathway controlling the pH response has been characterised at the molecular level. Environmental pH modulates the expression of *PHR1* and *PHR2* encoding proteins related to glycosidases which are required for cell wall assembly (Fonzi 1999). The expression of these two genes follow an opposite pattern. Indeed, *PHR1* is highly expressed at alkaline pH whereas *PHR2* is activated by acidic pH (Saporito-Irwin et al. 1995; Mühlschlegel and Fonzi 1997). Consistent with this result, in a mouse model, *C. albicans phr1* homozygous mutant is virulent in vaginal (where pH is acidic) but not systemic (where alkaline pH is found) infections. Opposite results were observed with *phr2* mutants (De Bernardis et al. 1998). The expression of both *PHR1* and *PHR2* is regulated by the zing finger transcription factor Rim101, which is the last effector in the pH-induced pathway. El Barkani et al. (2000) demonstrated that hyphal development induced by Rim101 is Efg1-dependant. Although it is still unknown whether these two proteins belong to the same signalling pathway regulating filamentation, the authors have proposed that Rim101 acts as an upstream regulator of Efg1. As *C. albicans* is able to sense and respond to variations in pH and CO_2 levels, it was possible to speculate that these two environmental factors regulate common target genes which affect morphogenesis of the pathogen. It has been recently reported that (Sheth et al. 2008), physiological levels of pH and CO_2 coregulate *HSP12* in a Rim101 and cAMP-dependent manner, respectively (Sheth et al. 2008).

V. Potential CO_2 Transporters or Receptors

Adenylyl cyclase has been identified as a CO_2 chemosensor in *C. albicans* but the mechanism of CO_2 diffusion and/or transport into the cell still remains unknown. The apolar properties of CO_2 indicate that it may spontaneously diffuse through the cell membrane.

Aquaporins are transmembrane proteins. Although they function mainly as water channels which prevent permeation of ions, they have also been shown to be involved in the transport of CO_2 across the membrane of plants and human erythrocytes (Tyerman et al. 2002; Blank and Ehmke 2003; Uehlein et al. 2003; Endeward et al. 2006). Interestingly, Carbrey et al. (2001) have identified in *C. albicans* a single gene encoding aquaporin (*AQY1*), but *aqy1* deletion mutants retain their ability to filament in response to 5% CO_2, revealing that aquaporin is dispensable for CO_2 transport (Klengel et al. 2005).

Furthermore, rhesus proteins, which were originally discovered as ammonium transporters, have more recently been discovered in *Chlamydomonas reinhardtii* as being CO_2 channels. Their expression has been shown to be enhanced under elevated CO_2 conditions. Moreover, rhesus mutants present a growth defect at high CO_2 compared to the wild-type strain as a result of intracellular CO_2 limitation (Soupene et al. 2004; Kustu & Inwood 2006). These proteins are structurally related to

the Mep/Amt family of ammonium transporters which have been identified in the fungal species *Saccharomyces cerevisiae* and *Hebeloma cylindrosporum* (Javelle et al. 2003; Marini et al. 2006). The role of these proteins as CO_2 channels in *C. albicans* remains to be investigated.

VI. Integration of Sensing and Metabolism

Besides being involved in sensing and signal transduction, bicarbonate is also a substrate for vital carboxylation reactions leading to the synthesis of phospholipids, arginine, purines and pyrimidines, and ATP via the citric acid cycle. Although the addition of carboxylation reaction products or metabolic cycle intermediates such as adenine, oleate or citrate to minimal growth medium does not restore the growth defect of carbonic anhydrase mutants, it is believed that there is a direct link between CO_2 signalling and metabolism (Klengel et al. 2005). Interestingly, carbonic anhydrase deletion strains of *C. neoformans* present a growth defect in its natural environment where CO_2 levels are low. Growth can be partially restored by addition of the fatty acid palmitate (Bahn et al. 2005). Therefore, due to the crucial role of the various biosynthetic pathways which require bicarbonate, it is hypothesised that carbonic anhydrase mutants of *C. albicans* and *Ccryptococcus neoformans* require a combination of metabolic intermediates or products to be able to survive in niches where CO_2 concentrations are limited.

VII. Conclusions

The success of *Candida albicans* in colonising and causing infection in its mammalian hosts is largely attributed to its ability to switch from hyphal to filamentous form. This trait allows the pathogen to adapt to environmental cues that dramatically change upon the sites of invasion, in particular carbon dioxide. Physiological concentrations of CO_2 directly activate the chemosensor adenylyl cyclase, which regulates the cAMP/PKA signalling cascade and the expression of hyphae-specific genes. These findings demonstrate the importance of the role of CO_2 sensing in host-pathogen interaction and virulence.

C. albicans is able to colonise diverse host niches where it senses and responds to a multitude of signals. Carbon dioxide has been proven to be a key signalling molecule affecting growth and morphogenesis of the pathogen. In addition, growth requires various metabolic processes and morphogenesis is also mediated by pH. Therefore, the integration of multiple signals seems crucial to the survival and the virulence of *C. albicans*. The connection between CO_2 signalling and metabolism as well as the potential co-regulation of CO_2 and pH sensing in *C. albicans* requires further investigation.

The increased resistance of *C. albicans* to antifungal agents is now a real concern and drives the medical community to finding new efficient treatments. The main effectors of the signalling cascades and metabolic pathways that are involved in and connected to CO_2 sensing may be promising targets for drug development.

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