Chapter 1

SIGNALING DYNAMICS AMONG MORPHO-PATHOGENIC DETERMINANTS AND MATING REGULATORS OF
CANDIDA ALBICANS
1. 0. INTRODUCTION

Systemic fungal infections have increased dramatically in prevalence and severity over last few decades, in concert with the number of patients living for extended periods with significant immune dysfunction, AIDS, cancer chemotherapy and organ transplantation all contributing to this rise, as has the widespread use of antibiotics. The most common systemic fungal infection is candidiasis, which accounts for well over half of these invasive mycoses. A single species, Candida albicans, causes the majority of these infections. Its success stems in part from its capacity to live as a benign commensal in a variety of body locations, most notably the oral cavity, genitalia and gastrointestinal tract. C. albicans is very much related to ‘model’ yeast Saccharomyces cerevisiae. The genome sequence of C. albicans has recently been completed. A unique critical feature of C. albicans, one that has attracted researchers for decades is its ability to switch between different morphological forms. C. albicans can grow as single-celled, budding yeast forms (blastospores) or as filamentous forms (including both pseudohyphae and true hyphae). A crucial component of this versatility is its ability to survive as a commensal in several anatomically distinct sites, each with its own specific set of environmental pressure. Thus, C. albicans must be able to adapt its growth to a range of physiological extremes. This fungus has evolved sophisticated mechanisms to sense and respond to environmental cues by activating developmental switches that result in coordinated changes in cell physiology, morphology and cell adherence. Many of the Candida-specific genes (as compared with S. cerevisiae) encode catabolic proteins, and it can be postulated that some of the genes could account for the survival of the organism as a commensal and as a pathogen. The major breakthrough is assessing the contribution of specific genes to morphogenesis and virulence occurred when transformation protocol for this diploid organism were developed along with techniques that could be used to delete both alleles of a specific gene sequentially. Whole-genome microarray analysis using oligonucleotide-based arrays (Affymetrix) has now become important to understand the signal transduction pathways in different developmental programs of this organism. The outcome of this research has led to the unraveling of many fundamental biological processes that take place in C. albicans. Progress in understanding many aspect of the biology of C. albicans has been hindered by the ability to carry out simple, large scale genetic screens because of absence of well characterized sexual cycle in this organism. More surprising and exciting discovery of mating behavior covered recently a new area about the adaptation of C. albicans. This review details the current state of knowledge of physiological and molecular mechanisms of different morpho-pathogenic determinants and mating regulators.
1. 1. MAJOR POSITIVE REGULATORS FOR MORPHOGENESIS AND VIRULENCE

*C. albicans* is a dimorphic fungus, in which the yeast-to-hyphal transition is triggered by different environmental cues such as serum, N-acetylglucosamine, neutral pH, high temperature and starvation. Disease progression is promoted by yeast-to-hyphal morphogenesis, which is linked to the expression of virulence factors (Brown and Gow, 1999; Calderone and Fonzi, 2001 see Table 1. 1). Research efforts have been directed to the characterization of the signal transduction pathways involved in this process. Most of the dimorphic transition signals converge into two parallel signal transduction pathways, defined by the transcription factors Efglp and Cphlp (Ernst, 2000) (Fig. 1. 1). The Efglp protein is thought to be the major regulator in hyphal development (Lo et al., 1997; Stoldt et al., 1997). Current models place *EFGl* in the cyclic AMP (cAMP) signaling pathway. Epistatic analyses are consistent with Efglp being situated downstream of the *C. albicans* isoforms of protein kinase A (PKA) (Bockmühl et al., 2001; Sonneborn et al., 2000), and Efglp itself could be a target of PKA phosphorylation (Bockmühl and Ernst, 2001). The Cphlp/Acprlp is part of a mitogen-activated protein (MAP) kinase-based signaling pathway. Cphlp/Acprlp is a transcription factor thought to be activated by this MAP kinase cascade (Malathi et al., 1994; Liu et al., 1994; Köhler and Fink, 1996, Leberer et al., 1996; Singh et al., 1997; Leberer et al., 1997; Csank et al., 1998). Additional pathways are the pH-responsive, adhesion and embedded pathways, which involve specific regulators but which are also dependent on the Efglp and Cphlp mediated pathways (Fig. 1. 1).

1. 1. 1. Mitogen Activated Protein Kinase Pathway

Multiple pathways have been shown to regulate the morphogenic transition between budding and filamentous growth. Like *S. cerevisiae*, a mitogen activated protein kinase (MAPK) pathway is involved in filamentation in *C. albicans*. The cascade consists of the kinases Cst20p (homologous to the p21-activated kinase [PAK] kinase Ste20), CaSte7p/Hst7p (homologous to the MAP kinase kinase Ste7p), and Ceklp (homologous to the Fus3p and Kss1p MAP kinases) (Clark et al., 1995; Kohler and Fink, 1996; Leberer et al., 1996; Singh et al., 1997; Whiteway et al., 1992). The transcription factor, Acprlp/Cphlp, which is homologous to Ste12p that regulates mating and pseudohyphal growth in *S. cerevisiae*, has been identified (Malathi et al., 1994; Liu et al., 1994). Null mutations in any of the genes in the MAP kinase cascade (Cst20p, Hst7p, or Ceklp) or the transcription factor Cphlp confer a hyphal defect on solid medium in response to many inducing
Signaling Dynamics among Morpho-pathogenic Determinants and Mating Regulators of Candida albicans

**Fig. 1.** Regulation of dimorphism in *C. albicans* by multiple signaling pathways. The Cph1-mediated MAPK pathway and the Efg1-mediated cAMP pathway are well characterized signaling pathways in dimorphic regulation. In *C. albicans* Ras1p is an important regulator of hyphal development and likely functions upstream of the cAMP-dependent protein kinase A pathway. In this pathway, two catalytic subunits or isoforms of protein kinase A (PKA), Tpk1p and Tpk2p have differential effects on hyphal morphogenesis under different hypha inducing conditions. Like in *S. cerevisiae*, Cph1p/Act1p, a homolog of Ste12p and MAP kinase cascade that includes Cst20p (p21 activated kinase; MAPKKK), Hst7p (MAP kinase kinase; MAPKK) and Cek1p (MAPK) are also involved in filamentation in *C. albicans*. Ras1p also function upstream of MAPK pathways. Transcription of hyphal regulator, TEC1 is regulated by Efg1p and Cph2p. Rim101p or Czl1p may function through Efg1p or act in parallel with Efg1p. Tup1p is the negative regulator of hyphal transition. Tup1p, recruited by Rfg1p or Nrg1p or Mig1p, and Rbf1p also implicated in dimorphic transition. GlcNAc inducible hexokinase, Hxk1p plays negative role in hyphal development in certain conditions. Cell wall proteins (*HWP1, ECE1* etc. which are involved in adherence) are also regulated by major transcription factor Efg1p. Transcription factors are shown in rectangular boxes.

conditions; however, all of these mutants filament normally in response to serum (Csank *et al.*, 1998; Kohler and Fink, 1996; Leberer *et al.*, 1996). Interestingly, although a *cekl/cekl* MAP kinase mutant strain forms morphologically normal filaments in response to serum, it has a minor growth defect on serum-containing medium (Csank *et al.*, 1998). The *cekl/cekl* mutant strain also
Differences between yeast, pseudohyphae and true hyphae

*Candida albicans* can exist in three forms that have distinct shapes: yeast cells (also known as blastospores), pseudohyphal cells and true hyphal cells.

(i) Yeast cells are round to ovoid in shape and separate readily from each other.

(ii) Pseudohyphae resemble elongated, ellipsoid yeast cells that remain attached to one another at the constricted septation site and usually grow in a branching pattern that is thought to facilitate foraging for nutrients away from the parental cell and colony.

(iii) True hyphal cells are long and highly polarized, with parallel sides and no obvious constrictions between cells. Actin is always localized at the tip of the growing hypha.

A basal septin band (green) forms transiently at the junction of the mother cell and the evaginating germ tube; the first true hyphal septum forms distal to the mother cell and well within the germ tube. The sub-apical cells become highly vacuolated and do not branch or bud until the ratio of cytoplasm to vacuolar material increases significantly. All three cell types have a single nucleus per cell before mitosis. Important differences between yeast, pseudohyphal and true hyphal cells include the degree of polarized growth, the positioning of the septin ring (green in diagram and micrographs, and black in light microscope images) and of the true septum relative to the mother cell, the movement of the nucleus (blue line in diagram; stained with DAPI, blue in micrographs) relative to the mother cell and the degree to which daughter cells are able to separate into individuals. GFP, green fluorescent protein.

**Conditions of yeast-to-hyphal transition**

- **Serum**
- **N-acetylglucosamine**
- **Spider medium (Mannitol used as a carbon sources)**
- **SLAD medium (Nitrogen starvation)**
- **Iron deprivation**
- **Macrophages/neutrophils engulfment**
- **Growth in agar matrix**
- **Anoxia**
- **Lee’s Media**

**Pseudohyphae**

- **Nitrogen starvation medium (SLAD)**
- **pH-7.0, 35 °C**

**Hyphae**

- **Serum, > 34 °C**
- **Lee’s Medium, 37 °C**
- **pH-7.0, 37 °C**
has a virulence defect that may be attributable to this growth defect (Csank et al., 1998, Guhad et al., 1998a). This indicates that the Ceklp MAP kinase may function in more than one pathway or that deletion of the gene causes aberrant cross talk between distinct MAP kinase cascades, similar to the altered signaling that occurs in a fus3/fus3 mutant of S. cerevisiae. The other elements of the pathway have small but varied effects on virulence. cst20/cst20 mutant strains have a modest virulence defect in a mouse model of systemic candidiasis (Leberer et al., 1996). However, hst7/hst7 and cphl/cphl mutant strains are able to cause lethal infection in mice at rates comparable to wild-type strains (Leberer et al., 1996; Lo et al., 1997). In addition to these components, a MAP kinase phosphatase, Cpp1p, has been identified which regulates filamentous growth in C. albicans (Csank et al., 1998). Disruption of both alleles of the CPP1 gene derepresses hyphal production and results in a hyperfilamentous phenotype. This hyperfilamentation is suppressed by deletion of the MAP kinase Ceklp (Csank et al., 1998). cpp1/cpp1 mutant strains are also reduced for virulence in both systemic and localized models of candidiasis (Csank et al., 1998; Guhad et al., 1998b).

1. 1. 2. Acid proteinase–related gene ACPR/CPH1 encodes an important transcription factor for morphogenesis and mating

Acpr/Cphl, a transcription factor homologous to S. cerevisiae, Ste12, was identified first from our laboratory by reaction of the encoded protein with secretory acid proteinase antibody and therefore we refer to this as acid proteinase-related protein (Acpr1p). The antigenic epitopes are localized in the carboxy-terminal two-thirds of Acpr1p. This product has a modular structure comprising a DNA binding domain followed by a negatively charged region, which has also a potential active site for acid proteinase and a domain rich in glutamine residues. In S. cerevisiae Ste12p has an important role in pseudohyphal development and mating. Interestingly ACPR1/CPH1 of C. albicans can complement both mating defect in haploids and pseudohyphae formation in diploid S. cerevisiae (Malathi et al., 1994; Liu et al., 1994). ACPR1 also affects the budding pattern in haploid S. cerevisiae. In S. cerevisiae Ste12p binds to a heptamer sequence TGAAAACA, referred to as pheromone responsive element (PRE), which is present in multiple copies in the regulatory regions of the genes that respond to pheromones. The ability of Acpr1p to bind PRE also demonstrated in vitro (Malathi et al., 1994). Further studies will determine how the functional domain of Acpr1p responded in filament formation and mating of C. albicans.

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Table 1.
Major Morpho-pathogenic Determinants in *Candida albicans*

<table>
<thead>
<tr>
<th><em>C. albicans</em> protein homologue</th>
<th><em>S. cerevisiae</em> homologue</th>
<th>Protein function</th>
<th>References</th>
</tr>
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<tr>
<td>Ras1p</td>
<td>Ras2p</td>
<td>GTPase</td>
<td>Feng <em>et al.</em>, 1999</td>
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<td>Cyr1p</td>
<td>Cyr1p</td>
<td>Adenylate cyclase</td>
<td>Rocha <em>et al.</em>, 2001</td>
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<td>Cap1p</td>
<td>Srv2p</td>
<td>Adenylate cyclase associate protein</td>
<td>Bahn and Sundstrom, 2001</td>
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<td>Pde1p</td>
<td>Pde1p</td>
<td>Low-affinity phosphodiesterase</td>
<td>Norman and Treisman, 1988</td>
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<td>Pde2p</td>
<td>Pde2p</td>
<td>High-affinity phosphodiesterase</td>
<td>Sass <em>et al.</em>, 1986</td>
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<td>Tpk1p/Tpk2p</td>
<td>Tpk1p/Tpk2p</td>
<td>Catabolic subunit of cAMP dependent protein kinase A</td>
<td>Bockmühl <em>et al.</em>, 2001; Cloutier <em>et al.</em>, 2003</td>
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<tr>
<td>Bcy1p</td>
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<td>Regulatory subunit of cAMP dependent protein kinase A</td>
<td>Cassola <em>et al.</em>, 2004</td>
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<td>Hst7p</td>
<td>Ste7p</td>
<td>MAP kinase kinase</td>
<td>Leberer <em>et al.</em>, 1996; Singh <em>et al.</em>, 1976</td>
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<td>Cek1p</td>
<td>Fus3p/Kss1p</td>
<td>MAP kinase</td>
<td>Csank <em>et al.</em>, 1998</td>
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<td>Cpp1p</td>
<td>Cpp1p</td>
<td>MAP kinase phosphatase</td>
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<td>Tec1p</td>
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<td>Efg1p</td>
<td>Sok2p/Phd1p</td>
<td>Transcription factor of HLH family</td>
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<td>Cph2p</td>
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<td>Fkh2p</td>
<td>Forkhead transcription factor</td>
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<td>Mcm1p</td>
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<td>Transcription factor of MADS box family</td>
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<td>Tuplp</td>
<td>Transcriptional repressor</td>
<td>Braun and Johnson, 1997; Murad et al., 2001b</td>
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<td>Nrglp</td>
<td>Transcription factor with zinc-finger domain</td>
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<td>Rfglp</td>
<td>DNA-binding partner of Tuplp</td>
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<td>Rbflp</td>
<td>RPG-box binding factor</td>
<td>Ishii et al., 1997; Sharkey et al., 1999</td>
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<td>Raplp</td>
<td>Transcriptional silencer</td>
<td>Biswas et al., 2003a</td>
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<td>Rad6p</td>
<td>Transcription repressing factor</td>
<td>Leng et al., 2000</td>
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<td>Hoglp</td>
<td>MAP kinase for oxidative stress</td>
<td>Alonso-Monge et al., 1999; Alonso-Monge et al., 2003; O'Rourke and Herskowitz, 1998</td>
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<td>Ssn6p</td>
<td>Form complex with Tuplp</td>
<td>Hwang et al., 2003</td>
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<td>Slp1p</td>
<td>Histidine kinases</td>
<td>Nagahashi et al., 1998</td>
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<td>Hxk1p</td>
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<td>Kumar et al., 2000; Yamada-Otaka et al., 2001, Singh et al., 2001</td>
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<td>Hwp1p</td>
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<td>Als1p</td>
<td>Adhesin factor</td>
<td>Fu et al., 2002, Zhao et al., 2003</td>
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<td>Aspertyl proteinases</td>
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<td>Pblp</td>
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<td>Leidich et al., 1998; Mukherjee et al., 2003</td>
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<td>Gcn4p</td>
<td>General amino acid control</td>
<td>Tripathi et al., 2002</td>
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<td>Gaplp</td>
<td>General amino acid permease</td>
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<td>Csylp</td>
<td>Amino acid sensor</td>
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<td>Rim20</td>
<td>Proteolysis of Rim101</td>
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<td>Zinc-finger transcription factor</td>
<td>Davis et al., 2000a, b; El Barkani et al., 2000, Lamb et al., 2003</td>
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<td>Czlp</td>
<td>Putative zinc-finger transcription factor</td>
<td>Giusani et al., 2002</td>
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1. 1. 3. cAMP-PKA Pathway

The cAMP-dependent protein kinase A (PKA) pathway plays a very important role in filamentation in *S. cerevisiae, C. albicans* and other fungi (Sonneborn *et al.*, 2000). Nitrogen starvation results in the formation of elongated buds termed pseudohyphae, in *S. cerevisiae* dependent on activation of cAMP pathway (Gimeno *et al.*, 1992; Lengeler *et al.*, 2000). In *C. albicans*, an increase in cAMP level accompanies the yeast to hyphal transition, and inhibition of the cAMP phosphodiesterase induces this transition (Sabie and Gadd, 1992). Moreover, two cell-permeating PKA inhibitors, myristoylated protein kinase inhibitor (myrPKI) amide and the small-molecule PKA inhibitor H-89, block hyphal growth induced by N-acetylglucosamine, but not in response to serum (Castilla *et al.*, 1998).

1. 1. 3. 1. cAMP level and Morphogenesis

Recent work from several laboratories has shown that Ras-cAMP-PKA pathway (Fig. 1.1) is required for inter conversions between yeast, hyphal and pseudohyphal growth forms of *C. albicans*. Previous reports on cAMP levels during yeast-to-hyphal transition (Egidy *et al.*, 1990; Sabie and Gadd, 1992; Bahn and Sundstrom, 2001) are difficult to compare because of differences in strain and indeed experimental conditions. During germ tube induction, the mutation of one adenylate cyclase-associate protein gene does not produce a transient increase in cAMP and exhibits profound defects in filamentous growth that can be rescued by exogenous cAMP (Bahn and Sundstrom, 2001). To characterize more fully effects of hyperactivation of the cAMP pathways, Sundstrom’s group very recently chose to disrupt the PDE2 gene encoding high-affinity phosphodiesterase (PDEase) (Bahn *et al.*, 2003; Jung and Stateva, 2003). Hyperactivation of the cAMP pathway has consequences that are correlated with virulence. The result shed light on the function of pleiomorphic morphologies that *C. albicans* takes in host tissues and show that tight regulation of cAMP levels is essential for the normal growth and virulence of *C. albicans*.

1. 1. 3. 2. Adenylate Cyclase-Associate Protein gene regulates bud-hyphae transition, filamentation and virulence

*C. albicans* has a single gene homologous to the *S. cerevisiae* adenylate cyclase gene (*CDC35/CYRI*). The cyclase is not essential for growth in *C. albicans*, but is required for hyphal development (Rocha *et al.*, 2001). Recently, the adenylate cyclase associated protein (*CAP1*) has
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been identified and disrupted (Bahn and Sundstrom, 2001). An increase in cytoplasmic cAMP is seen to precede germ tube emergence in wild-type strains, but not in *cap1/cap1* mutants. The *cap1/cap1* mutant is defective in germ tube formation and hyphal development, the defects being suppressible by exogenous cAMP or dibutyryl cAMP. *cap1/cap1* strains are avirulent in a mouse model for systemic candidiasis.

1.3.3 Phosphodiesterase *PDE2* gene expression counteracts *CAP1*

Degradation of cAMP and the subsequent down-regulation of PKA are catalyzed by cAMP Phosphodiesterases (*PDE*ases). *PDE1* and *PDE2* encode the low- and high-affinity cAMP *PDE*ases, respectively in *S. cerevisiae* (Sass et al., 1986; Nikawa et al., 1987). Higher sensitivity to heat shock and nutrient starvation has been reported for *pde1/pde1* and *pde2/pde2* mutants. Exogenous cAMP greatly affects cAMP levels in *pde2/pde2* mutants, suggesting a role for *pde2p* in breaking down exogenous cAMP (Wilson et al., 1993). Cell-wall-related phenotypes, such as lysis upon hypo-osmotic shock and enhanced transformability have also been reported for *pde2/pde2* mutants, suggesting a role for *Pde2p* (and/or cAMP) in maintenance of cell-wall integrity in *S. cerevisiae* (Heale et al., 1994; Tomlin et al., 2000). Although *C. albicans* also has a low-affinity PDEase encoded by *CaPDE1* (Hoyer et al., 1994), *CaPDE2* was well studied for morphogenesis and virulence because pseudohyphal formation in *S. cerevisiae* is inhibited by *PDE2* (Kubler et al., 1997; Lorenz and Heitman, 1997; Pan and Heitman, 1999; Lengeler et al., 2000). An advantage of studying cAMP hyperactivation by disrupting *PDE2* rather than overexpressing *TPK1* or *TPK2* is that the *pde2/pde2* mutant displays phenotypes that result solely from increasing cAMP, thereby pre-empting any unpredictable effects of inequitable overexpression of one cAMP signaling component relative to the others.

Under conditions in which wild-type strains forms smooth colonies composed of budding yeasts on agar media, the homozygous *pde2/pde2*, but not the heterozygous *PDE2/pde2* mutant or the reintegrant, formed colonies that appeared wrinkled in *C. albicans*. The wrinkled colonies exhibited mixtures of elongated yeasts and filamentous forms (pseudohyphae and true hyphae). The presence of germ tube that extends into true hyphae was confirmed by detection of hyphal wall protein Hwp1p, which is not found on pseudohyphae. Germ tube formation by the homozygous *pde2/pde2* mutant was accelerated in liquid media compared with wild-type strains. But interestingly constitutive overexpression of *PDE2* blocked bud-hypha transitions and filamentous growth of *C. albicans*. To investigate the relationship between *PDE2* and *CAP1*, *PDE2* genes were disrupted in *C. albicans cap1/cap1* mutant by Bahn et al, 2003. *cap1/cap1* null mutant produce
basal levels of cAMP; however, pulses of cAMP are not generated, and bud-hyphae transition are
defective (Bahn and Sundstrom, 2001). If PDE2 regulates filamentous growth by degrading cAMP, deletion of PDE2 in the cap1/cap1 null mutant background should accumulate cAMP in the cap1/cap1 pde2/pde2 double mutant, thus correcting the bud-hypha transition defect generated by disruption of CAP1 gene. This prediction turned out to be correct by Bahn et al., 2003 because normal germ tube formation and Hwp1p production were restored in the cAMP deficient, germ tube-defective cap1/cap1 mutation by deletion of PDE2. This fact was also accompanied by continuously increasing intracellular cAMP level over 3 h periods and was identical to the effect of addition of exogenous cAMP (Bahn and Sundstrom, 2001). Again the pde2/pde2 mutant was invasive on both YPD and YNB agar plates, whereas the wild-type, cap1/cap1 and cap1/cap1 pde2/pde2 mutant were not. But the pde2/pde2 mutants are sensitive to nutrient deprivation and defective in entry into stationary phase. Even pde2/pde2 mutant is avirulent in a murine model of systemic candidiasis. The avirulent phenotype of pde2/pde2 mutant was correlated with the detrimental effects of hyperactivation of cAMP signaling including defective entry into stationary phase and increased sensitivity to nutrient starvation. The mechanism of cAMP induction of PDE2 is unknown but may involve a cAMP response element (CRE) motif (Nehlin et al., 1992) in the 5' untranslated region (UTR) of the C. albicans PDE2 promoter. They speculate that PDE2 expression is induced in response to increase in cAMP during bud-to-hyphal transition through the interaction of an unknown CRE-binding (CREB) protein to the CRE-like site in the PDE2 promoter. A similar mechanism involving cAMP-driven upregulation of a PDEase gene through a CRE in the promoter has been described in mammalian cells (Le Jeune et al., 2002) but not in fungi, to our knowledge.

1.1.3.4. Target of Protein kinase A

Growth and cellular differentiation of eukaryotic cells depends to a large extent on the activity of
CAMP-dependent protein kinases (protein kinase A, PKA). PKAs are structurally conserved,
consisting of two catabolic subunits that are inactivated by the binding of a homodimer of
regulatory subunits. External cues elevate intracellular levels of cAMP, whose binding to the
regulatory subunits liberates and thereby activates the catabolic subunits. A comparison of PKA
subunit isoforms in the fungal pathogen C. albicans and the related well-studied S. cerevisiae
reveals several significant differences. (i) The genome set of genes encoding PKA isoforms in S.
cerevisiae comprises three paralogues (TPK1-TPK3) (Toda et al., 1987b), whereas only two
homologues (TPK1 and TPK2) are present in the genome of C. albicans. (ii) In C. albicans, both
isoforms have a positive, stimulatory function on the formation of true hyphae, whereas in \textit{S. cerevisiae}, Tpk2p has a positive function and Tpk1p and Tpk3p have negative functions in pseudohyphal development (Robertson and Fink, 1998; Pan and Heitman, 1999). In \textit{C. albicans} Ras1 protein is likely to activate two protein kinase A (PKA) isoforms Tpk1p and Tpk2p (Bockmühl \textit{et al.}, 2001; Cloutier \textit{et al.}, 2003). \textit{tpk1/tpk1} mutants are defective in hyphal formation on solid media but not so much in liquid (Sonneborn \textit{et al.}, 2000) (Fig. 1. 2A). Hyphal formation in \textit{tpk2/tpk2} mutants, on the other hand, is partially affected on solid media but is blocked in liquid. Because Tpk1p and Tpk2p differ in their N-terminal domains of approximately 80-90 amino acids, while the catabolic portion are highly homologous, the functions of hybrid Tpk proteins with exchanged N-terminal domain were tested by Bockmühl \textit{et al.} (Bockmühl \textit{et al.}, 2001). The result suggested that the catabolic portions mediate Tpk protein specificities with regard to filamentation, whereas agar invasion is mediated by the N-terminal domain of Tpk2p. The regulatory subunits of PKA in both \textit{S. cerevisiae} and \textit{C. albicans} are encoded by the \textit{BCY1} (Toda \textit{et al.}, 1987a, b; Cassola \textit{et al.}, 2004).

Recently Cantore’s group examined the morphogenetic behaviour of \textit{C. albicans} yeast cells lacking the protein kinase A regulatory subunit and generated \textit{bcyl/bcyl/tpk2/tpk2} double mutant strain because a homozygous \textit{bcyl/bcyl} mutant in a wild-type genetic background could not be obtained. In the \textit{bcyl/bcyl/tpk2/tpk2} mutant, protein kinase A activity (due to the presence of the \textit{TPK1} gene) was cyclic AMP independent, indicating that the cells harbored an unregulated phosphotransferase activity. This mutant has constitutive protein kinase activity and displayed a defective germinative phenotype in N-acetylglucosamine and in serum-containing medium. It was also found the in strains having Bcy1p, the Tpk1-GFP fusion protein localizes predominantly to the nucleus. This was not in case of \textit{bcyl/bcyl/tpk2/tpk2} double mutant, where it was observed to be dispersed throughout the cell. Coimmunoprecipitation of Bcy1p with the Tpk1-GFP fusion protein demonstrated the interaction of these proteins inside the cell. So one of the roles of Bcy1p is to tether the protein kinase A catabolic subunit to the nucleus (Fig. 1. 2B). Taken together, these finding suggest that \textit{C. albicans} Bcy1p could play a pivotal role in regulating the enzymatic activity and the availability of protein kinase A, in the nucleus or in the cytoplasm, depending on its own, growth phase-related physiological requirements (Cassola \textit{et al.}, 2004).

A strain mutated for \textit{TPK2} containing a single allele of \textit{TPK1} under a regulatable promoter is unable to grow properly at low expression levels. Efg1p, the basic helix-loop-helix (bHLH) protein, that functions downstream of the PKAs. Overexpression of \textit{TPK2} is unable to suppress the
mutant phenotype of efg1/efg1, whereas overexpression of EFG1 can nullify the filamentation defect in tpk2/tpk2 (Sonneborn et al., 2000). The suppression activity of EFG1 depends on a threonine residue at the position 206, a potential phosphorylation site for a PKA (Bockmühl and Ernst, 2001).

![Diagram of PKA functions in C. albicans](image)

Fig. 1. 2. (A) Model of PKA functions in the morphogenesis of C. albicans. A single PKA regulatory subunit (Bcy1p) is activated by elevated cAMP level, which is triggered by filament-inducing conditions and produced by adenylate cyclase (AC). Inactivation of the regulatory subunit activates two catabolic PKA isoforms subunit in C. albicans (Tpk1p, Tpk2p); in addition, it is proposed that different cAMP-independent pathways determine the cellular activities of the catabolic subunits depending on the nature of the inducing medium (liquid or solid media) (Bockmühl et al., 2001). (B) In absence of regulatory subunit (Bcy1p), Tpk1p distributed throughout the cell and activates through an unknown cAMP independent pathway (Cassola et al., 2004).

1. 1. 4. Role of calmodulin in morphogenesis

Ca^{2+} and calmodulin may play a pivotal role in the regulation of yeast-to-mycelial transition in C. albicans (Paranjape, 1990). Although the molecular mechanism behind such regulation is still not elucidated, a calmodulin inhibitor, trifluoperazine (TFP), is reported to block GlcNAc-induced germ tube formation in C. albicans (Gupta Roy and Datta, 1986). Similar inhibition with TFP was also found in C. albicans grown in CaCl_2 containing defined media. Also, germ tubes and hyphae had more calmodulin activity than yeast cells, irrespective of the inducing condition used. The C. albicans calmodulin-encoding gene, CMD1, has been isolated (Saporito and Sypherd, 1991).
1.1.5. Ras1p, the master hyphal Regulator

A single Ras homolog, Ras1p, has been identified in *C. albicans*, which is not essential for survival (Feng et al., 1999). The *ras1/ras1* mutant strains have a severe defect in hyphal growth in response to serum and other conditions (Feng et al., 1999). In addition, while a dominant negative Ras1p mutation (Ras1A16) caused a defect in filamentation, a dominant active Ras1p mutation (Ras1V13) enhanced the formation of hyphae (Feng et al., 1999). The *in vitro* defects in morphological transition were reversed by either supplementing the growth media with cAMP or over expressing components of the filament inducing MAP Kinase cascade. The *ras1/ras1* mutant strains exhibit a filamentation defect similar to that of *efgL/efgL cph1/cph1* mutant strains, and evidence from *S. cerevisiae* indicates that Ras1p lies upstream of both the cAMP-PKA and MAP kinase pathways regulating pseudohyphal growth (Leberer et al., 2001; Lorenz and Heitman, 1997; Masch and Fink, 1997; Pan and Heitman, 1999; Roberts et al., 1997).

1.1.5.1. Coordination of G-protein with Ras1 signaling

Genes similar to *GPA2*, encoding α subunit of G-protein, and *GPR1*, coding for G-protein receptor of *S. cerevisiae* have been identified in the *C. albicans* genome-sequencing project, Stanford University. *GPA2* has been reported to act upstream to MAP kinase pathway in *C. albicans* (Sanchez-Martinez and Perez-Martin, 2002). Deletion of both the alleles of *GPA2* causes *in vitro* defects in filamentation formation in nitrogen starvation conditions and in embedded conditions but not in serum containing media. These defects cannot be reversed by exogenous addition of cyclic AMP. However over expression of *HST7* can complement the filamentation defect showing that *GPA2* is required for MAP kinase signaling pathway (Sanchez-Martinez and Perez-Martin, 2002). They have also analyzed the genetic interactions between the GTP-binding proteins Ras1p and Gpa2p. Their epistasis analysis using *RAS1* and *GPA2* hyperactive alleles further supports a role for Gpa2p upstream of the MAP kinase cascade. The expression in Gpa2p-defective cells of the *RAS1*<sup>G13V</sup> allele suppresses the hyphal growth defect in nitrogen starvation medium, while the expression of the *GPA2*<sup>Q355L</sup> allele in Ras1p-defective cells suppresses only slightly the defect in hyphal growth in the same growth conditions (Sanchez-Martinez and Perez-Martin, 2002).
1. 1. 6. EFG1, the major transcriptional Regulator

The *C. albicans* EFG1 gene encodes a member of APSES family fungal specific regulators that are involved in morphogenetic processes (Leng et al., 2001). This class includes StuAp of *Aspergillus nidulans*, Asm1p in *Neurospora crassa* as well as the Phd1 and Sok2 proteins of *S. cerevisiae*. StuAp is involved in the formation of conidiophores in *A. nidulans* (Miller et al., 1992), while Asm1p of *Neurospora crassa* has a role in ascospore maturation (Aramayo et al., 1996). Phd1p enhances and Sok2p suppresses pseudohyphal formation in *S. cerevisiae* (Gimeno and Fink, 1994; Ward et al., 1996).

1. 1. 6. 1. Role of EFG1 in morphogenesis in *C. albicans*

Similar to Phd1p, heterologous overexpression of Efg1p can induce pseudohyphal growth in *S. cerevisiae* (Stoldt et al., 1997). Overexpression of Efg1p can also induce filamentous growth in *C. albicans* (Stoldt et al., 1997). An efg1/efg1 mutant strain has a moderate but not complete defect in hyphal growth in response to many environmental conditions (Lo et al., 1997). efg1/efg1 mutant strains also show an aberrant morphology in the presence of serum. And overexpression of Efg1p forms exclusively pseudohyphae instead of the true hyphae that are produced by wild-type strains (Lo et al., 1997). An efg1/efg1 cph1/cph1 double mutant strain has a much more severe defect in filamentous growth and does not filament under almost any conditions tested, including the presence of serum (Lo et al., 1997). In addition, while the efg1/efg1 mutant strain has a minor reduction in virulence and the cph1/cph1 mutant has little or no defect, an efg1/efg1 cph1/cph1 double mutant strain is essentially avirulent (Lo et al., 1997). Thus, Cph1p and Efg1p define elements of two separate pathways that together are essential for both filamentation and virulence in *C. albicans*. Chlamydospore formation of the fungal pathogen *C. albicans* was found to depend on the Efg1 protein. Efg1p residue T206 is essential for chlamydospore formation. Recent studies have shown the involvement of EFG1 in normal biofilm formation (Ramage et al., 2002).

1. 1. 6. 2. Adaptation of Efg1p morphogenetic pathway

In *C. albicans*, the phenotypes of efg1/efg1 mutants and EFG1 overexpression are consistent with an activator and repressor function of Efg1p on gene expression. Hyphal morphogenesis induced by serum required Efg1p. But overexpression of EFG1 induced pseudohyphae rather than true hyphae, suggesting that Efg1p may not be required or even be inhibitory for late phases of filament
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**Fig. 1.3.** Model for morphogenetic regulation by Efglp. In hypha-inducing conditions (e.g. serum, GlcNAc) Efglp is induced and activated but microaerophilic condition it is repressed. The activated Efglp (by PKA isoforms Tpk1p and Tpk2p) initiates hypha formation by inducing genes involved in hypha formation and/or repressing genes directing the yeast form. Efglp also induces the cell wall proteins (*HWP1*, *HWP2*, *RBE1*) which are involved in adherence. The phase specific genes (at the white to opaque phase transition period) are also induced by Efglp. In parallel, Efglp in conjunction with Sin3p-Rpd3p deacetylase complex, silences chromatin, and thereby down-regulates *EFG1* promoter activity (Tebarth et al., 2003; Doedt et al., 2004). The 3.2 kb major transcript of *EFG1* is expressed in the white phase and less abundant 2.2 kb transcript is expressed in opaque cells. Hdplp-Rpd3p deacetylase complex regulates white to opaque phase transition as well as *EFG1* down regulation.

formation. So *EFG1* expression is downregulated rapidly following hyphal induction (Tebarth et al., 2003). Ernst's group also showed that Efglp mutants lacking a potential Protein kinase A (PKA) phosphorylation site is defective in filament formation (Bockmuhl and Ernst, 2001). When *EFG1* transcript levels were examined in transformants overexpressing *TPK1* or *TPK2* a reduction (about fourfold) of *EFG1* mRNA was observed (Tebarth et al., 2003). This finding suggested that Efglp is downregulated by its immediate upstream regulator PKA. Tebarth et al. also showed that overexpression of *EFG1* increases its 2.1 kb transcript, but level of the endogenous 3.2 kb-major *EFG1* transcript declined dramatically. Serial deletion analysis of the promoter region revealed that the TATA box region is required for *EFG1* autoregulation. Binding of Efglp to the *EFG1* transcriptional initiation region by chromatin immunoprecipitation was also shown. In summary, it appeared that levels and/or activities of Efglp, Tpk1p and Tpk2p were able to down-regulate the major *EFG1* promoter. In addition, they found that a homologue of Efglp, named Efhlp, also affected Efglp down-regulation, indicating that at least four proteins can contribute to *EFG1* expression. Interestingly *efg1*/*efg1* null mutant showed hyperfilamentous phenotype in an *efg1*/*efg1*
mutant background under embedded or hypoxic conditions indicating the cooperation of the Efg1p and Eh1p in suppression of an alternative morphogenetic signaling pathway (Doedt et al., 2004). Negative autoregulation and PKA-mediated downregulation is probably mediated though the Sin3p-Rpd3p containing histone deacetylase complex (Fig. 1.3).

During hyphal induction, EFG1 transcript levels declined to low levels; downregulation is affected at the level of transcriptional initiation as shown by an EFG1 promoter-LAC4 fusion. Under standard induction conditions, using serum or GlcNAc as inducers in liquid or on solid media, there was a complete block of hyphal formation in efg1/efg1 null mutants. Contrastingly, under microaerophilic or embedded conditions hyphal formation is not defective at all in homozygous efg1/efg1 mutants, but rather appeared to be stimulated (Giusani et al., 2002). These results indicate that depending on environmental cues (and depending on the genetic background); Efg1p has a dual role as a transcriptional activator and repressor, whose balanced activity is essential for yeast, pseudohyphal and hyphal morphogenesis of C. albicans.

1. 1. 6. 3. EFG1 and Phenotype Switching

Transcription of EFG1 is regulated in a unique fashion in the white-opaque transition (Srikantha et al., 2000). It is expressed in both the phases but levels of expression and the size of the transcript differ. Trans-acting factor EFG1 is expressed as a more abundant 3.2 kb transcript in the white phase and less abundant 2.2 kb transcript in the opaque phase of the transcript (Fig. 1.3). Overexpression of EFG1 in strain WO-1 of C. albicans stimulates opaque phase cells to switch to the white phase, and reduced expression results in an elongate cellular morphology similar to that of the opaque phase phenotype, but lacking opaque-specific pimples (Sonneborn et al., 1999a). Deletion of EFG1 results in cells that at first appeared to be blocked in the opaque phase phenotype (Srikantha et al., 2000). However, a shift from 25 °C to 42 °C caused mass conversion of these cells to the white phase phenotype, including deactivation of opaque phase specific genes and activation of white phase-specific genes (Srikantha et al., 2000). White phase cells of the EFG1 null mutant, still formed elongate cells that were shaped like opaque phase cells, but lacked opaque phase pimples (Srikantha et al., 2000). EFG1 is therefore not involved in the actual switch event. Rather, it functions downstream of the switch event in the regulation of a subset of white-phase specific genes involved in the generation of the round white cell shape. To begin to understand how C. albicans differentially transcribes alternative phase specific mRNA from the same gene locus, Soll’s group functionally characterized the 2320 bp upstream regulatory region of EFG1 by
analyzing the activity of deletion derivatives in a luciferase-based reporter system and identified a major white phase-specific activation sequence (AR2) (Srikantha et al., 1996). Srikantha et al (2000) also presented the evidence that, although the cis-acting sequence necessary for the transcription of the 3.2 kb EFG1 mRNA was distal to the 1.2 kb leader sequence immediately upstream of the EFG1 translation start site, cis-acting sequence regulating the 2.2 kb transcript was in the 1.2 kb leader sequence. The results suggested that the upstream region of EFG1 contained overlapping promoters for the expression of the white phase specific and opaque-phase specific transcripts. Overlapping promoter also have been demonstrated for the α and β mRNA of the EFG1 homologue StuA in A. nidulans (Wu and Miller, 1997). It should be noted that EFG1 is not the only phase regulated genes expressed as alternative molecular weight transcript in the white-opaque transition, the deacetylase HOS3 is also transcribed as a 2.5 kb transcript in the white phase and as a less abundant, lower molecular weight 2.3 kb transcript in the opaque phase (Srikantha et al., 2001).

1. 1. 6. 4. EFG1 and cell wall dynamics

Recently, the cell wall mannoprotein Hwp1p was described as being regulated by EFG1 (Sharkey et al., 1999; Braun and Johnson, 2000). In addition to HWP1, other cell wall genes such as HYR1 and ALS8 were also described as being controlled by EFG1 (Braun and Johnson, 2000), indicating that cell wall regulation at the transcriptional level plays an important role for virulence in C. albicans. In order to analyze cell wall dynamics and the regulatory function of CPH1 and EFG1 in the transcriptional control of cell wall genes in a systemic manner, Sohn et al have established a DNA microarray comprising known and as yet not further characterized putative cell wall genes (Sohn et al., 2003). They used this microarray to determine transcriptional profiles from wild-type cells, cph1/cph1, efg1/efg1 and cph/cph1efg1/efg1 double mutant strains of C. albicans cultured under various yeast or hyphae-inducing conditions (Fig. 1. 3). Overall, their data demonstrated that EFG1 plays a major role in the regulation of cell wall genes analyzed under both yeast and hyphae-inducing conditions. CPH1 seems to be of minor importance in the transcriptional control of cell wall genes during yeast and hyphal growth. During induction of filamentation, many hyphae-specific genes are expressed in wild type cells that are important for adhesion and virulence. Among those genes are HWP1 as well as HWP2 (hyphal wall protein 2). Concomitant with the upregulation of hyphal wall proteins, yeast specific proteins such as YWP1 are downregulated during the yeast-to-hyphae transition. In an efg1/efg1 mutant, the transcription of YWP1 is greatly reduced in YPD at 30 °C, whereas genes such as RBE1 (another not yet characterized cell wall
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protein) that are normally repressed by *EFG1* in wild-type cells are greatly upregulated. Instead of hyphae-specific genes, *RBE1* is now also expressed under hyphae-inducing conditions, resulting in a significantly different cell wall structure in the *efg1/efg1* mutant strains compared with wild-type cells (Fig. 1.4). Thus, not only the lack of hyphae-specific genes but also the change in the overall organization of the cell wall in the *efg1/efg1* mutant under both yeast- and hyphae inducing conditions might contribute to the reduced virulence of this mutant that has been observed before (Lo et al., 1997).

**Fig. 1.4. Model of cell wall composition in wild-type cells and *efg1 efg1* mutant strains of *C. albicans* under yeast and hyphae-inducing condition.**

1. 1. 7. Tec1p, one of the downstream effector of Efg1p

Recently, a new member of the TEA/ATTS family of transcription factors, Tec1p, has been shown to regulate hyphal development and virulence in *C. albicans* (Schweizer et al., 2000). TEA/ATTS family members AbaA in *A. nidulans* and Tec1p in *S. cerevisiae* are involved in the regulation of conidiophore formation and filamentous growth, respectively (Andrianopoulos and Timberlake, 1994; Gavrias et al., 1996). In *S. cerevisiae*, *TEC1* transcription is regulated by Ste12p, and cooperation between Tec1p and Ste12p is important for pseudohyphal growth (Madhani and Fink, 1997). In *C. albicans*, however, *TEC1* transcription is not regulated by *CPH1* (Lane et al., 2001b). *tecl/tecl* mutants exhibit suppressed filamentation in liquid serum-containing media. *EFG1* overexpression does not suppress the morphological defect of the *tecl/tecl* mutant, whereas *TEC1* overexpression has a partial phenotype in the *efg1/efg1* mutant (Schweizer et al., 2000). These
results, coupled with the fact that efg1/efg1 strains have a more severe defect in hyphal development than do tec1/tec1 strains, suggest that TEC1 is one of the downstream effectors of Efg1p.

1.1.8. Cph2p regulate hyphal development via Tec1p

Cph2p, a myc family bHLH protein, has been found to regulate hyphal development in C. albicans (Lane et al., 2001b). cph2/cph2 mutant strains are impaired in hyphal development and in the induction of hypha-specific genes in liquid Lee’s media, and Cph2p is necessary for the transcriptional induction of TEC1 (see Fig. 1.1 and Fig. 1.5). Cph2 binds directly to two sterol-regulatory-element-1-like elements upstream of TEC1. Furthermore, the ectopic expression of TEC1 expression suppresses the defect of cph2/cph2 in hyphal development. The function of Cph2p in hyphal transcription is therefore mediated, in part, through Tec1p. Lane et al examined the levels of expression of hypha-specific genes in the cph2/cph2 strains by Northern hybridization. The levels of ECE1, HWPl, HYR1, RBT4, and SAP5 expression in the cph2/cph2 strains were similar to those in the wild-type strains. Interestingly, they also observed that in some serum-containing media, Cph2p appeared to have repressive activity for a group of hyphae-specific genes, including RBT4 and SAP5.

Recently a microarray approach has been applied to determine the role of CPH1, CPH2 and EFG1 in the convergent transcriptional regulation of downstream factors and to identify differentially regulated proteins (Lane et al., 2001a). It was shown that TEC1 expression is regulated by Efg1p and Cph2p. Thus the regulation of TEC1 transcription provides one example of how C. albicans cells can integrate two different upstream signals into a single downstream output (Fig. 1.5). They have discovered that Efg1p, Cph2p, and Cph1p all regulate the expression of hydrolytic enzymes SAP5 and SAP6, which may be important for the organism to invade and damage the oral
epithelium, present at sites of oropharyngeal candidiasis in humans. All pathways that were analyzed also effected the transcription of other hyphae-specific genes HWP1, RBT4 and RBT1. Interestingly, other Tup1p-repressed genes are not regulated by Efg1p and Cph1p, and they are not required for virulence either.

1.1.9 Small acidic protein Bmh1 and pseudohyphal induction

Recently a 14-3-3 gene, BMH1 has been isolated from *C. albicans*. In *S. cerevisiae*, there are two such genes BMH1 and BMH2, which are essential for normal pseudohyphal induction and normal bud cell development. In *C. albicans*, however, there is just one copy of this gene and is essential for survival. The heterozygous mutant is defective in growth and morphogenesis (Cognetti *et al.*, 2002). The Bmh1 proteins function in the cAMP-dependent RAS/MAPK and rapamycin-sensitive signaling cascades.

1.1.10 Cph1p or Efg1p independent filamentation by Cdc5

*CaCDC5*, a cell cycle regulator polo-like kinase in *C. albicans* has been identified. Cells lacking this gene are blocked early in nuclear division. The cell cycle defects are accompanied by the formation of hyphal like filaments under yeast growth conditions. The filaments resembled serum induced hyphae. Filament formation is not dependent on Cph1p or Efg1p but requires Cdc35p (Bachewich *et al.*, 2003).

1.1.11 Contribution of Fkh2p in pseudohyphae formation and virulence

In *S. cerevisiae*, two forkhead transcription factors, *ScFKH1* and *ScFKH2* regulate the expression of B-cyclin genes. They also influence morphogenesis. *C. albicans* has only one homologue, *CaFKH2*. Cells lacking this gene formed constitutive pseudohyphae under all yeast and hyphal growth conditions tested. Under hyphal growth conditions levels of hyphae specific mRNA were reduced, and under yeast growth conditions levels of several genes encoding proteins likely to be important for cell wall separation were reduced. Together these results imply that Fkh2p is required for morphogenesis of true hyphae as well as yeast cells. Cph1p and Efg1p were not required for pseudohyphal morphology of fkh2 mutants, implying that it acts in pathways downstream or parallel to them. Cells lacking Fkh2p were unable to damage human epithelial and endothelial cells in vitro, suggesting that Fkh2p contributing to *C. albicans* virulence (Bensen *et al.*, 2002).
1.1.12. Role of CaMcm1p in morphogenesis

In *S. cerevisiae* MCM1 is an essential gene of the MADS box transcription factor family involved in a variety of cellular processes including chromatin remodeling, arginine response and mating as well as cell cycle regulation (Mai et al., 2002). *C. albicans* homologue of MCM1 was identified while screening for genes which could activate FLO11::lacZ expression in *S. cerevisiae*. Both over expression and repression of this gene led to the induction of hyphae. A hyphae-specific gene *HWP1* was induced by repression of *CaMCM1*. But the change in expression was not dependent on NRG1 or TVP1 (Rottmann et al., 2003). Thus *CaMCM1* is a component of a hitherto unknown regulatory mechanism of hyphal growth. In a distinct strain background, WO-1, *CaMCM1* mRNA level have been shown to be differentially regulated in white and opaque cells (Srikantha et al., 2001). Furthermore, several phase-specific genes contain potential MADS box binding sites (Lockhart et al., 2003b), indicating regulation by CaMcm1p.Opaque cells that show an elongated shape had reduced levels of *CaMCM1* mRNA (Rottmann et al., 2003).

1.1.13. Cdc42 and Cdc24 are required for invasive hyphal growth and pathogenicity

The function of different polarity establishment proteins in dimorphism was studied. Ectopic expression of a Rho G-protein, *CaCDC42* (Ushinsky et al., 2002; Hazan and Liu, 2002) or it's exchange factor Cdc24p were unable to form invasive hyphal filaments and germ tubes in response to serum or elevated temperature and yet could grow normally as a budding yeast. Further these mutants were avirulent in a mouse model for systemic infection. These results suggest that these proteins are required for invasive hyphal growth and pathogenicity of *C. albicans* (Bassilana et al. 2003).

1.1.14. G1 Cyclin is necessary for maintenance of filamentous Growth

*S. cerevisiae* has three major G1 cyclins. Cln1p and Cln2p are more homologous to each other in protein sequence, and their expression is cell cycle regulated, while Cln3p is only distantly related to Cln1p or Cln2p, and its expression does not vary as dramatically through the cell cycle. Two putative G1 cyclins, CaCln1p and CaCln2p, have been cloned from *C. albicans* by functional complementation in *Saccharomyces* (Loeb et al., 1999). The Cacnl/Cacnl mutants were found to be slower than wild type cells in cell cycle progression. The Cacnl/Cacnl mutants were also defective in hyphal colony formation on several solid media. The expression of hyphae-specific
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genes in the CaCln1/CaCln1 mutant is consistent with the observed morphological phenotypes. The induction of three known hyphae-specific genes, HYR1, ECE1, and HWP1 in CaCln1/CaCln1 cells in response to serum is slightly reduced (about 50%) in comparison to that in wild-type cells. In liquid Lee's medium, the CaCln1/CaCln1 strain shows a profound defect in the transcriptional activation of all three hyphae-specific genes. So CaCln1p may coordinately regulate hyphal development with signal transduction pathways in response to various environmental cues (Loeb et al., 1999).

1. 1. 15. Farnesoic acid has a role in filamentous to yeast form transition

Studies on the morphological transition from a filamentous to a budding yeast form in C. albicans have shown that this organism excretes an autoregulatory substance into the culture media. This was identified as farnesoic acid. This substance inhibited filamentous growth and might be involved in developmental signaling (Oh et al., 2001).

1. 2. MAJOR NEGETIVE REGULATORS FOR MORPHOGENESIS AND VIRULENCE

1. 2. 1. Transcriptional Repressor Tup1p

The Tup1p transcription factor may be involved in constituting the hyphae-repressed state in the presence of glucose and other non-inducing conditions. In S. cerevisiae, the Tup1p 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 and Johnson, 1997). Tup1p contains seven conserved WD40 repeats at the C terminus, which could anchor it 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 and Johnson, 1997). A homozygous C. albicans tup1/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/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 and Johnson, 1997). Tup1p had activities besides repression of filamentation, because tup1/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/tup1 mutation, was abolished by the presence of an
efgl/efgl mutation, while a cphl/cphl mutation had very little effect. A comparison of a tup1/tup1
efgl/efgl double mutant with a tup1/tup1 efgl/efgl cphl/cphl triple mutant showed a slight
influence of the cphl/cphl mutation on hyphal morphogenesis (Braun and Johnson, 2000). An
analysis of transcript levels of hyphae-specific genes including HYR1, ALS1, HWPl and ECE1
showed no differences between tup1/tup1 and tup1/tup1 cphl/cphl mutants, only the HWPl
transcript was lowered slightly in the tup1 efgl/efgl cphl/cphl mutant compared to the tup1/tup1
efgl/efgl mutant. These results indicate that Efglp is the main, and Cphlp a minor contributor
to the tup1/tup1 hyphal phenotype. Genes repressed by Tuplp have been identified recently, of
which some are expressed in a filament-specific manner (Braun and Johnson, 2000).
Braun et al identified six genes termed “repressed by TUP1” (RBT) whose expression is regulated
by TUP1 using subtractive hybridization (Braun et al., 2000). One of the genes (HWPl) has
previously been characterized, and a seventh TUP1-repressed gene (WAPI) was recovered due
to its high similarity to RBT5 (Fig. 1.1). These genes all encode secreted or cell surface proteins, and
four out of seven (HWPl, RBPl, RBTL, and WAPI) encode putatively GPI-modified cell wall
proteins. The remaining three, RBT2, RBT4, and RBT7, encodes respectively, an apparent ferric
reductase, a plant pathogenic related protein (PR-1), and a putative secreted RNase T2.

1.2.1.1. CaTuplp repression mode of action with CaNrglp and CaMiglp

A DNA binding protein, Nrglp that represses filamentous growth in C. albicans has been
identified. It contains a Zinc finger domain that is conserved in transcriptional regulators from
fungi to human. It is most closely related to ScNrglp which represses transcription in Tuplp-
dependent fashion. The nrgl/nrgl mutant cells are predominantly filamentous under non-filament
inducing conditions. They also show attenuated virulence. Nrglp represses several filament
specific genes such as ECE1 and HWPl. Most of these genes contain Nrglp response element
(NRE) in their promoter. These genes constitute a subset of those under Tuplp control, providing
further evidence that Nrglp acts by recruiting Tuplp to target genes (Braun et al., 2001; Murad et
al., 2001a, b). In S. cerevisiae another transcriptional repressor ScMiglp targets the
ScTuplp/ScSsn6p complex to the promoters of glucose repressed genes to repress their
transcription. Murad et al., 2001b provided new insights into the regulatory functions of CaTuplp,
CaNrglp and CaMiglp, as well as the differential regulation of secreted aspartyl proteinase (SAP)
and agglutinin-like sequence (ALS) gene family members. Their transcript profiling data set for C.
albicans supports current models concerning the mode of action of CaMiglp, CaNrglp and
CaTuplp. These models suggest that CaNrglp and CaTuplp regulate a different set of C. albicans
genes from CaMiglp and CaTuplp. This is consistent with the idea that CaMigl and CaNrgl target the CaTuplp repressor to specific subset of C. albicans genes (Fig. 1.6). However, CaMiglp and CaNrglp repress other C. albicans genes in a CaTuplp-independent fashion. The target of CaMiglp and CaNrglp repression, the phenotypic analysis of nrgl/nrgl and migl/migl mutants, indicate that these factors play differential roles in the regulation of metabolism, cellular morphogenesis and stress responses (Murad et al., 2001a, b).

**Fig. 1.6.** Model summarizing the transcriptional repression mediated by CaTuplp, CaNrglp and CaMiglp in C. albicans.

### 1.2.1.2. Ssn6p-Tuplp complex

In *S. cerevisiae*, Tuplp forms a transcriptional co-repressor complex in concert with Ssn6 and regulate a diverse set of genes controlled by mating type (Fig. 1.17), glucose, oxygen and DNA damage (Smith and Johnson, 2000). Although neither Tuplp nor Ssn6p itself has any DNA-binding activity, they are recruited to a specific promoter through the interaction with distinct upstream DNA-binding proteins such as Miglp, Roxlp, Ctrlp and Nrglp (Smith and Johnson, 2000). *C. albicans* CaSsn6p encodes a putative global transcriptional co-repressor. It is highly homologous to the *S. cerevisiae* Ssn6p (Hwang et al., 2003). Its expression level declines significantly in response to strong hyphal inducer such as serum. The mutant lacking Ssn6p displayed a stubby pseudohyphal growth pattern, derepressed filament specific genes in response to increased temperature and failure to develop true hyphae. Such morphological defects were not rescued by overexpression of Tuplp, Cphlp and Efglp. Overexpression of Ssn6p led to increased...
filamentation and decreased virulence (Hwang et al., 2003). So CaSsn6p may act as an activator as well as repressor of filamentous growth.

1.2.2. Recruitment of hypoxic gene, Rfglp in the general repression complex

Rfglp is a HMG protein which was cloned in an attempt to characterize the C. albicans homologue of S. cerevisiae Roxlp, a repressor of hypoxic genes. When a homologous deletion was generated, the cells became constitutively filamentous. So deletion of RFG1 results in a similar filamentous growth phenotype as reported for the deletion of TUP1. Rfglp function required Mot3p. Mot3p's role in enhancing repression by Roxlp does not involve cooperative binding, but rather helps in either the recruitment or function of the Tup1p/Ssn6p complex. Mot3p is not capable of repression in the absence of Roxlp, indicating that it is not capable of Tup1/Ssn6 recruitment (or function) on its own. Therefore, since Rfglp repression of the hypoxic reporter gene in S. cerevisiae was Mot3p dependent, it is likely that repression was also Tup1p dependent. Thus, Rfglp appears to fulfill many of the criteria that would be predicted for the regulon-specific DNA binding protein that recruits Tup1p to the hyphal growth genes. (Khalaf and Zitomer, 2001; Kadosh and Johnson, 2001)

1.2.3. CaRaplp, repressor of pseudohyphal growth under yeast growth condition

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 homologue of Rap1p has been identified which is not essential for survival. The rap1/rap1 mutants formed budding as well as pseudohyphal cells under conditions that promote budding yeast growth in wild type strain. The phenotype was reverted upon reintroduction of a functional copy. Thus this gene is required to repress pseudohyphae formation under conditions favouring growth as budding yeast (Biswas et al., 2003a).

1.2.4. RPG-box binding factor, Rbf1p

A C. albicans protein, Rbf1p, was identified 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/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). Thus, Rbf1p seems to
be involved exclusively in pseudohyphal, but not true hyphal growth. Interestingly, 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/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/rbf1 mutant in the mouse model of systemic infection was significantly attenuated (Ishii et al., 1997). 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 WHI1 gene, which in phenotypic switching between a white and an opaque phenotype is specifically expressed in the white phase (Soll, 1997); the level of WHI1 transcripts is reduced in homozygous rbf1/rbf1 mutants compared to wild-type cells (Ishii et al., 1997).

1. 2. 5. Role of Rad6p in repression of hyphal growth

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).

1. 2. 6. Hog1p and oxidative stress response

It is evident that the mechanisms of defense against oxidative stress are especially relevant for many pathogenic fungi, such as C. albicans, since the neutrophil–macrophage system is crucial in the control and outcome of the infections that they cause (Vazquez-Torres and Balish, 1997) through oxidative killing mechanism. Hog1 MAP Kinase is reported to be essential in oxidative stress response and chlamydomospore formation in C. albicans (Alonso-Monge et al., 2003). Alonso-Monge et al. showed previously the HOG pathway represses the serum-induced yeast-to-hyphal transition in C. albicans and describe its role as a major determinant of virulence. The hog1/hog1 mutant strains are more hyper invasive than wild-type (Alonso-Monge et al., 1999). The repressive role of the HOG pathway in C. albicans could be its involvement in the activation of RBF1, a transcription factor whose deletion generates hyphal forms (Ishii et al., 1997), or alternatively, a putative SSN6-TUP1 complex in C. albicans. In fact, it has been recently shown that the S. cerevisiae Ssn6p-Tup1p repressor complex (Keleher et al., 1992) plays a role in the repression of
different osmolarity-inducible genes in *S. cerevisiae* (Some of which are HOG dependent) and that ssn6p or tup1p mutants partially suppress the characteristic osmotic sensitivity associated with hog1/hog1 mutant. Hog1p was activated by phosphorylation in presence of H2O2. Increase in external osmolarity is signaled by Hog1p phosphorylation. Cap1p, homologue of Yap1p of *S. cerevisiae* and Cat1p are the only proteins that have been associated with the oxidative stress response in *C. albicans*. It was demonstrated that Hog1p and Cap1p play separate roles in oxidative stress defense and operate through different mechanisms (Fig. 1.7). A bZIP transcription factor, Sko1p, has been shown to be a repressor of the transcription factor of some oxidative stress response genes. Alonso-Monge *et al.* proposed a molecular model for the oxidative stress response in *C. albicans* (Alonso-Monge *et al.*, 2003).

![Diagram](Image)

**Fig. 1.7.** *C. albicans* seems to respond to oxidative stress in a manner different from *S. cerevisiae*. N, nucleus. The black bar inside the nucleus represents DNA. (Alonso-Monge *et al.*, 2003)

### 1.3.1. N-ACETYLGLUCOSAMINE CATABOLIC PATHWAY OF *C. ALBICANS*

The response of the organism to induction by GlcNAc involves dramatic changes in the enzyme levels of the aminosugar catabolic pathway. This inducible pathway consists of four enzymes viz. GlcNAc permease, GlcNAc kinase, GlcNAc-6-phosphate deacetylase, and GlcN-6-phosphate deaminase, which act sequentially on GlcNAc, to generate Fructose-6-phosphate that is fed into the glycolytic pathway. The notable trait of this metabolic pathway is that it is inducible by GlcNAc, and there exists a strong correlation, that GlcNAc can be utilized as a sole carbon source by the pathogenic strains of *C. albicans*.

Investigations into the GlcNAc catabolic pathway had begun by the study on the induction and regulation of N-acetylg glucosamine kinase (Bhattacharya *et al.*, 1974a, b), N-acetylglucosamine-6-phosphate deacetylase in *C. albicans* (Rai and Datta, 1982). The pathway in *C. albicans* was found
not to be repressed by glucose (Singh and Datta, 1978), though it was found that glucose does repress the catabolism of GlcNAc in *C. albicans*, giving an explanation that strain variations could cause such a conclusion (Niimi *et al.*, 1987). Synthesis of N-acetylglucosamine-catabolic enzymes, namely permease (high-affinity uptake system), kinase and deaminase was induced by N-acetylglucosamine (Singh and Datta, 1979a, b; Gopal *et al.*, 1982) during germ tube formation, and was dependent on concomitantly new protein synthesis as the inducer operated at a transcriptional level. As a related branch of the pathway, N-acetylmannosamine catabolism was also found to be inducible, by either aminosugar N-acetylmannosamine or N-acetylglucosamine and evidence was found that both the pathways converged at GlcNAc (Singh and Datta, 1979a, b). N-acetylmannosamine could also induce the enzymes for N-acetylglucosamine utilization (Sullivan and Shepherd, 1982). However, there is evidence that the germ tube formation, induced by GlcNAc and N-acetylglucosamine metabolism may be mutually exclusive events. Even GlcNAc utilization by the cells is not required for germ tube induction with immobilized-GlcNAc unavailable to the cells (Shepherd and Sullivan, 1982), which suggests that GlcNAc signaling may initiate at the cell surface. Protein synthesis is reduced in the GlcNAc induced germ tube stage, but it is not so in a high amino acid containing medium. The yeast-form cells of *C. albicans* efficiently convert to the mycelial form even under lowered rate of protein synthesis, and initiation of hyphal morphogenesis in the presence of N-acetylglucosamine is separated from cellular growth (Torosantucci *et al.*, 1984). The level of activity of the N-acetylglucosamine catabolic enzymes in germ tube stage is lower as compared to yeast phase cells. A strain of *C. albicans* that did not form germ tubes was endowed with a pronounced ability for induction of N-acetylglucosamine catabolic enzymes. (Natarajan *et al.*, 1984). It was also found that mRNA for the catabolic enzymes are either absent or present at much lower levels in glucose-grown as compared to GlcNAc grown cultures (Natarajan & Datta, 1993). These studies gave us the idea that GlcNAc not only act as an inducer of hyphal formation but also regulates the expression of a number of genes within the cells. In order to determine this, a differential screening of *C. albicans* genomic library was carried out in our lab in order to clone the differentially expressed genes. Through differential screening of *C. albicans* genomic library in Yep13 the GlcNAc inducible clones were obtained. Further studies will determine how the differentially expressed genes regulate the *C. albicans* yeast-to-hyphal transition in presence of N-acetylglucosamine. The DNA micro array studies in *C. albicans* with cDNA probes derived from GlcNAc-grown cultures are vital to understand the crosstalk of multiple GlcNAc-induced signaling pathways regulating the expression of common set of differential expressed genes.
1.3.2. The Nag Regulon

The most interesting feature of this pathway in *C. albicans* is that all the genes involved in the catabolism of GlcNAc exist in a cluster (Kumar *et al.*, 2000). This cluster houses a set of six genes (Fig. 1.8). Three of these genes are glucosamine-6-phosphate deaminase (*NAG1/CaNAG1*), GlcNAc-6-phosphate deacetylase (*DAC1/CaNAG2*) and GlcNAc kinase (*HXK1 CaNAG5*). The other three genes *NAG3/TMP*, *NAG4/TMP2* and *NAG6*, of which *NAG3/TMP* and *NAG4/TMP2* encodes for transmembrane proteins and *NAG6* for a catabolic protein with a possible GTP-binding site (Yamada-Okabe *et al.*, 2001). *CaNAG3* and *CaNAG4* share more than 80% homology with each other and have significant sequence homology with *S. cerevisiae* Ypr156C and Ygr138C (Yamada-Okabe and Yamada-Okabe, 2002, Kumar *et al.*, 2000). It could be that GlcNAc in yeast is recognized by catabolic enzymes specific for other sugars. Whatever the phenomenon, the genes responsible for uptake, phosphorylation and deacetylation of GlcNAc are yet to be defined in yeast. The most unusual feature of this cluster is that *NAG1* reading frame is opposite to that of *DAC1*, indicating that the two genes are transcribed divergently from a bi-directional promoter (Kumar *et al.*, 2000). The functional analysis of the bidirectional promoter was also confirmed by expressing two reporter genes *GFP*, encoding green fluorescent protein, and *LAC4*, encoding β-galactosidase in-vitro as well as in-vivo conditions using bidirectional promoter, replacing the catabolic pathway genes *DAC1* and *NAG1*, respectively (our unpublished data). Deletion analyses revealed that the active promoter extends to at least -400-bp upstream, with respect to *NAG1* start codon, and transcription starting at -8 and -29 (Kumar *et al.*, 2000). The removal of the distal (-291 to -281, BoxB) and proximal (-164 to -150, BoxA) regulatory regions abolished GlcNAc induction, whereas removal of only BoxB reduced induction by ~50% (Kumar *et al.*, 2000). There could be a synergistic effect of the two regions on transcriptional activation of *NAG1* and *DAC1*. *CaNAG3, CaNAG4, CaNAG5* and *CaNAG6* are transcribed from the distal to the proximal region, while
CaNAG1 and CaNAG2 are transcribed from the proximal to the distal region of the cluster (Yamada-Okabe et al., 2001). GlcNAc catabolic pathway is also reported from bacteria such as *Escherichia coli* (Vogler and Lengeler, 1989), *Klebsiella pneumoniae* and *Vibrio* species (Yamano et al., 1997). Recently it was found that N-acetylglucosamine catabolic pathway is important for intestinal colonization of *V. cholerae*. The nagA and nagB mutant of *V. cholerae* shows a statistically significant decrease in colonization as compared to the wild type strain (unpublished data). Surprisingly, NAG1, DAC1 and HXK1 are not present in *S. cerevisiae*, suggesting that either the pathway is absent or is present in a different form in the yeast. But interestingly *S. cerevisiae* strain 3059 is one exception which grew well on both glucose and the GlcNAc media. (Singh and Datta, 1979b). But how this GlcNAc catabolic pathway activated in this *S. cerevisiae* clinical isolates is not yet known.

1.3.3. Role of Nag regulon in morphogenesis and virulence

It has been reported from our laboratory that disruption of a part of this cluster involving DAC1, NAG1, and HXK1 has shown the resultant mutant unable to grow on aminosugars and avirulent in a murine model of systemic candidiasis (Singh et al., 2001). Hyphal formation under GlcNAc-inducible conditions is attenuated in the mutant, but it shows hyperfilamentation and change in colony morphology under other filament-inducing conditions like Spider (where mannitol used as carbon source) and SLAD (nitrogen starvation condition) (Fig. 1.9). Thus, disruption of the pathway leads to changes in both virulence and morphogenesis. TMP1 and TMP2 were not required for GlcNAc uptake, since there was no difference in the growth of tmp1/tmp2 mutant in GlcNAc containing medium as compared to the wild type strain (Sengupta and Datta, 2003). When checked for their ability to form germ tubes in response to GlcNAc, it was found that unlike Δnag1Δdac1Δhxk1, tmp1/tmp2 strain was competent enough to form elongated germ tubes. It was also found that tmp1/tmp2 mutant became sensitive to cycloheximide, 4NQO and 1-10 phenanthroline. The Northern analysis revealed that there was an upregulation of TMP1 and TMP2 genes when 4NQO and cycloheximide was added to the medium. When 1-10 phenanthroline was added to the medium, there was an increase in the transcription level of TMP1 but the TMP2 transcript could not be detected under similar conditions. The above observations indicate that though TMP1 and TMP2 are present in the same gene cluster as the catabolic pathway genes, they are not indispensable for GlcNAc metabolism or GlcNAc mediated signaling. Both the genes were shown to have a role in multidrug resistance (Sengupta and Datta, 2003). When the genes (*NAG1, DAC1* or *HXK1*) were disrupted individually, it was observed that the growth pattern remains the
same, i.e., all the three mutants show impaired growth in GlcNAc, but normal growth in glucose. There was difference in hyphal formation phenotype however. Germ tube formation was reduced in dac1/dac1 and hxkl/hxkl mutants but nag1/nag1 mutants retained the ability to form hyphae on plates containing serum. Thus, the hyperfilamentation that was observed in the case of the triple mutant (nag1/nag1 dac1/dac1 hxkl/hxkl) appears to be brought about by the disruption of the NAG1 gene. Virulence in a murine model of systemic candidiasis was affected in various degrees in the three individual mutants (Singh et al., 2001, Yamada-Okabe et al., 2001). The highest attenuation of virulence (>1000-fold) was shown by the hxkl/hxkl mutant, followed by dac1/dac1 (100-fold), and moderately by nag1/nag1 (10-fold).

GlcNAc 37°C 4 hours  
Wild-type Strain  Heterozygous Mutant  Homozygous Mutant  Heterozygous Revertant

SLAD 37°C 10 days

Fig. 1. Morphology of GlcNAc catabolic pathway mutants under GlcNAc inducing medium and solid SLAD (nitrogen starvation condition) plates. After induction with 2.5 mM GlcNAc, the homozygous mutant exhibited a complete block of germ tube formation. Homozygous mutant was hyperfilamentous on solid SLAD plate. Even heterozygous mutant and revertant strains failed to exhibit an intermediate phenotype in the nitrogen starvation condition (Singh et al., 2001).

1. 3. 4. Regulation of the NAG cluster genes by GlcNAc

Synthesis of GlcNAc-catabolic enzymes, namely permease (high-affinity uptake system), kinase and deaminase was observed to be induced by GlcNAc during germ tube formation, and was dependent on concomitant new protein synthesis as the inducer operated at transcriptional level. GlcNAc induces germ tubes in C. albicans within 3 hours. The increase in the levels of the catabolic enzymes is reported to reach saturation within this time. When Northern blots were carried out, it was seen that the transcripts for these genes were induced within minutes of each other (Kumar et al., 2000). NAG1 mRNA, absent in uninduced cells, is detected at 8 minutes of
induction by GlcNAc, and reaches steady-state by the first 30 minutes of growth. Similarly, \textit{DAC1} mRNA appeared at 16 minutes and \textit{HXK1} mRNA at 4 minutes upon induction by GlcNAc and reached steady state at 30 minutes of growth in GlcNAc. These observations indicate the activation of trans-acting factor(s), enabling the coordinated expression of the catabolic pathway genes. GlcNAc was unable to induce the other 3 genes in the cluster. It could be that a master switch is involved in the regulation of the whole process, activation of which leads to the turning-on of the genes concerned. Previous work in the lab has revealed that the regulation of the genes of this pathway is brought about by at least two inducible putative transcription factors. These factors may be regulated in turn via protein-protein interacting mechanisms mediated through GlcNAc induction (Kumar et al., 2000).

1. 3. 5. \(\beta\)-N-acetylglucosaminidase and virulence

\(\beta\)-N-acetylglucosaminidase, essentially a chitobiase and probably acting together with chitinase, is involved in the release of GlcNAc from chitin by hydrolysis (Barrett-Bee K and Hamilton, 1984). This could have two effects: first, it could provide an appropriate carbon source to the fungus, and secondly, it might help in the adhesion of \textit{Candida} cells to host tissues by bringing about conformational changes in the cell wall (Cannon et al., 1994). The enzyme is reported to be induced by GlcNAc and repressed by other sugars such as glucose (94-fold lower), fructose, galactose and mannose (Niimi et al., 1997). Thus, GlcNAc not only regulates its own catabolic pathway, but also regulates the availability of GlcNAc to the cell. The enzyme is secreted and deposited into various parts of the cell wall of both the yeast and hyphal forms. It is also secreted into the culture medium, more so in the case of mycelia, probably due to the more porous nature of the hyphal cell wall.

A mutant, deficient in \(\beta\)-N-acetylglucosaminidase, is reported to exhibit attenuated virulence in a mouse model of candidiasis (Jenkinson and Shepherd, 1987). However, the same mutant shows normal growth. The gene encoding \(\beta\)-N-acetylglucosaminidase, \textit{HEX1} has been cloned (Cannon et al., 1994). It has been observed that the \textit{HEX1} transcript is 200 nucleotides longer when isolated from GlcNAc-grown cells as compared to the one from glucose-grown cells, implying that alternate transcription termination sites are used depending upon the growth conditions. Temperature and pH changes were also seen to affect the level of \textit{HEX1} transcript and \(\beta\)-N-acetylglucosaminidase activity.
1. 4. KINASES IN SEARCH OF MORPHOGENESIS AND VIRULENCE

A number of genes encoding potential 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 histamine 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 Ssk1p regulator. Although the *C. albicans* Sln1p and Ssk1p proteins are the direct homologues of *S. cerevisiae* Sln1p and Ssk1p proteins, they are not essential in sensing hyperosmolarity. However, hyphal development of *sln1/sln1* and *ssk1/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/ssk1* mutant on nitrogen starvation condition did not allow formation of hyphae, invasive growth was stimulated significantly compared to the wild-type strains (Calera et al., 2000). A similar phenotype, i.e. a filamentation defect and hyperinvasive growth on solid media, was observed for *C. albicans hog1/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 till 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 and Herskowitz, 1998). Thus, the hyper invasive characteristics of *ssk1/ssk1* and *hog1/hog1* mutants are 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 Cos1p protein is a homologue of the *Neurospora 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 and Calderone, 1999). Interestingly, deletions of *SLN1* or *COS1* alleles in an *hk1/hk1* mutant restored filamentation and virulence, suggesting that Sln1p and Cos1p act upstream of Hk1p, via a negative regulator (Yamada-Okabe et al., 1999). So histidine kinase pathways including the Sln1p and Cos1p possibly 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 *pkcl/pkcl* 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 *pckl* mutation on Spider media (where mannitol used as a carbon sources) 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-Garcia *et al.*, 1995, 1998). Homozygous *mkcl/mkcl* 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.*, 1999b). 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 *chn1/chn1* 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 *CRKI* has been identified, which has a major effect on hyphal growth (Chen *et al.*, 2000). The protein sequences of Crk1p is most similar to those of *S. cerevisiae* Sog1p and human Pkl1p/Cdk9p. *crk1/crk1* mutants are impaired in the induction of hyphae specific genes and avirulent in mice. Ectopic expression of Crk1p kinase domain (*CRKIN*) promotes filamentous or invasive growth in *S. cerevisiae* and hyphal development in *C. albicans*. Crk1p promotes filamentation through a route independent of Cph1p and Efg1p in *C. albicans*. *RAS*V13 can also activate filamentation in a *cph1/cph1efg1/efg1* double mutant. Interestingly CRK1N produces florid hyphae in *ras1/ras1* mutant strains, while RASV13 generates feeble hyphae in *crk1/crk1* strains. To address the regulatory mechanisms of Crk1p, Chen’s group searched for Crk1p interacting proteins by two-hybrid screening (Ni *et al.*, 2001) and showed a *CDC37* ortholog (*CaCDC37*) interacted preferentially with the kinase domain of Crk1p (*Crk1N*) (Ni *et al.*, 2004). Recently Bachewich *et al* identified another *CaCDC5*, a cell cycle regulatory polo-like kinase (*PLK*) in *C. albicans* and demonstrate that shutting off its expression induced cell cycle defects and dramatic changes in morphology (Bachewich *et al.*, 2003). Very
recently Sudbery's group has investigated the role of the Nimlp kinases, Gin4p and Hsl1p, in the formation of these septin structures. They found that both gin4/gin4 and hsl1/hsl1 mutants form pseudohyphae constitutively, in a fashion that in the case of gin4/gin4, is partly independent of Swe1p. Gin4p-depleted pseudohyphae are unable to form hyphae when challenged with serum, but this can be overcome by ectopic expression of Gin4p from the MET3 promoter. So Gin4p plays an important role to regulate the developmental switch from pseudohyphae to hyphae (Wightman et al., 2004).

1.5. ADHESINS

The virulence factors expressed or required by Candida species, and in particular C. albicans, to cause infections may well vary depending on the type of the infections (i.e., mucosal or systemic), the site and stage of infection, and the nature of the host response. It seems apparent that a panel of virulence attributes is involved in the infective process, but no signal factor accounts for Candida virulence and not all expressed virulence attributes may be necessary for a particular stage of infection (Fallon et al., 1997). One of the important virulent attribute is adhesion. Most studies investigated the role of three well characterized adhesins (ALS family, Hwp1p, Int1p) in morphogenesis, pathogenicity and phenotype switching of C. albicans.

1.5.1.1. C. albicans adherence and HWP1

Proadhesive and proinvasive factors of C. albicans contribute to disease by mediating penetration of host tissues. Filamentous forms, particularly true hyphae, embed themselves within the superficial, keratinized layer of stratified squamous epithelium and grow by apical extension (Staab and Sundstrom, 1996). The true hyphae are extensions of germ tube that emerge from yeasts. To explore the role surface protein in tissue invasion, the function of HWP1, a developmentally regulated gene in germ tubes and true hyphae was studied (Staab and Sundstrom, 1996). HWP1 encodes an outer mannoprotein, Hwp1p, with a cell surface-exposed NH2-terminal domain and COOH-terminal features conferring covalent integration into cell wall β-glucan. It may belong to a unique subset of GPI-anchored proteins characterized by the presence of a conserved structural motif suggest that it imparts a general property, e.g., interaction with specific surface protein or wall polysaccharides. The surface localization of Hwp1p is compatible with diverse functions, from cell wall assembly to cell signaling. HWP1 has no known S. cerevisiae homolog.
1.5.1.2. Role of Hwp1p in morphogenesis and pathogenicity

*HWP1* was originally isolated as a hyphal- and germ tube-specific gene from a differential screen (Staab *et al.*, 1999; Sundstrom, 1999). Deletion analysis revealed that *HWP1* is conditionally required for hyphal formation. The ability to form hyphae on solid media was severely reduced in the *HWP1* heterozygous mutant and essentially eliminated in the null mutant. In presence of serum, colonies of the null mutant were able to produce peripheral hyphae, but at reduced levels compared to the wild type. All mutants of *HWP1* maintained the ability to invade the agar directly beneath the colony and to form germ tubes in liquid suppression cultures. *hwpJlhwpJ* null mutant was less virulent than parental or single-gene-deleted strains in a hematogenously disseminated murine model (Staab *et al.*, 1999). Also, an *HWP1*-deficient mutant caused reduced mortality in mice, germinated less readily in the kidneys of infected mice and caused less endothelial cell damage (Tsuchimori *et al.*, 2000). These data confirm the role of *HWP1* in morphogenesis and virulence. When tested *in vitro*, the *hwpllhwpJ* mutants germinated normally in liquid media containing 10% serum but exhibited reduced hyphal development when grown on serum-containing agar (Sharkey *et al.*, 1999). In the murine kidney, the organisms were almost certainly exposed to serum constituents. The finding that the null mutants germinated very poorly *in vivo* suggests that the signal transduction pathway(s) that induces germination on solid media may also regulate germination in the murine kidney. Staab *et al.* also reported in 1999 that the Hwp1 protein may act as an adhesin by serving as a substrate for host cell transglutaminases and very recently they have shown that Hwp1p on germ tubes and true hyphae of *C. albicans* forms covalent cross-links to buccal epithelial cells in vitro by functioning as a substrate for mammalian transglutaminases (Sundstrom *et al.*, 2002). Illness correlated with extensive alterations of the lingual and esophageal mucosa that were absent in mice given the *hwpllhwpJ* mutant. So *HWP1* is a promising target for development of antifungal drugs for treatment of oroesophageal candidiasis.

1.5.1.3. *HWP1* and cellular signaling

To date, at least two distinct signal transduction pathways that induce the yeast to hyphal transformation in *C. albicans* have been identified. One pathway contains the transcription factor encoded by *EFG1*, and the other is the mitogen-activated protein (MAP) kinase pathway (Csank *et al.*, 1998; Kohler *et al.*, 1996; Leberer *et al.*, 1996; Liu *et al.*, 1994; Lo *et al.*, 1997; Stoldt, 1997). *HWP1* expression is dependent on *EFG1* *in vitro*, because there is no detectable *HWP1* expression in a homozygous *efg1/efg1* null mutant (Sharkey *et al.*, 1999). *In vivo*, *HWP1* may be regulated by
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Fig. 1. Transcriptional and functional regulation of *HWP1*. Transcription of *HWP1* is regulated by major transcription factor *Efg1p*. Interestingly, hyphal gene repressor *Tup1p* and *Rbf1p* are positive regulator of *Hwp1p* whereas MADS box transcription factor *CaMcm1p* (Rottmann et al., 2003) negatively regulates *HWP1* transcription. pH regulator, *Mds3p* (Davis et al., 2002) and *Dfg5p* (Spreghini et al., 2003) are required for expression of *HWP1* in alkaline medium. *HWP1* is expressed in conjugation tubes of *a/a* cells by α-pheromone of opposite mating type cells. The cell surface protein *Hwp1p* is involved in adherence and plays a role in mating.

Factors other than *Efg1p* because the *hwp1/hwp1* mutants had markedly reduced virulence, whereas the virulence of *efg1/efg1* mutants appears to be diminished to a lesser extent (Lo et al., 1997). *HWP1* also appears to be regulated independently of the MAP kinase pathway *in vitro* and *in vivo*. Tsuchimori et al., 2000 have found that, *in vitro*, *HWP1* is expressed at normal levels in a homozygous *cph1/cph1* mutant, which lacks the terminal transcription factor of the MAP kinase pathway (Sharkey et al., 1999). In the murine model, the *hwp1/hwp1* null mutants also had a different phenotype from any of the MAP kinase mutants. The *hwp1/hwp1* mutants also had deficient germination in the murine kidney. In contrast, although some of the MAP kinase mutants have reduced virulence *in vivo* (Csank et al., 1998), in the one study where histopathology was performed, no reduction in filamentation was seen in the tissues (Leberer et al., 1996). Thus, the reduction in virulence of the MAP kinase mutants may be caused by factor(s) other than decreased filamentation. *HWP1* expression is also influenced by *TUP1* and *RBF1*, both negative regulators of hyphal development (Braun et al., 1997; Ishii et al., 1997). *HWP1* was partially derepressed under non-inducing conditions in a *TUP1* null mutant. The lack of complete derepression indicates that expression of *HWP1* is under multiple controls and probably requires an additional positive signal(s). This may be supplied via the *EFG1*-dependent pathway. Since *TUP1* influences *HWP1* expression but *CPH1* does not, *CPH1* cannot be acting upstream of *TUP1*. Unexpectedly, deletion of *RBF1* resulted in reduced expression of *HWP1* under inducing conditions, indicating that *RBF1* is a positive regulator of *HWP1*. *ECE1* exhibits a developmental expression pattern similar to *HWP1*, but *ecel/ecel* mutants are not affected in filamentation ability. However, as with *HWP1*, expression of *ECE1* was abrogated in an *EFG1* null mutant but occurred normally in a *cph1/cph1* background. *TUP1* also had no effect. In contrast, *ECE1* was not regulated by *RBF1*. 36
Recently *Candida* homologue of *Mcm1p* was identified as being important for morphogenesis (Rottman *et al*., 2003). It was found that expression of *HWP1* was induced by repression of *CaMCM1*. But this change of *HWP1* expression was not the result of change in expression levels of *NRG1* or *TUP1*. Two newly identified conserved regulator of pH, *MDS3* and *DFG5* are required for expression *HWP1* in alkaline media (Davis *et al*., 2002; Spreghini *et al*., 2003) (Fig. 1. 10).

### 1. 5. 1. 4. Role of *HWP1* in mating

Recently Lockhart *et al* (2003b) reported that *HWP1* was expressed only by opaque-phase a/a cells in response α-pheromone. Lachke *et al* (2003) also recently reported that when opaque phase cells of natural a/a and α/α strains were mixed and incubated on skin, up to 50% of the cells fused, a proportion significantly higher than that observed *in vitro* (Lachke *et al*., 2003). They entertained the possibility that cell surface molecule involved in adherence may play a role in mating and may even regulated by the mating process. They also investigated *HWP1* was expressed in conjugation tubes induced by pheromone of opposite mating type. Selective fluorescence staining techniques, including immunostaining with anti-Hwp1p antibody, reveal that Hwp1p is expressed exclusively on conjugation tube of a/a cells (Fig. 1. 10), not α/α cells, in mating mixtures. In addition, the first daughter bud formed in the mating progress emerges from the a/a contribution to the conjugation bridge, which selectively contains Hwp1p. So contiguous conjugation bridge of a/a and α/α cells are functionally distinct. But to understand how *HWP1* is regulated in conjugation tube, a functional analysis of the *HWP1* promoter is necessary.

### 1. 5. 2. Role of *INT1* in adhesion

In *C. albicans*, multiple adhesins mediate attachment to epithelium, endothelium, or platelets (Bendel *et al*., 1995). *C. albicans* gene *INT1* encodes a surface expressed glycoprotein, Int1p. Int1p is implicated in adhesion to human epithelial cells, morphologic switching to filamentous forms, and virulence (Gale *et al*., 1996; Gale *et al*., 1998; Bendel *et al*., 1999). The *C. albicans INT1* gene was originally cloned because of its similarity to vertebrate leukocyte integrin (Gale *et al*., 1996), adhesins that bind extracellular matrix proteins and induce morphogenic changes in response to extracellular signals (Ruoslahti *et al*., 1987). *INT1* expression in the budding yeast *S. cerevisiae* triggers a morphologic switch to filamentous growth (Gale *et al*., 1996). *C. albicans int1/int1* strains have a reduced ability to form hyphae on Milk-Tween and Spider media but form apparently normal hyphae in presence of serum (Gale *et al*., 1998). In addition, *int1/int1* strains
have attenuated virulence in a mouse model of systemic candidiasis (Gale et al., 1998). Recently Asleson’s group exploited the ability of \( C. \) \( \textit{albicans} \) \( INT1 \) to induce filamentous growth in \( S. \) \( \textit{cerevisiae} \) to ask about the cytoskeleton components required for \( INT1 \)-induced filamentous growth and indicated that \( \text{Sla2p} \), a cytoskeleton protein, might interact with \( \text{Int1p} \) to mediate morphogenesis by modulating the actin skeleton. (Asleson et al., 2001). \( S. \) \( \textit{cerevisiae} \) \( \text{Sla2p} \) protein was closely related to mouse talin. In addition, they determined that \( \text{Sla2p} \) does not trigger \( INT1 \)-induced filamentous growth exclusively through the morphogenesis checkpoint mediated by \( \text{Swe1p} \) kinase (McMillan, 1998). \( \text{Swe1p} \) phosphorylates \( \text{Clb2/Cdc28} \), thereby preventing the switch to isotropic growth that occurs early in the cell cycle (Sia et al., 1996). \( \text{Sla2p} \) and \( \text{Swe2p} \) contribute independently to polarized growth in \( S. \) \( \textit{cerevisiae} \) (Fig. 1.11). In \( C. \) \( \textit{albicans} \), \( \text{Int1p} \) is important in axial budding pattern and colocalizes with \( \text{Cdc3p} \) septin in a ring at the mother-bud neck of yeast and pseudohyphal cells. Under conditions that induce hyphae, both \( \text{Cdc3p} \) and \( \text{Int1p} \) localizes to a ring distal to the junction of the mother cell and germ tube. Thus placement of the \( \text{Int1p/sep} \) ring with respect to the mother daughter cell junction distinguishes yeast/pseudohyphal growth from hyphal growth (Gale et al., 2001).

Fig. 1.11. \( \text{Int1p} \) triggers filamentous growth through at least two pathways. \( \text{Sla2p} \) and \( \text{Swe1p} \) contribute independently to polarized growth in \( S. \) \( \textit{cerevisiae} \) cell expressing \( INT1 \). \( \text{Swe1p} \) may also trigger the morphogenesis checkpoint via \( \text{Swe1p} \) (Asleson et al., 2001)
The ALS family of *C. albicans* encodes large cell-surface glycoproteins with three domain structure that are implicated in the process of adhesion to host surfaces (Hoyer, 2001). The ALS (Agglutinin-Like Sequence) family includes eight genes named ALS1 to ALS7 and ALS9. Recent work showed that ALS3 and ALS8, which were presumed to represent separate loci, are the same gene, eliminating ALS8 from the family nomenclature (Zhao et al., 2003). Each Als protein has a relatively conserved N-terminal domain, a central domain consisting of a tandem copies of a highly conserved 108bp unit of repeated motif, and a serine-threonine-rich C-terminal domain that are relatively variable across the family. Within a given ALS gene, the size of tandemly repeated region varies considerably between alleles due to differences in the number of copies of 108 bp sequence present. Several members of ALS family genes have been cloned on the basis of the presence of their CX2 motifs (Chen and Chen, 2000). They are differentially regulated and exhibit variability in size and expression. Also examination of various *C. albicans* isolates showed that certain strains are lacking ALS genes that are absent in others (Hoyer and Hecht, 2000, 2001). Finally, allelic sequence polymorphisms can be found outside of repeated regions. These sequence difference are more pronounced within the 3’ end of the gene, which encodes the heavily glycosylated portion of the mature protein (Hoyer and Hecht, 2001). Very recently Zhang et al analyzed 66 *C. albicans* strains, representing a worldwide collection of 266 infection-causing isolates, and discovered 60 alleles of the ALS7 open reading frame (ORF) (Zhang et al., 2003). Molecular biological techniques have offered several examples of *C. albicans* allelism. Allelic sequence variation has been detected outside coding regions as demonstrated by Yesland & Fonzi (2000) who showed that nucleotide differences in regions flanking PHR1 were responsible for biased targeting of disruption cassettes during mutant construction. Staib et al. (2002) documented allelic sequences variation in the promoter region of SAP2 and showed that the divergent sequence resulted in differential regulation of the alleles. To date, the largest published example of *C. albicans* allelic variation is between α and α alleles of PAP, OBP and PIK in the mating type locus (Fig. 1. 16), where alleles are only approximately 60% identical (Hull and Johnson, 1999). The net effect of this sequence divergence on protein function has yet to be determined. Zhao et al recently published the phylogenetic tree of nucleotide sequence from the 5’ domain of *C. albicans* (SC5314) ALS genes (Zhao et al., 2003). In *C. albicans*, ALS1 genes are found on three different chromosomes: chromosome 3 (ALS6 and ALS7), R (ALS3) and 6 (ALS1, ALS2, ALS4, ALS5) (Wickes et al., 1991; Hoyer et al., 2001). Work with *C. albicans* genomic fosmid library indicated that ALS1 and ALS5
were found on the same fosmid and, therefore located within approximately 50 kb of each other (Hoyer & Hecht, 2001).

### 1. 5. 3. 1. Role of \( ALSI \) in morphogenesis, pathogenicity

Fu et al constructed a genomic library of \( C. albicans \) DNA in an \( S. cerevisiae \) expression vector. By screening for adherence to human endothelial cells, they identified first time the clone of \( ALSI \) gene which has homology to \( S. cerevisiae AG\alpha1 \) and is a member of the immunoglobulin gene superfamily. The adherence of the clone to endothelial cells was over 100-fold greater than that of control \( S. cerevisiae \) transformed with empty plasmid. Fu et al studied elaborately about the three lines of \textit{in vitro} evidence which demonstrate that Als1p mediates adherence of \( C. albicans \) to endothelial cells. First, deletion of both alleles of \( ALSI \) significantly reduced endothelial cell adherence. Secondly, overexpression of \( ALSI \) enhanced adherence. Thirdly, the enhanced adherence of the overexpression strain could be blocked with a monoclonal antibody directed against the N-terminal of Als1p. Their further studies revealed that this gene acts downstream to the morphogenetic regulator Efglp, as overexpression of Als1p in \( EFG1 \) mutant strain can complement its filamentation defect (Fu et al., 2002). Cphlp has no role in expression of \( ALS1 \). Furthermore, Als1p is reported to be essential for virulence in hematogenously disseminated infection in a murine model (Calderon and Fonzi, 2001).

### 1. 5. 3. 2. \( ALSI \) and cellular signaling

Als1p is the first cell surface protein identified that functions as a downstream effector of filamentation in \( C. albicans \). It was also found that the \( ALS1 \) overexpression strain flocculated expensively, forming large aggregates of cells that rapidly sedimented. This result demonstrated the functional similarity between \( S. cerevisiae Flo11p \) and \( C. albicans \) Als1p. Both proteins function as effector of filamentation and mediate adherence and flocculation (Fu et al., 1998; Guo et al., 2000). In \( C. albicans \), Als1p functions as an effector of the cAMP-protein kinase \( \Lambda \) pathway via Efglp, but does not appear to be an effector of the MAP kinase pathway. On the other hand, \( HWP1 \) also encodes a cell surface protein and is regulated by Efglp (Sharkey et al., 1999; Staab et al., 1999). Even \( HWP1 \) in the \( efg1/efg1 \) mutant fails to restore filamentation (Sharkey et al., 1999). Therefore, Hwp1p alone does not function as an effector of filamentation downstream of \( EFG1 \). The regulatory elements controlling \( INT1 \) expression are not yet known, similar epistasis experiment should be performed.
1. 5. 3. 3. Other ALS1 genes

Gaur and Klotz (Gaur and Klotz, 1997) described one member of ALS gene family, ALA1/ALS5. This gene was identified by its ability to cause S. cerevisiae to bind to extracellular matrix proteins. S. cerevisiae expressing ALA1 also exhibited enhanced adherence to human buccal epithelial cells. ALA1/ALS5 causes attachment and aggregation in S. cerevisiae, similar to C. albicans, when expressed from a high copy vector (Gaur et al., 1999). Target recognition studies of this gene were done. It was reported that Ala1p/Als5p recognizes patches of certain amino acids, such as serine, threonine and alanine which must be accessible before adherence (Gaur et al., 2002). Recently they also indicated that interaction in SRS (stable, reversible, specific) adherence of Ala5p have striking similarities with those of the molecular chaperone Hsp70, which specifically binds to non-native proteins and resists denaturation (Gaur and Klotz, 2004).

1. 6. EXTRACELLULAR HYDROLYTIC ENZYMES

In susceptible hosts, C. albicans enters the blood stream and causes deep-seated infection in target organs. Because the organisms must cross the endothelial cell lining of the blood vessels to enter these organs, the interaction between C. albicans and vascular endothelial cells is likely to be a critical step in the initiation of a disseminated infection. One factor that contributes to this process of virulence is hydrolytic enzyme production. The three most significant extracellular hydrolytic enzymes produced by C. albicans are the secreted aspartyl proteinases (Sap), phospholipase B enzymes, and lipases. Different roles have been suggested for the Saps based on in vitro experiments; these roles include nitrogen supply (Staib, 1965), adherence (Watts et al., 1998), degradation of host barriers (Colina et al., 1996), and invasion of host defense mechanisms (Kaminishi et al., 1995; Ruchel, 1986).

1. 6. 1. Molecular and Biochemical properties of SAP

All proteinase catalyze the hydrolysis of peptide bonds (CO-NH) in proteins but can differ markedly in specificity and mechanism of action (Barrett and Rawlings, 1991). Aspartyl proteinases are ubiquitous in nature and are involved in a myriad of biochemical processes (Davies, 1990). The Sap proteins encoded by a family of 10 SAP genes (Monod et al., 1994; Monod et al., 1998) have been the most comprehensively studied as key virulence determinants of C. albicans.


**C. albicans** is not the only *Candida* species known to produce extracellular proteinases. Many of the pathogenic *Candida* species have been shown to possess *SAP* genes, including *C. dubliniensis* (Gilfillan et al., 1998), *C. tropicalis* (Monod et al., 1994; Togni et al., 1991), and *C. parapsilosis* (de Viragh, 1993). All 10 *SAP* genes of *C. albicans* encode preproenzymes approximately 60 amino acids longer than mature enzyme, which are processed when transported via the secretory pathway. Most Sap proteins contain putative N-glycosylation site, but it remains to be determined which Sap proteins are glycosylated. Unlike Sap1p to Sap8p, Sap9p and Sap10p both have C-terminal consensus typical for glycosylphosphatidylinositol (GPI) proteins (Monod, 1994). Newly synthesized mRNA of *SAP* genes are transferred into the preproenzymes on the rough endoplasmic reticulum. The N-terminal signal peptide is removed in the rough endoplasmic reticulum by a signal peptidase and the proenzyme transferred to the Golgi apparatus, where it is further processed after Lys-Arg sequences by a kex2 proteinase (Newport et al., 1997; Togni et al., 1996). Once activated, the enzyme is packed into secretory vesicles and transported to the plasma membrane and either remains attached to the cell membrane, is incorporated into the cell wall via a GPI anchor (Sap9p and Sap10p) or is released into the extracellular space. The Sap1p to Sap10p proteins are between 35 to 50 kDa in size and account for all of the extracellular proteolytic activity of *C. albicans*. Distinct difference in pH optima is evident between the heterologously expressed proteinases Sap1p to Sap6p, with Sap1p to Sap3p having highest activity at lower pH values and Sap4p to Sap6p having highest activity at higher pH values, with a pH range of activity between 2.0 and 7.0.

### 1.6.1.1. Role of *SAP* genes in Pathogenicity

Aspartyl proteinases are secreted by pathogenic species of *C. albicans* *in vivo* during infection. (Morschhauser et al., 1997; Staib et al., 2000). This enzyme is also secreted *in vitro* when the organism is cultured in presence of exogenous protein (usually BSA) as nitrogen source (Banerjee et al., 1991a and b). The *C. albicans* isolates with highest proteinase activities could adhere most strongly to buccal epithelium and were most pathogenic (Ghannoum and Abu-Elteen, 1986; De Bernardis et al., 1996). The secreted aspartyl proteinases are thought to contribute to virulence through their effects on adherence, invasion, and pathogenicity. As a protease, the enzyme may have a spectrum of substrates, depending upon the host organ, e.g., skin or blood that is colonized or infected. An *in vivo* expression technology was used that is based on genetic recombination as a reporter of gene expression to monitor the differential activation of individual members of this gene family at various stages of the infection process. It was shown that *SAP* expression depends
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on the type of infection, with different *SAP* isogenes being activated during systemic disease as compared to mucosal infection. In addition, the activation of individual *SAP* genes depends on the progress of the infection, some members of the gene family being induced immediately after contact with the host, whereas others are expressed only after dissemination into deep organs. In the latter case, the number of invading organisms determines whether induction of a virulence gene is necessary for successful infection (Staib *et al.*, 2000). The importance of Saps in establishing systemic and vaginal *C. albicans* infection was demonstrated by the protective role of the proteinase inhibitor, pepstatin A, or aspartic proteinase antibodies in experimental animal models (De Bernadies *et al.*, 1997, Fallon *et al.*, 1997). The importance of Saps for disseminated infections was demonstrated by an increasing survival rate of mice and guinea pigs infected intravenously with Δsap1, Δsap2, Δsap3 (Hube *et al.*, 1997) or Δsap4-6 (Sanglard *et al.*, 1997). The contribution of Sap2p to systemic infection could be explained partially by processing a role for this enzyme in endothelial cell damage. However, adherence of Δsap1, Δsap2 and Δsap3 mutants to endothelial cells was not affected (Ibrahim *et al.*, 1998). The expression of the closely related genes SAP4-6 was observed by *C. albicans* in murine macrophages, which exhibited a 53% more effective killing towards the sap4-6 mutant compared with the wild-type strains (Borg-von Zepelin *et al.*, 1998). Using the same mutants, an important role of SAP4-6 but not SAP1-3 was also shown in a mouse peritonitis model (Kretschmar *et al.*, 2002). In contrast, Δsap2 mutants were almost avirulent during experimental vaginal infection in the rat, and the virulence of Δsap1 and Δsap3, but not Δsap4-6 was attenuated in comparison with the parental strain (De Bernardis *et al.*, 1999). The expression of SAP1-3 and SAP6 was also detected in samples from patients with oral candidiasis (Schaller *et al.*, 1999, 2000). A possible role of SAP1-3 during oral infection may be the attachment to the epithelial surface, as a reduced adherence of the single mutants (Δsap1, Δsap2, Δsap3) to buccal epithelial cells was observed, where as Δsap4-6 was significantly increased in adherence (Watts *et al.*, 1998)

Bernhard Hube's group clearly confirmed a correlation of the *SAP* gene expression with epithelial lesion and showed that *SAP1*, *SAP2*, and *SAP3* contribute significantly to the tissue damage and invasion of oral epithelium and cutaneous epidermis (Schaller *et al.*, 1999;2000) while *SAP4*, *SAP5*, and *SAP6* are important for systemic infection (Felk *et al.*, 2002, Sanglard *et al.*, 1997). But still it is not clear whether the murine model is representative of proteinase expression during human vaginal infection. Since it is known that vaginal candidiasis is an extremely common fungal disease affecting nearly three-quarter of all woman (Sobel, 1998), the expression and role of *SAP* gene family in an *in vivo* model of vaginal candidiasis was studied based on reconstituted human
vaginal epithelium by using RT-PCR and immunoelectron microscopy (Schaller et al., 2003). Then Hube’s group compared the SAP expression profile observed in their in vitro studies to that found in sample from patients suffering from vaginal candidiasis. Significant epithelial damage was observed after 12h, concomitant with the additional expression of SAP1, SAP4, and SAP5. Additional transcript of SAP6 and SAP7 were detected at a later stage of the artificial infection. Similar SAP expression profiles were observed in three samples isolated from human patients with vaginal candidiasis. Further more, SAP null mutant lacking either SAP1 or SAP2 had a drastically reduced potential to cause tissue damage even through SAP3, SAP4, and SAP7 were up-regulated in these mutants. In contrast the vaginopathic potential of mutants lacking SAP3 or SAP4 to SAP6 was not reduced compare to wild-type cells. They have also shown that the virulence potential of the sap/sapl and the sap2/sap2 mutants was fully restored by plasmid-borne expression of SAP1 and SAP2 in these strains, indicating the observed reduced virulence of these mutants was in fact due to the disruption of SAP1 and SAP2. So in particular, Sap1p and Sap2p, but not Sap3p to Sap6p, seem to play an important role during tissue damage in the vaginal model as demonstrated by the use of proteinase inhibitor and of mutants sap1/sap1 and sap2/sap2. It is not clear how the proteinase contribute to the tissue damage, since the pH optima of Sap1p and Sap2p are 4.5 to 3.5 respectively (Borg-von et al., 1998). One possible explanation would be local pH differences within the tissue during infection.

1.6.1.2. SAP genes and Phenotype Switching

Most strains of C. albicans are capable of switching at extremely high frequencies between two and more general phenotypes that are distinguishable by different colony morphologies. The switching process regulates the expression of a number of phase-specific genes including PEP1(SAPI) (Kvaal et al., 1999), SAP3 (Hube et al., 1994, OPI(Morrow et al., 1992; 1993), CDR3 (Balan et al., 1997) CDR4 (Kvaal et al., 1999), NIK1 (Srikantha et al., 1998), WHI1 (Srikantha et al., 1995), and EFG1 (Srikantha and Soll, 1993) The levels of the Sap1p, Sap2p, and Sap3p isoenzymes were monitored under a variety of growth conditions for several C. albicans strains (White et al., 1993), including strain WO-1, which alternates between two phenotypes, white (W) and opaque (O) (Soll, 1992). These studies revealed that the specific Sap isoenzyme produced is determined by the cell type (strain) whereas the level of Sap production is affected by environmental factors. It was further shown that both the yeast-to-mycelium transition and phenotypic switching can determine the type of Sap isoenzymes produced. SAP1 and SAP3 levels were regulated during the phenotypic transition between W and O forms (Fig. 1. 20). Soll’s group
have examined the role of the opaque-phase-specific gene \textit{PEP1} (\textit{SAP1}) in the switching process, in the genesis of the opaque-specific phenotype and in phase specific virulence in two animal models. \textit{PEP1} (\textit{SAP1}) has been demonstrated to be first \textit{SAPs} transcribed by \textit{C. albicans} upon interaction with reconstructed human epithelium. The \textit{PEP1} (\textit{SAP1}) open reading frame was inserted downstream of the promoter of the white-phase specific gene \textit{WHII} in the transforming vector, and resulted transformants were demonstrated to misexpress \textit{PEP1} (\textit{SAP1}) in the white phase. Misexpression conferred upon white-phase cells the increased capacity of opaque-phase cells to grow in medium in which protein was the sole nitrogen source (Fig. 1. 22). Again misexpression of \textit{PEP1} (\textit{SAP1}) had no effect on the white-phase cells in a systemic mouse model, in which white-phase cells were already more virulent than opaque-phase cells. So misexpression of \textit{SAP1} conferred upon white-phase cells two dissociable opaque-phase characteristics increased adhesion and the capacity to cavitate skin (Kvaal \textit{et al}., 1997). So the cell-associated \textit{Pep1p} (\textit{Sap1p}) protein causes an increase in the adhesive properties of white-phase cells, while released \textit{Peplp} (\textit{Saplp}) protein facilitates tissue cavitation. In strain WO-1, these functions are regulated by high-frequency phenotype switching.

1.6.1.3. \textit{SAP} production and Adherence

Adherence of \textit{Candida} to host tissues allows the fungus to attain a foothold and to colonize a specific niche environment. Adherence of \textit{C. albicans} to host cells is a complex, multifunctional process involving several types of candidal Adhesins on a morphologically changing cell surface. Implication of Sap proteins in adherence has also come from recent studies by Hube, Sanglard and colleagues. The authors constructed strains harboring disruptions in a number of \textit{SAP} genes, including \textit{SAP1}, \textit{SAP2}, and \textit{SAP3} (Hube \textit{et al}., 1997) and a triple-knockout of \textit{SAP4}, \textit{SAP5}, and \textit{SAP6} (Sanglard \textit{et al}., 1997). In all cases, mutants showed decreased virulence in an animal model of disseminated candidiasis. Interestingly, \textit{Sap4p}, \textit{Sap5p}, and \textit{Sap6p}, are produced by \textit{C. albicans} cells after phagocytosis by macrophages. A \textit{sap4/sap4}, \textit{sap5/sap5}, \textit{sap6/sap6} null mutant was killed more effectively by 53\% after contact with macrophages, than the wild-type strain. (Von Zepelin \textit{et al}., 1998). It has been reported that inhibition of Saps by HIV protease inhibitor reduces oropharyngeal candidiasis (Monod and Borg-von, 2002). However, expression of \textit{Sap2p} as a sole putative virulence factor did not cause \textit{S. cerevisiae} to become virulent and constitutive overexpression of \textit{SAP2} did not augment virulence of \textit{C. albicans} in experimental oral or systemic infection (Dubois \textit{et al}., 1998). The majority of studies linking Sap production with \textit{C. albicans} adherence have been performed using proteinase inhibitor pepstatin, which inhibit \textit{Sap2p} (and
probably Sap1p and Sap3p) very efficiently (Pichova et al., 2001). Borg and Ruchel demonstrated a marked reduction in C. albicans adhesion and invasion of human mucosa by pepstatin, and a similar reduction of C. albicans adherence using pepstatin was also shown with human epidermal cells (Borg and Ruchel, 1988).

1. 6. 1. 4. SAP expressions in yeast to hyphal transition

SAP2 was the dominant transcript in the yeast form, and its expression was autoinduced by peptide products of its own enzymatic activity and repressed by amino acids (Hube et al., 1994). SAP4 and SAP6 expression was observed only at neutral pH during morphogenetic conversion from yeast to hyphae induced by serum. SAP4-6 genes are differentially expressed during hyphal production (Chen et al., 2002). Expression of SAP7 was not detected under any of the experimental conditions used throughout the study. SAP8 is the third gene of the family to be expressed in the opaque phenotype (Soll, 1992). Since the hyphae-deficient efg1 mutant was, in contrast to the cph1/cph1 mutant, not able to invade or damage tissue, question was raised whether this was due solely to block in dimorphism or to the loss of factors associated with hyphal cells. The proteinase genes SAP4 to SAP6 have been shown to regulate during the dimorphic transition (Hube et al., 1994; Schroppel et al., 2000; White and Agabian, 1995). Therefore Hube’s group investigated the detailed expression of SAP1 to SAP3 and SAP7 to SAP10. In order to investigate how genes are expressed under hyphal-inducing condition, hypha production in wild type, Efg1 p and Cph1 p-deficient strains were induced by incubation of 5 % serum or by pH- and temperature-regulated transition. Using Northern blot analysis, Schroppel et al found that expression of SAP4 to SAP6 was undetectable in efg1/efg1 mutant in serum containing medium, which is a strong environmental signal for hyphal growth of wild-type cells. Using RT-PCR, they were able to confirm reduced expression of SAP4, SAP5, and SAP6 in the efg1/efg1 mutant under such conditions (Schroppel et al., 2000). However, expression of all three genes was even enhanced in both efg1/efg1 and cph1/cph1 mutants when hyphal formation was induced via a pH and temperature shift protocol, suggesting that the transcription factor Efg1p and Cph1p can be either positive or negative regulators of SAP4 to SAP6 expression. Since efg1/efg1 mutants do not produce hyphal cells under these conditions, it can also be concluded that expression of SAP4 to SAP6 is not strictly linked to the hyphal morphology but regulated by a factor which is also regulated by a factor that regulates hyphal formation. Expression of Sap antigen during systemic infections has long been recognized in antigen-antibody studies (Macdonald and Odds, 1980; Ruchel et al., 1991). Staib et al demonstrated a high expression of SAP5 in all phases of infection.
with wild type cells and a late induction of SAP2 and SAP6 using in vivo expression technology. With Sap-specific antibodies, Sap1p to Sap3p proteinase were detected on both yeast and hyphal wild-type cells, while the Sap4- to Sap6-specific antigen was identified mostly on penetrating hyphal cells, conferring hyphae specific expression of Sap4p and Sap6p. Recently it has been reported that the transcription factors Cphlp and Efglp are involved in the expression of SAP5 as the avirulent cphl/cphl efgl/efgl mutant did not express this gene (Staib et al., 2002). Although expression of SAP2 was high in all in vivo samples investigated and large amount of Sap1p to Sap3p antigen were found on all types of wild-type Candida cells, the level of expression did not correlate with the importance of the corresponding gene for invasion. Only mutants lacking SAP6 had strongly reduced abilities to invade and damage parenchymal organs despite the fact that hyphal production was normal and all other proteinase genes were still expressed (Felk et al., 2002). It can be concluded that the reduced virulence of hyphae-deficient mutants is not only due to the inability to form hyphae but also due to modified expression of SAP genes normally associated with the hyphal morphology.

1.6.2. Phospholipase B

Phospholipases are important pathogenicity determinants in C. albicans. They play a significant role in damaging cell membranes and invading host cells. High phospholipase production is correlated with an increased adherence and a higher mortality rate in animal models (Mayser et al., 1996). Various phospholipases reported from C. albicans include phospholipases A, B, C, and D (Niewerth and Korting, 2001). Phospholipase A and lysophospholipase activities have been found in the cell wall of yeast cells and hyphae. Enzyme activity was more in the walls of older yeast cells than of younger cells and was more prominent at the tip of growing hyphae (Goyal and Khuller, 1992).

The virulence of strains deleted for the C. albicans phospholipase B gene Caplb1, for hematogenously disseminated candidiasis, was significantly attenuated, compared to the isogenic wild-type parental strain (Ghannoum, 1986, 1998). Although deletion of CapLB1 did not produce any detectable effects on candidal adherence to human endothelial or epithelial cells, the ability of the Caplb1/Caplb null mutants to penetrate host cells was dramatically reduced. Reintroduction of PLB1 restored the virulence (Mukherjee et al., 2001). Thus, phospholipase B may well contribute to the pathogenicity of C. albicans by abetting the fungus in damaging and traversing host cell membranes, processes which likely increase the rapidity of disseminated infection (Leidich et al.,
1998). Studies show that the expression of *PLB1* is regulated by nutritional supplementation, environmental factors and the growth phase of the *C. albicans* cells, as well as by physiological conditions. The differential expression of *PLB1* in response to environmental factors may be correlated to host-specific components available to *C. albicans* (Mukherjee et al., 2003). These data prove that phospholipase B is essential for *Candida* virulence, and paves the way for studies directed at determining the mechanism(s) through which phospholipase modulate virulence in this organism. The phospholipase D1 has been reported to be important for morphological transition under certain conditions (Hube et al., 2001).

1.7. pH REGULATION IN *C. ALBICANS*

*C. albicans* is able to cause infections in a broad range of host niches which show significant differences in ambient pH. For example, the mouse systemic pH is ~7.3 whereas the pH in the rat vagina is ~4.5. This suggested that the ability of *Candida* to react appropriately to (among other environmental variable) rather different pH environments is crucial for its pathogenicity. The environmental pH acts as a manipulator for many physiological functions including morphogenesis (Fonzi, 2002). Under optimal (37°C) temperature conditions, filamentation is favoured by ambient pHs close to neutrally and is considerably reduced at pH lower than 6. In contrast, the yeast form predominates almost exclusively at pH 4 (Buffo et al., 1984).

1.7.1. Genes involves in pH regulation

Fonzi et al first isolated *PHR1*, a prototypical alkali-expressed gene encoding a 548-residues membrane-anchored glycosylphosphatidylinositol by using differential screening techniques. Sequence analysis of *PHR1* demonstrated that it was homologous to the *GGP1/GAS1* gene, encoding GPI-anchored protein of *S. cerevisiae* cell surface (Nuoffer et al., 1991). *PHR1* was strongly expressed under condition of alkaline pH but was not expressed at any pH below 5.5 (Saporito-Irwin et al., 1995). Consequently Fonzi's group searched for a functional homolog of *PHR1* active at low pH. This resulted in the isolation of a second pH regulated gene, designated *PHR2* (Muhlschlegel and Fonzi, 1997).

*PHR2*, expressed at an ambient pH below 5.5, play a role in morphogenesis (Saporito-Irwin et al., 1995). The virulence of the organism also is affected in this pattern, when either or both of the genes are disrupted. Deletion of *PHR1*, results in pH-conditional defects in growth, morphogenesis,
and virulence, evident at neutral to alkaline pH, but absent at acidic pH. Conversely, a phr2/phr2 null mutant exhibited pH-conditional defects in growth and morphogenesis analogous to those of phr1/phr1 mutants, but manifests at acid rather than alkaline pH values. Engineered expression of PHR1 at acidic pH in a phr2/phr2 mutant strain and PHR2 at alkaline pH in a phr1/phr1 mutant strain complemented the defects in the opposing mutant. Deletion of both PHR1 and PHR2 resulted in a strain with pH-independent, constitutive growth and morphological defects (Ghannoum et al., 1995; Muhlschlegel and Fonzi, 1997). When these strains were tested for pathogenicity in various niches of the host with different pH (systemic pH is near neutrality and vaginal pH is around 4.5, the virulence phenotype paralleled the pH dependence of the in vitro phenotypes. The phr1 null mutant was avirulent in a mouse model of systemic infection, but uncompromised in its ability to cause vaginal infection in rats. The virulence phenotype of a phr2/phr2 null mutant was the inverse. The mutant was virulent in a systemic infection model, but avirulent in a vaginal infection model. Heterozygous mutants exhibited partial reductions in their pathogenic potential, suggesting a gene dosage effect (De Bernardis et al., 1998).

Another pH regulatory gene of C. albicans whose maximal expression occurs at neutral pH, with no expression detected below pH 6.0, has been cloned (Sentandreu et al., 1998). This gene was designated as PRA1, for pH regulated antigen. The protein predicted from nucleotide sequence was 299 amino acids long, with motif characteristics of secreted glycoproteins. The predicted surface localization and N-glycosylation of the protein were demonstrated directly by cell fractionation and immunoblot analysis. The PRA1 protein was homologous to surface antigens of Aspergillus species, which react with serum from aspergillosis patients, suggesting that the PRA1 protein may have a role in the host-parasite interaction during candidal infection.

1.7.2. pH response regulators

The pathway controlling pH-responsive gene expression has been most extensively dissected for the ascomycete Aspergillus nidulans (Penalva and Arst, 2002). Central to the pH response is the pH-dependent activation of the zinc finger transcription factor encoded by pacC (Tilburn et al., 1995). PacC is synthesized in an inactive form, which is activated at alkaline pH by proteolytic removal of the carboxy-terminus (Mingot et al., 1999). Proteolysis is dependent upon six genes palA, -B, -C, -F, -H, and -I (Tilburn et al., 1995). The activated form of PacC induces the expression of alkaline-expressed genes and repressed acid expressed genes (Tilburn et al., 1995). This regulatory pathway is apparently conserved, as various elements have been identified in
Yarrowia lipolytica and S. cerevisiae. The Y. lipolytica YIRIM101, is activated by carboxy-terminal truncation and is required for alkaline-dependent expression of XPR2-encoded protease (Lambert et al., 1997). In S. cerevisiae the PacC homolog, RIM101, was initially identified as controlling meiosis and haploid invasiveness (Li and Mitchell, 1997; Su and Mitchell, 1993). RIM101 and the yeast homology of palB, CPL1, have been implicated in a pH-dependent growth response of yeast (Futai et al., 1999). Gene homologous to palA and palI are also present in S. cerevisiae (Denison et al., 1998; Negrete-Urtasun et al., 1997). C. albicans sequences homologous to palA/RIM20 and pacC (RIM101) were first reported from Mitchell’s lab (Wilson et al., 1999). To investigate the regulation of gene expressions in C. albicans, Fonzi et al have isolated and characterized PRR1 (pH response regulator) which is the C. albicans homolog of palF/RIM8. Expression of PRR1/RIM8 was pH dependent, and mutant lacking PRR1 was defective in pH-dependent regulation of gene expression. PHR1 was no longer induced at alkaline pH, and PHR2 was no longer repressed in both alkaline and acidic pH. Thus, PRR1 is a component of the pH response pathway in C. albicans. A prr1/prr1 null mutant exhibited no morphological abnormalities at either pH; however, it lost the ability to form hyphae on medium 199 and of 10% serum (Porta et al., 1999).

The ability to form hyphae on serum was not restored by forced expression of PHR1, indicating that additional PRR1-dependent genes are required for hyphal development. Multiple regulatory genes influence the process of hyphal development. HWP1 was also identified as a downstream target induces HWP1 expression, and HWP1 is required for normal filamentation. Expression of HWP1 was induced in absence of PRR1, indicating that pH response pathway is not required for the expression of EFG1-dependent genes. This suggests the pH response pathway is distinct from the EFG1-dependent signaling pathway (Porta et al., 1999).

Fonzi’s group also reported about characterization of the full-length homolog of pacC, which they have designated PRR2/CaRIM101 (for pH response regulator). PacC, CaRIM101p, ScRIM101p have expensive amino acid sequence identity in their tridactyl zinc finger regions but otherwise diverge substantially. In absence of PRR2/RIM101, alkaline pH and acid-expressed genes were no longer repressed (Ramon et al., 1999). The same phenotype was observed in PRR1 mutant, which lacks the C. albicans homolog of palF/RIM8 (Porta et al., 1999).
1. 7. 3. *PacC* related transcription factor, *RIM101*-Dependent and –independent pathways

*Rim101p/PacC* activity is controlled by proteolytic processing. In acidic conditions, Rim101p exists primarily in a full length “long” form which has no known function (Li and Mitchell, 1997). In alkaline conditions, a carboxyl-terminal portion is cleaved to yield the active “short” form. Proteolysis is controlled by pH through the action of a number of gene products, including Rim20p/PalA, Rim8p/PalF, Rim13p/PalB, and Rim9p/PalII (Denison et al., 1995 and 1998; Li and Mitchell, 1997) (Fig. 1. 12). Mitchell’s group has used the partial *C. albicans* genomic database to identify *RIM101* pathway members in *C. albicans* and to determine their role in alkaline responses. *C. albicans* *RIM101* specifies a protein of 604 amino acids. The *RIM101/PacC* family has limited sequence identity over the entire protein sequence; *C. albicans* and *S. cerevisiae* Rim101p are approximately 20 % identical. However, they do share three structural features. First, the zinc finger domain of Rim101p/PacC family is highly conserved; *C. albicans* Rim101p has 57 % identity and 87% similarity to the other homolog. Second, *C. albicans* Rim101p has an 84-amino acid C-terminal region with 32 % D/E-residues which is conserved in other member of this family. Third, these proteins are similar in size, ranging from 585 to 668 amino acids. They also identified two another pH regulatory genes, *RIM8/PalF* and *RIM20/PalA* and showed that Rim101p, Rim8p, and Rim20p are positive regulators of the alkaline-induced genes *PRA1, PHR1,* and *RIM101* itself. The loss of *RIM101* pathway function blocks inductions of these genes at alkaline pH. So these proteins are negative regulators of the alkaline-repressed gene *PHR2*. But these proteins are positive regulators of alkaline-induced filamentation, because loss of *RIM101* pathway function prevents filamentation at alkaline pH.

However, unlike *Aspergillus* and *Yarrowia*, *C. albicans* remains pH responsive in the absence of the *RIM101* pathway. For example, the dominant Rim101-405 allele, which complements the filamentation defect of the *rim101/rim101* null mutant, promotes filamentation very weakly at acidic pH. Thus the uncoupling of Rim101p processing from the upstream regulators does not completely bypass the control of filamentation by external pH. In addition, *PHR2* becomes an alkaline-induced gene in cells that lack *RIM101* pathway functions. Thus, both morphological and gene expression responses suggests about the existence of a *RIM101*-independent pH response pathway (Fig. 1. 12). This pathway has two roles: to stimulate *PHR2* expression at alkaline pH, and act in conjugation with Rim101p to activate filamentation (Davis et al., 2000a). Davis et al., 2000a also found that the RIM101 pathway is necessary in vivo for pathogenesis. They showed both *rim101/rim101* and *rim8/rim8* mutant have a significant reduction in virulence using the mouse.
model of hematogenously disseminated systemic candidiasis. Again, these mutants showed a marked reduction in kidney pathology. But rim101/rim101 mutant was less virulent than rim8/rim8 mutant. The rim8/rim8 mutant has a virulence defect because of absence of processed Rim101p. However, in the rim101/rim101 mutant, no Rim101p present. Thus, one simple model to explain the difference in virulence is that both processed and unprocessed Rim101p function during infection. So this is the new hypothesis that unprocessed Rim101p has a functional role in target gene regulation (Davis et al., 2000b).

![Diagram](image.png)

**Fig. 1. 12.** Alkaline responses in Rim101p regulation. Alkaline pH stimulates RIM101p activity through increased expression and proteolytic activation, both of which require Rim8p and Rim20p. Full length Rim101p-long does not have a known activity. Processed Rim101p-short is required for the alkaline response, which includes activation of alkaline-induced genes, repression of alkaline-repressed genes, and filamentation. Since Rim101p is an alkaline-induced gene, its expression may depend on autoregulation by Rim101p-short. Alkaline pH also stimulates a RIM101-independent pathway (Davis et al., 2000b). This pathway activates PHR2 expression and stimulates filamentation in conjugation with Rim101p.

1. 7. 4. Alkalinity Mimicking Mutations truncating CaRIM101p

In *C. albicans* the extragenic suppressors in two independent revertant were identified as nonsense mutation in the pH response regulator *CaRIM101 (PRR2)* that resulted in the carboxy-terminal truncation of the open reading frame (El Barkani et al., 2000). In RIM101-1426, a C-to-T transition at nucleotide 1426 introduced an ochre codon at position 476 of the coding region, truncating the 661-residue native protein by 186 amino acids. Similarly a C-to-A transversion at position 1751 of RIM101-1751 converted the codon for Ser-584 to an ochre stop codon truncating the protein by 78 residues. This dominant allele conferred the ability to filament at acidic pH, to express PHR1*, an alkaline-expressed gene, at acidic pH, and to repress the acid-expressed gene PHR2. It was also found that both the wild-type and dominant mutant alleles could act as multicopy suppressors of the temperature restriction on filamentation, allowing extensive filamentation at 29 °C. An
$efg1/efg1$ mutation was epistatic to RIM101-1426 and prevented filamentation. So the ability of the activated alleles to promote filamentation was depended upon the development regulator $EFG1$. Even the $EFG1$ mutation did not prevent RIM101-1426 activation of $PHR1$ expression demonstrated that $EFG1$ is not interposed within the pathway controlling pH-dependent gene expression. This does not rule out the possibility of their functioning within the same pathway to control filamentation. In this regard it might be noted that $EFG1$ lies downstream of $TPK2$, which encodes a cAMP-dependent protein kinase (Sonneborn et al., 2000). Rim101p of $S. cerevisiae$ contains a functionally significant recognition site for cAMP-dependent protein kinases (Su and Mitchell, 1993). Although this site is not conserved in the $C. albicans$ homolog, two potential phosphorylation sites are present, and this could provide a regulatory connection between $TPK2$, RIM101, and $EFG1$.

1. 7. 5. Diverged Binding Specificity of Rim101p

Very recently it was reported from Fonzi's laboratory that Rim101p is a DNA binding protein and that, unlike $S. cerevisiae$ but like $A. nidulans$, it activates transcription of the alkaline pH-expressed gene $PHR1$ through its binding upstream (Ramon and Fonzi, 2003). In $S. cerevisiae$, Rim101p acts primarily as a repressor (Lamb and Mitchell, 2003). In vitro DNA binding has been demonstrated for $PacC$ from three filamentous fungi, and each recognizes the consensus sequence 5'-GCCARG-3' (Saporito-Irwin et al, 1995; Suarez and Penalva, 1996; Tilburn et al., 1995). But the DNA sequence (5'-NCCAAG-3') recognized by Rim101p is not the same as that recognized by $PacC$. Rim101p binding site was sufficient to confer pH-conditional expression to an artificial promoter, demonstrating that Rim101p can independently activate expression. By microarray analysis and RT-PCR data, six genes were detected whose expression was enhanced at least twofold in Rim101p-expressing verses non-Rim101p-expressing cells at pH 7.5 (Fig. 1. 13). These, in addition to two previously reported genes, RIM101 and $PRA1$ were examined for the presence of Rim101p binding sites within 1 kb upstream of the coding region. Of the eight putative binding sites, only one was preceded by a G residue and thus conformed to the $PacC$ consensus binding site. Since RIM101 negatively regulates the expression of $PHR2$, genes that were derepressed in absence of the regulator were also examined. Six of 14 genes identified contained an upstream Rim101p binding site (Ramon and Fonzi, 2003).
### RIM101 Regulated genes

#### Positively Regulated genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ENA2)</td>
<td>Plasma membrane-type ATPase involved in Na(^+) efflux</td>
</tr>
<tr>
<td>(FRE4)</td>
<td>Ferric reductase</td>
</tr>
<tr>
<td>(FRE5)</td>
<td>Ferric reductase transmembrane component</td>
</tr>
<tr>
<td>SKN1</td>
<td>β-1,6-Glucan synthesis</td>
</tr>
<tr>
<td>PHO8</td>
<td>Repressible alkaline phosphatase</td>
</tr>
<tr>
<td>PHR1</td>
<td>β-1,6-Glucanotransferase</td>
</tr>
<tr>
<td>PRA1</td>
<td>pH-regulated antigen</td>
</tr>
<tr>
<td>RIM101</td>
<td>Transcription factor; regulation of pH response</td>
</tr>
<tr>
<td>WAP1/CSA1-1</td>
<td>TUP1-regulated cell wall protein</td>
</tr>
</tbody>
</table>

#### Negatively Regulated genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCP1</td>
<td>Cytochrome c peroxidase</td>
</tr>
<tr>
<td>CHA1</td>
<td>L-Serine/L-threonine deaminase</td>
</tr>
<tr>
<td>EMC33</td>
<td>Glycosylphosphatidylinositol-anchored cell surface protein</td>
</tr>
<tr>
<td>FET99(FET3)</td>
<td>Iron transport multicopper oxidase</td>
</tr>
<tr>
<td>FRE7</td>
<td>Ferric reductase transmembrane component</td>
</tr>
<tr>
<td>GIT99 (GIT1)</td>
<td>Glycerophosphoinositol transporter</td>
</tr>
<tr>
<td>(MNN1)</td>
<td>α-1,3-Mannosyltransferase</td>
</tr>
<tr>
<td>(MNN4)</td>
<td>Phosphorylation of manno-oligosaccharide</td>
</tr>
<tr>
<td>PHO11</td>
<td>Secreted acid phosphatase</td>
</tr>
<tr>
<td>PHO99(PHO87)</td>
<td>Phosphate Transporter</td>
</tr>
<tr>
<td>PHR2</td>
<td>β-1,3-Glucanotransferase</td>
</tr>
</tbody>
</table>

Fig. 1. 13. *RIM101* Regulated genes. The summary of the genes listed is those detected by microarray analysis and verified by RT-PCR (Ramon et al., 2003).

### 1.8. AMINO ACID CONTROL REGULATES MORPHOGENESIS OF *C. ALBICANS*

Nitrogen availability has profound significance in fungal biology. In response to nitrogen limitation fungi initiate morphological changes, sexual and asexual sporulation and expression of virulence determinants (Marzluf, 1997a, b). For instance, haploid MATα cells of *Cryptococcus neoformans*, which typically grows as yeast, will develop hyphae and fruiting bodies in nitrogen deficient media and these processes are inhibited by ammonia (Wickes et al., 1996). In *S. cerevisiae*, starvation for a single amino acid stimulates the expression of genes on all amino acid biosynthetic pathways in a phenomenon termed general amino acid control (GCN response) (Hinnebusch, 1988). Nitrogen catabolic repression, the overriding control phenomenon in relation to nitrogen availability, has been demonstrated in numerous fungi (Marzluf, 1997a). Though somewhat of a misnomer (Magasanik, 2003), this global control mechanism prevents expression of the many genes required to utilize various secondary nitrogen sources when an adequate supply of preferred nitrogen source
Nitrogen regulation is controlled by global positive and negative transcription factors as well as pathway specific transcription factor.

1.8.1. Role of Gcn4 in morphogenesis in *C. albicans*

Tripathi *et al.* (2002) reported first time that amino acid starvation stimulates morphogenesis in *C. albicans,* and confirm that a GCN-like response does exist in this fungus. They also showed that both responses are dependent upon CaGcn4p, a functional homologue of *S. cerevisiae* transcription factor ScGcn4p. Hence, CaGcn4p plays a central role in co-ordinating morphogenetic and metabolic responses to amino acid starvation in *C. albicans* (Tripathi *et al.*, 2002). But amino acid starvation is not a strong as morphogenetic stimulus as serum, and it leads to the formation of pseudohyphae rather than true hyphae. Nevertheless, amino acids partially suppress the stimulatory effect of serum, and they do so in a reproducible manner, suggesting that amino acid starvation might comprise one component of the serum stimulus. They also reported that CaGcn4p activated by GCRE (Gcn4 regulatory element) element in an Efg1p-dependent fashion, but ectopic expression of *CaGCN4* of *C. albicans* in a Cph1p-, Efg1p- and Ras1p-independent manner. Even constitutive RAS*V13* (Feng *et al.*, 1999) signaling did not activate the GCN-like response. Therefore, neither MAPK nor Ras-cAMP signaling is implicated in the GCN4 responses in *C. albicans.* Instead, by analogy with *S. cerevisiae* (Hinnebusch, 1988), the GCN-like response might be regulated by CaGcn2p signaling. A ScGcn2p homologue is present in the *C. albicans* genome. However, ScGcn4p as not been implicated in pseudohyphal development (Lengeler *et al.*, 2000; Gancedo, 2001) and transcript profiling of GCN response has not revealed any obvious link with pseudohyphal development in *S. cerevisiae* (Natarajan *et al.*, 2001). In contrast, Tripathi’s group demonstrated that CaGcn4p does influence *C. albicans* morphogenesis. Therefore, there have been some divergences in the cellular roles of Gcn4p between *S. cerevisiae* and *C. albicans.*

1.8.2. Phagocytosis by neutrophils induces an amino acid deprivation response in *C. albicans*

Ingestion of a microorganism by mammalian cells exposes that organism to a novel environment. Transcriptional profiling of a microorganism with a well annotated genome sequence offers a unique window into the response of that organism to phagocytosis. Recently, Fink’s group used transcriptional profiling of *S. cerevisiae* and *C. albicans* to analyze the phagosomal microenvironment of the human neutrophil (Rubin-Bejerano *et al.*, 2003, Ruchel, 1986). They also suggest that the human neutrophil phagosome is amino acid-deficient, and indicate an amino acid-
deprivation response that is conserved between *S. cerevisiae* and *C. albicans*. In both the organisms the transcriptional response is dominated by induction of the methionine and arginine amino acid biosynthesis (Rubin-Bejerano *et al.*, 2003). The response of yeast cell upon deprivation of external amino acids was determined by growing the cells in medium containing or lacking amino acids. Clustering analysis showed that the cells grown in medium without amino acids, like those ingested by neutrophils, induced only the methionine and arginine biosynthetic pathways. To determine whether the induction of the methionine and arginine genes under conditions of amino acid deprivation is under control of Gcn4p, they analyzed the transcriptional profiles of *gcn4*/*gen4* mutant that had been transferred from amino acid-rich medium to minimal medium. Induction of methionine genes by deprivation is independent of Gcn4p, whereas induction of the arginine genes is dependent on Gcn4p. Neither the methionine nor the arginine pathway was induced by exposure to hydrogen peroxide, a critical component of neutrophil-mediated host defense (Babior *et al.*, 1973). Genes induced by hydrogen peroxide (e.g., *SOD2*, *CCP1*, and *CTA1*), were not consistently expressed in response to neutrophils. A number of methionine biosynthesis genes are transiently expressed during S-phase of the cell cycle (Spellman *et al.*, 1998), raising the possibility that the induction of methionine genes in response to neutrophils is a consequence of cell cycle arrest. *Candida* phagocytosed by neutrophil responds to oxidative stress. Even *C. albicans* exhibits a more obvious transcriptional antioxidant response to human neutrophils than does Saccharomyces. After 1 hour of exposure to human neutrophils, when 52% of the *C. albicans* cells were alive, no Candida filaments were observed. But inside the macrophages *Candida* yeast-form cells form filaments (Lo *et al.*, 1997). So the difference in the transcriptional response of *Candida* to ingestion by macrophages and neutrophils could have biological consequences. Whether this is consequences of the amino acid free environment or other factors that differ between these two types of immune cells are not yet known.

1. 8. 3. Amino acid incorporation and general amino acid permease

The potential importance of nitrogen regulation in dimorphism and virulence of *C. albicans* prompted the present studies in our laboratory also. In order to identify and characterize the genes that could be involved in the regulation of morphogenesis and virulence induced by N-acetylglucosamine, an important hyphal regulator of *C. albicans*, we identified the general amino acid permease gene (*CaGAP1*) from *Candida* (Biswa *et al.*, 2003b). We also demonstrated the general amino acid permease activity in *S. cerevisiae* as well as *C. albicans*. In *cagap1* null mutant the citrulline uptake rate was 2 times lower than the wild type strain and a revertant strain in *C.
albicans. Interestingly the citrulline uptake of transformants (Δgap1::CaGAPI) was increased 2.5 fold than the gap1 mutant of S. cerevisiae. So CaGAPI of C. albicans is a functional homologue of S. cerevisiae GAP1.

![Diagram](https://via.placeholder.com/150)

Fig. 1. Probable GlcNAc inducible signaling pathway which regulates general amino acid permease activity in C. albicans. CaGAPI is induced GlcNAc through Cph1p-mediated Ras1p signaling which leads to a morphological changes (Biswas et al. 2003b).

CaGAPI induced by GlcNAc at yeast to germtube transition period. GlcNAc induction of the CaGAPI gene is less in cph1/cph1 and ras1/ras1 null mutants while no striking change of expression was found in efg1/efg1 and tpk2/tpk2 mutant strains. GlcNAc induction of CaGAPI was repressed by calcium calmodulin dependent protein kinase C (PCK) inhibitor TFP, indicating that PCK plays a role in CaGAPI expression (unpublished data). It was also reported that the NH2-terminal region of Acprp/Cph1p can recognize and bind pheromone responsive elements (PRE) in vitro like Ste12p of S. cerevisiae (Malathi et al., 1994). Interestingly one PRE sequence TGAAACA is also present in CaGAPI promoter. This clearly showed the role of Cph1p dependent Ras1p signaling in GlcNAc induced CaGAPI expression (Fig. 1. 14). We have shown that a null mutant of CaGAPI has defect in filamentation in solid spider and nitrogen starvation condition. Despite the defect, mutant strain could not block the induction of filaments by serum response, but we found less hyphal clump formation in GlcNAc inducing conditions. Defective morphology and less filamentation of both the heterozygous and homozygous mutant in nitrogen starvation strongly suggest that the GlcNAc inducible CaGAPI is regulated by external nitrogen source. Thus one interpretation of this data is that GlcNAc induced hyphal formation is sensitive to the dosage of the CaGAPI gene under nitrogen source control. Herein lies the importance of GlcNAc, which not only acts as an inducer of hyphal formation (Mattia et al., 1982; Simonitti et al., 1974) but also
regulates the expression of a number of genes within the cell. Through the induction of CaGAP1, GlcNAc might indirectly alter the nutritional status of the cell, by causing an increased uptake of amino acids. Again, depending on the source of nitrogen in the extra cellular medium, CaGAP1 is induced or repressed. In poor nitrogen source like proline or nitrogen starvation condition CaGAP1 is induced by GlcNAc through Cphlp mediated Raslp signaling pathway, which leads to a morphological change. This interplay between GlcNAc and different nitrogen sources probably brings about a coordinated regulation of CaGAP1 expression and morphogenesis. Recently it was found in S. cerevisiae that Gap1p act as an amino acid sensor for rapid activation of fermentable growth signaling pathway which controls the PKA (protein kinase A) targets (Donaton et al., 2003).

1.8.5. Transcriptional profile of CaGap1p

Interestingly there is one 5'GAATAG 3'sequence (at -646 position) and several TTGTT/TGGTT sequences upstream of CaGAP1 promoter. GAATAG /GATA type sequences which are binding target of transcription factor Gln3p were also found in GAP1 promoter of S. cerevisiae (Miller & Magasanik, 1991). TTGTT or TGGTT plays an auxiliary role in activation of nitrogen regulated gene by Gln3p (Stanbrough et al., 1995). The regulation of CaGAP1 at the level of transcription is comparable to GAP1 regulation in yeast, where the transcription factors Gln3p (in presence of glutamate) and Nil1p (in presence of urea or proline) are activators (Stanbrough et al., 1995), while Dal80p (Cunningham & Cooper, 1993) and Nil2p (Lodish, 1988; Rowen et al., 1997) are inhibitors. In the presence of ammonium, Ure2p, another transcriptional repressor sterically hinders Gln3p from activating GAP1 (Blinder et al., 1996). These factors bind to an upstream regulatory sequence containing a motif surrounding a core GATA sequence (Springael & Andre, 1998). The obvious similarity between the CaGAP1 promoter and nitrogen regulated gene promoter like GAP1, GLN1, GDH2 etc. of S. cerevisiae is the presence of GAATAG sequence (Cunningham & Cooper, 1993). Another feature common to the CaGAP1 and GAP1 promoter is the presence of TTGTT or TGGTT, which plays an auxiliary note in activation by Gln3p (Miller & Magasanik, 1991). Five GATA-type transcription factors and one gene homologous to URE2 have been reported from Candida albicans Genome Sequencing Project, Stanford. One can therefore presume that the regulation of CaGAP1 might be brought about by all of them.
1.8.6. Role of *GAT1* in virulence

Very recently Fonzi's group identified one of the GATA factors, *GAT1* which is required for nitrogen catabolic repression (Limjidaporn *et al.*, 2003). The most notable feature of Gat1p, the predicted 755 amino acid protein, was the presence of a single type IVa zinc-finger motif, C-x$_7$-C-x$_4$-C-x$_2$-C (Teakle and Gilmartin, 1998). Sequence comparisons showed Gat1p was most similar to AREA and closely related orthologues of other filamentous fungi. The most similar yeast homologue was Gat1p, which like AREA is a transcription activator involved in nitrogen catabolic repression (Stanbrough *et al.*, 1995; Coffman *et al.*, 1996). Coffman *et al.* also examined the expression of *GAP1*, *UGA4*, and *DAL5* in repressing and non-repressing nitrogen source in *S. cerevisiae* (Coffman *et al.*, 1996). *UGA4* encodes a putative γ-aminobutyric acid. Mutation of *GAT1* and *GLN3* individually reduces *GAP1* expression approximately 50%, expression is completely eliminated in the double mutant (Stanbrough *et al.*, 1995; Coffman *et al.*, 1996). *UGA4* and *DAL5* of yeast are also regulated by both *GAT1* and *GLN3*, but expression is strongly influenced by *GAT1* similar to the effects seen by Coffman *et al.* *GAT1* had no discernable influence on cell morphology. The null mutant formed normal chlamydospore and filamentation as well as the control strain. The absence of filamentation defects in the mutant is not surprising since filamentation is not sensitive to nitrogen catabolic repression. But virulence of a strain lacking *GAT1* was grossly attenuated in a murine model of disseminated infection. This was surprising for two reasons. Dimorphic ability, a strong virulence determinant (Braun and Johnson, 1997) was unaltered *in vitro* and based on preliminary experiments, *in vivo* as well. Secondly, the growth rate of the mutant in serum at 37°C was comparable to the control and it utilized the full range of amino acids present in the serum. One factor likely to contribute to attenuation of the *gat1/gat1* mutant is the lack of, or reduction in, secreted aspartyl proteinase expression. These are encoded by *SAP* genes, a multigene family in *C. albicans*, and some members are sensitive to nitrogen catabolic repression (Hube *et al.*, 1994). So *GAT1* has been identified as a mediator of nitrogen regulation in *C. albicans* and its function is important for virulence (see Fig. 3.30 of Chapter 3).

1.8.7. Csy1, a nutrient sensor important for hyphal morphogenesis

Serum and -amino acid based media are known to induce filamentous growth in *C. albicans*. However, the mechanism by which amino acids induced filamentation is not well established. Tripathi and colleagues have shown that amino acid starvation promotes pseudohyphal, but not hyphal, growth in *C. albicans* and this response is dependent on CaGcn4p (Tripathi *et al.*, 2002).
Very recently Brega’s group showed that one of the primary amino acid sensors of *C. albicans*, Csy1, plays an important role in amino acid sensing and filamentation (Brega et al., 2004). Loss of Csy1p results in a lack of amino acid-mediated activation of amino acid transport and lack of induction of transcription of specific amino acid permease genes. Their study also demonstrated that *C. albicans* cells lacking Csy1p show altered colony morphology and hyphal formation in serum- and amino acid-based solid media, but not in N-acetylglucosamine, spider, and SLAD media, which do not contain amino acids. So the amino acid sensor Csy1p is an important regulatory membrane protein playing critical role in amino acid transport and filamentation in *C. albicans*. But how Csy1p sense amino acids, activates the transcription of AAP genes, and regulates the *C. albicans* yeast-hyphae morphogenesis is not yet known.

1.9. EMBEDDED/MICROAEROPHILIC CONDITIONS

Although homozygous *efg1/efg1* 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., 2000). A limited supply of oxygen, as occurs under a cover slip during induction of chlamydospores, allows wild-type cells to form filaments, which is enhanced in *efg1/efg1* mutants (Sonneborn et al., 1999b). Similarly, growth of wild-type colonies embedded in agar stimulates filamentation, which still occurs in homozygous *efg1/efg1 cph1/cph1* mutants (Riggle et al., 1999; Giusani et al., 2002). 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/efg1* mutants (Sonneborn et al., 2000), 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/efg1* mutants does not depend on the Cph1 MAP kinase, because a homozygous *efg1/efg1 cph1/cph1* strain is as hyperfilamentous as the homozygous *efg1/efg1* mutant (Sonneborn et al., 2000). It is possible that agar embedding generates microaerophilic conditions, which activate the same Efg1p-independent pathway of morphogenesis under both conditions. The putative transcription factor Czf1p 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 Czfl p contribute to filamentation under embedded conditions. The defective phenotype of a czf1/czf1 mutant is exacerbated by the presence of a cph1/cph1 mutation, which by itself shows defects in the types of media used for monitoring the czf1/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/czf1 mutation. Hyperfilamentation of efg1/efg1 single and efg1/efg1 cph1/cph1 double mutants suggests that Efg1p is a negative modulator of the Czflp pathway under microaerophilic/embedded conditions. Thus, these data once again suggest that positive and negative functions are combined in the Efg1p protein. Conceivably, Efg1p and Czflp 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 Czflp 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.

1. 10. THIGMOTROPISM/CHEMOTROPISM AND SURFACE HYDROPHOBICITY

Vascular endothelium is a barrier against hyphal entry into the host tissue, which C. albicans has to penetrate in order to establish itself within the host. Blood-borne blastospores that adhere to human vascular endothelium give rise to germ tubes, which emerge at the point of adherence and grow down directly into the endothelial cell. This process is assisted by the phagocytic activity of the host tissue. This is in stark difference to its hyphal growth behavior on epithelium. Hyphae produced by surface blastospores grow over the surface prior to penetration, which suggests that, for epithelium, thigmotropism might be associated with both surface growth and the invasion process. Another school of thought however proposes that while thigmotropism may aid invasion of tissue invaginations, chemotropism can explain C. albicans hyphal invasion patterns of both endothelium and epithelium (Davies et al., 1999).
Surface hydrophobicity is a nonspecific factor that can govern mutual adhesion of cell types via van der Waals forces (Klotz *et al.*, 1985). It has been observed that hydrophobic *C. albicans* cells cultured at 25°C are more grossly virulent than are their more hydrophilic counterparts cultured at 37°C. Thus, *C. albicans* cells have different degrees of hydrophobicity according to their growth temperature (Antley and Hazen, 1988), and these differences correspond with differences in epithelial cell adhesion (Hazen, 2001) and virulence in terms of mouse lethality (Antley and Hazen, 1988). This cell surface hydrophobicity is due to the direct contribution of multiple surface proteins and the indirect contribution of surface protein N-mannosylation groups. The degree of surface hydrophobicity of *C. albicans* cells contributes not only to epithelial adherence but also to the speed of hyphal germ tube formation. Germ tubes and strongly hydrophobic *Candida* cells adhere better to epithelia than do yeast forms and hydrophilic cells, but hydrophobicity also influences germ tube formation. Also, hydrophobic cells seem to be more resistant to phagocytosis (Hazen, 2001).

### 1.11. MATING PATHWAY IN *CANDIDA ALBICANS*

#### 1.11.1. Identification of Mating Type-Like Locus in *C. albicans*

Sexual reproduction in fungi is typically controlled by genes that reside in a genetic called a mating-type, or *MAT*, locus. In *S. cerevisiae*, there are three loci containing mating type genes. Two of these loci are silent (*HML* and *HMR*). One contains the *MATa* genes (*MATa1* and *MATa2*), and the other contains the *MATα* genes (*MATα1* and *MATα2*). The third locus (*MAT*) contains either *MATa* or *MATα* gene and is expressed. Here the *MAT* locus switches reversibly from a to α or from α to a by site specific recombination with a copy of the silent *HML* or *HMR* gene (Soll *et al.*, 2003). The *C. albicans* mating type-like locus (*MTL*) closely resembles with the *MAT* locus of *S. cerevisiae* (Hull and Johnson, 1999) (Fig. 1.16). In both the organisms, this locus can exist as one of two alleles, either a or α. Cells carrying only a-allele mate as a cells, and cells carrying α-allele mate as α cells, and cells carrying both alleles (a/α cells) do not mate (Fig. 15). *MTLa* gene cluster was obtained by chromosome walking with a lambda library of *C. albicans* genomic fragments. The beginning probe for the walk was based on a sequence trace from the Stanford *C. albicans* Sequencing project that resembled a portion of the *S. cerevisiae* *MATa1* gene. *MTLα* was obtained by walking downstream of the *MTLa* to its flanking DNA sequence and then back into and through *MTLa* (Hull *et al.*, 1999).
The configuration of mating loci and a comparison mechanism for generating mating types and the mating processes between *Candida albicans* and *Saccharomyces cerevisiae*. *S. cerevisiae* contains a cassette system that includes two silent loci and one expressed locus but *C. albicans* is normally heterozygous for mating type at one locus. *C. albicans* has inserted an extra developmental step, the switch from white to opaque, into the mating process. In *S. cerevisiae*, a and α cells are immediately mating competent, and all α-specific and α-specific genes are upregulated. In *C. albicans*, a hemizygous a or α cell is not mating competent unless it switches to the opaque phenotype. In *C. albicans*, upregulation of α-specific and α-specific gene expression is divided between the transition to a hemizygous state and the transition from white to opaque. HML, homothallic mating locus left; HMR, homothallic mating locus right; MAT, mating type locus; MTL, mating type-like locus. This figure is based on Soll *et al.*, 2003; Bennett *et al.*, 2003; Lockhart *et al.*, 2003; Miller *et al.*, 2002.

The clusters of genes in the *MTL* locus are much larger than those of the *S. cerevisiae*. Hull and Johnson demonstrated that in contrast to *S. cerevisiae*, *C. albicans* strain SC5314, which is diploid, possesses only one *MTL* locus on chromosome 5. The locus is heterozygous, containing only one homolog of the *MATα* gene *MATα1* which is homologous to *S. cerevisiae* *MATα1*, on the other homolog *MTLα1* and *MTLα2*, which are homologous to *S. cerevisiae* *MATα1* and *MATα2*. Both
the MTLα and MTLα loci contain three additional genes (encodes a poly (A) polymerase, a phosphatidyl inositol kinase and an oxysterol binding protein) not found in the MATα and MATα loci of S. cerevisiae (Fig. 1. 16). C. albicans does not possess a S. cerevisiae like cassette system for mating type switching. Rather, it carries opposing MTLα and MTLα alleles at the same locus on the chromosome 5 homologs.

**S. cerevisiae MAT locus**

```
| “a” | a1 |
| “α” | α2 α1 |
```

**C. albicans MTL locus**

```
| “a” | PAPA OBPA PIKα α2 a1 |
| “α” | α2 OBPA PIKα α1 PAPA |
```

Fig. 1. 16. The MAT locus of S. cerevisiae and the MTL locus of C. albicans resembles each other in the arrangement, direction of transcription encoding transcriptional regulators: a1, α1, and α2. In C. albicans MTL encodes an extra transcriptional regulator, α2 (HMG box protein) relative to that S. cerevisiae. In addition, each MTL allele encodes a poly (A) polymerase, a phosphatidyl inositol kinase, and an oxysterol binding protein.

In laboratory strains of S. cerevisiae, a and α cells are generally haploid, and hence contain only one of the two MAT loci, while a/α cells are generally diploid. Higher ploidy states also exist in nature. In C. albicans the three cell types exists only in diploid state (Hull et al., 2000; Lockhart et al., 2002; Magee and Magee, 2000). Naturally occurring diploid a and α cells are homozygous at their MAT loci (Lockhart et al., 2002) and it is thought that C. albicans undergoes a diploid-tetraploid parasexual cycle (Bennett and Johnson, 2003) (Fig. 1. 15). This discovery implies that to become a functional homozygote, C. albicans would have to be come genetically MTL homozygous.

The genes encoded by the mating locus of different fungi, specially ascomycetes generally fall into three specific categories: DNA binding protein that regulate the expression of sexual cycle genes, structural genes that code for mating pheromones, and structural genes that code for mating pheromone receptors (Casselton and Olesnicky, 1998; Kronstad and Staben, 1997, Turgeon, 1998). The C. albicans mating type locus (MTL) closely resembles the S. cerevisiae MAT locus. The genes in the MAT locus of sexually reproducing yeast S. cerevisiae have been well characterized (Johnson, 1995). Here MATa codes for a single regulatory protein (the homeodomain protein a1),
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and *MATα* codes for two proteins (Homeodomain protein α2 and the alpha domain protein α1). Homeodomain proteins constitute a superfamily of DNA binding proteins that play critical roles in gene regulation and development in many eukaryotic species. Homeodomain were first identified in Drosophila regulatory proteins that specify developmental patterns (Scott *et al.*, 1989). Homeodomains consist of three α helices and an NH₂-terminal arm. They bind DNA by inserting the third of these three α helices into the major groove of the DNA, while the NH₂-terminal arm contacts base in the adjacent minor groove (Li *et al.*, 1995). Another transcription regulator, Mcm1p belongs to protein family SRF-like protein (Norman and Treisman, 1988) also involved in transcription regulation of sexual cycle gene. The SRF-like domain was originally identified in the serum response factor (SRF), mammalian protein that activates transcription in response to serum (Norman and Treisman, 1988). These three proteins (αI, α2 and Mcm1p) work in pair wise combination to turn off transcription of two set of cell type-specific genes (Fig. 1.17C). In a/α diploid cells of *S. cerevisiae*, the gene product of *MATα* and *MATα* are both expressed. αI and α2 form a heterodimer which represses the transcription of haploid specific genes, including H0, which is a nuclease for mating type switching; RME, a repressor of early meiosis; several genes encoding components of the mating-specific mitogen activated protein kinase pathways. In both the haploid α cell type and diploid a/α cell type, transcription of genes coding for a cell type functions (a specific genes) are marked for repression by the cooperative binding of α2 and Mcm1p to the conserved DNA sequence (the α2-Mcm1p operator) (Fig. 1.17A). Matα1, which encodes an activator of α specific gene, is also repressed by the a1-α2 repressor complex. In haploid a cell, ‘a’ specific genes are expressed due to absence of the α2-Mcm1p repressor complex, and α specific genes are not expressed due to the absence of the α1-Mcm1p activator complex. In haploid α cells α specific genes are expressed through activation by α1-Mcm1p complex. Several additional genes are also known whose inactivation leads to inappropriate expression of a-specific genes in both the haploid α cells and diploid a/α cells. Two such genes are SSN6 (Schultz and Carlson, 1987) and *TUP1* (Braun and Johnson, 1997). AD Johnson’s group also demonstrated that Ssn6 acts as a transcriptional repression when brought to a promoter by fusion with the LexA bacterial DNA-binding domain. Repression by the LexA-Ssn6 fusion requires Tup1p (Braun and Johnson, 1997) (Fig. 1.17B). So Ssn6p and Tup1p are brought to the promoter by interaction with the promoter-specific, DNA-binding proteins (a1, α2 and Mcm1p).

In *C. albicans* another additional gene *MATα2* is present. *MATα2* encodes a putative DNA binding protein (α2) of 201 amino acids that shows significant sequence similarity to HMG box proteins.
present in mating type loci of other fungi, including \textit{C. parasitica}, \textit{K. lactis}, \textit{N. crassa}, and \textit{P. anserina} (Astrom et al., 2000; Coppin et al., 1997). The HMG box family of DNA binding protein constitutes a large class of well characterized transcription regulators found in many eukaryotes (Grosschedl et al., 1994).

![Diagram of \( \alpha_2 \)-DNA Complex](image1)

![Diagram of General Repression by Tup1-Ssn6 Complex](image2)

\textbf{C} Gene set

\begin{tabular}{|c|c|c|}
\hline
\textbf{Gene set} & \textbf{Repressed} & \textbf{Diagram of Repressed Promoter} \\
\hline
\textit{a}-specific genes & Ssn6 & \textbf{Repression Conditions} \\
\textit{a1} & Tup1 & \textbf{Promoter specific} \\
\hline
\textit{a2/Mcm1 operator} & \( \alpha_2 \) and \( a/\alpha \) cell type & \( \alpha_2, Mcm1 \) \\
\textit{a1/a2 operator} & \( a/\alpha \) cell type & \( a1, a2 \) \\
\hline
\end{tabular}

\textbf{Fig. 1.17.} Model of mating regulation by Ssn6-Tup1 complex in \textit{S. cerevisiae}. (A) Complex formed in vivo by \( \alpha_2 \) and MCM1 with 31 bp \textit{STE6} operator. (B) Generally Ssn6-Tup1 complex associates with the portion one fusion protein LexA. (C) The two sets of genes shown in the first column are repressed under the conditions shown in the third column. In second column, Ssn6 and Tup1 are brought to the promoters by interaction with the promoter specific, DNA-binding proteins listed in the fourth column.

1.11.2. Evidence of Mating of the "Asexual" Yeast, \textit{C. albicans}

It has always been presumed that the fungus \textit{C. albicans} only reproduces asexually (Whelan and Magee, 1981). This organism is naturally diploid and has not been observed to undergo mating.
spontaneously. However, two reports by Hull et al (2000) and Magee and Magee (2000) reveal that C. albicans does have a sex life after all. Apparently, this organism can be “forced” to mate, suggesting that matting may occur naturally (albeit rarely). Diploid organisms are still viable even if one copy of a gene is inactive or imperfect. Hypothesizing that mating may take place between homozygous MTLα and homozygous MTLα cells in nature, Hull et al generated hemizygous MTLα (a/-) and hemizygous MTLα (α/-) strains from the laboratory strain SC5314 and infected mice with a mixture of two. Alternative adenine and uridine auxotrophy was introduced into two strains, so that the MTLα and MTLα strain were ade2URA3 or ADE2ura3, respectively. Fusants could hence be selected through complementation by plating mixed population on medium lacking adenine and uridine. In in-vivo experiments Yeast cells from the kidneys of infected mice were then plated on selection medium and rich medium. For all test mating, about 10<sup>3</sup> C. albicans colonies per kidney were formed on rich medium, conditions under which all the starting strains rapidly grow. 44 of 10<sup>3</sup> total CFU retrieved from the kidneys of mice infected with mixture of a and α cells proved to be ADE2URA3 (MATα/mtlα1mtlα2 + mtlα1/MTLα). None of 10<sup>3</sup> total CFU retrieved from mice injected with mixture of ade2 URA3 a/α cells and ADE2 ura3 a/α cells proved to be ADE2URA3 (<10<sup>-3</sup>). Hence, mixture of homozygous a and homozygous α cells fused, but mixtures of heterozygous a/α cells did not, or did so at undetectable rates. Treatment with serum elicited rapid germ tube formation, indicating that the Ade+Ura+ prototrophs were indeed C. albicans. Fluorescence activated cell sorting (FACS) analysis suggested that cells from most of the colonies tested had substantially increased DNA content compared with that of starting strains. Images of DAPI (4',6'-diamidino2-phenylindol) stained prototroph cells further suggested that the fusants were mononuclear. These data were consistent with mating system in which homozygous a/a and homozygous α/α strains fused to form tetraploids. However, meiosis and recombination were not demonstrated.

1. 11. 3. Mating-Type Locus control White-Opaque switching in Candida albicans

In addition to bud-filament transition, C. albicans is capable of undergoing a different type of morphological change that has been termed as “phenotypic switching” (Fig. 15). This switching is most easily observed in the morphology of colonies (Soll, 1992; Soll et al., 1993). A single cell can divide and in absence of environmental signals, give rise to several distinct types of colonies. This switching occurs spontaneously at frequencies well above those produced by point mutation and has been reported to be reversible. In addition, cells isolated from each type of colony usually produce the same type of colony on replating, indicating that variant colony morphology once
formed, is heritable. Two examples of phenotypic switching have been described in *C. albicans*: 3153A-type switching and the white opaque transition first described in WO-1 strain. 3153A-type switching was first observed in standard laboratory strain 3153A (Slutsky et al., 1985), and later in other laboratory strains and clinical isolates (Pomes et al., 1985; Soll et al., 1987). This type of switching produces at least seven different colony morphologies. The predominant colony type is smooth, but variant colonies arise at the frequency of $10^{-4}$. A low dose of UV light, which killed less than 10% of the cell population, stimulated a 200-fold increase in this initial frequency (Morrow et al., 1989) (Fig. 1.22).

The white-opaque transition was first described in the strain WO-1, isolated from blood and lungs of a bone marrow transplant patient at the University of Iowa Hospitals and Clinic (Slutsky et al., 1985; Rikkerink et al., 1988). It is an alternation between two quasistable, heritable transcriptional states. The white-phase cells appear relatively round, they form white, dome-shaped colonies on solid agar, and they express a set of white-specific genes. Opaque cells are more elongated and exhibit peculiar budding patterns. They contain a dominant vacuole and show prominent pimples on the cell wall. The colonies appear darker and grow flatter against the agar, and they express a set of opaque-specific genes (Soll, 1997). In addition, white and opaque cells have been demonstrated to differ in antigenicity (Anderson, et al., 1990), constraints on bud-hypha transition (Anderson et al., 1989), polypeptide synthesis (Soll et al., 1991), lipid and sterol content (Ghannoum et al., 1990), adhesiveness, susceptibility to antifungal agents (Soll et al., 1991), assimilation of sugars and uptake of dyes (Anderson and Soll, 1987). Thus it is clear that switching involves the regulation of a number of unrelated genes. Several opaque-phase-specific genes have been identified such as, *OP4* (Morrow, 1994; Morrow, 1993), the secreted aspartyl proteinase genes, *SAP1* and *SAP3* (Hube et al., 1994; Morrow et al., 1994; Morrow et al., 1992; White et al., 1993), the drug resistance gene, *CDR3* (Balan et al., 1997), and the two-component regulator gene *CaNIK1* (Srikantha et al., 1998). Similarly White-phase-specific gene, *WHII* (Srikantha and Soll, 1993) was identified which is homologous to the glucose lipid-regulated protein *GLP1* of *S. cerevisiae* (Stone et al., 1990) (Fig. 1.20). It is turned on in white budding phase and turned off in the opaque phase. Efg1p, a morphogenesis regulator is shown to be expressed in the white-phase (Sonnenborn et al., 1999a; Srikantha et al., 2000). Fusion of *WHII* promoter and *SAP1* promoter to GFP reporter gene showed that these genes were activated only in white phase and opaque phase cells respectively showing that expression of these genes are tightly linked to the cell type. It was demonstrated the *WHII* is regulated through two unique upstream activation sequences (Srikantha et al., 1995;
Srikantha et al., 1997). OP4 is regulated primarily through a MADS box consensus sequence (Lockhart et al., 1998). Therefore, phase specific genes appear to be regulated by phase specific transacting factor.

Recently it was shown by Miller and Johnson, that the white-opaque switching is controlled by the mating-type (MTL) locus of C. albicans. C. albicans is typically diploid and is heterozygous for the MTL locus. It was observed that a and α strains, constructed in CAI4 background, by disruption of MTLα and MTLα genes respectively, routinely formed dark, flat sectors at the colony edge that superficially resembled opaque sectors of the classical white-opaque switching strain WO-1 (Miller and Johnson, 2002). Genetically unrelated clinical isolates which could switch frequently were homozygous at MTL locus were either MTLα or MTLα (Lockhart et al., 2003a). The opaque colonies could be stained red by phloxine B stain, and showed typical morphology. It was observed that white-opaque switching was negatively regulated by combined action of two homeodomain proteins, MTLα1, encoded by MTLα locus and MTLα, encoded by the MTLα locus. Thus loss of either gene product converts non-switching type to switching type. They also demonstrated that the opaque cells are actually mating competent cells and white opaque switching is required for mating process. Expression studies of WO-1 strain showed that affected genes represented functions as diverse as metabolism, adhesion, cell surface composition, stress response, signaling, mating type and virulence. Approximately one third of the differences between cell types are related to metabolic pathways, opaque cells expressing a transcriptional profile consistent with oxidative metabolism and white cells expressing a fermentive one. This bias was obtained regardless of carbon source, suggesting a connection between phenotypic switching and metabolic specialization of switch phenotypes enhance selection in relation to the nutrients available at different anatomical sites (Lan et al., 2002).

1.1.4. Mating pheromone and C. albicans

In S. cerevisiae, mating between haploid cells is signaled by binding of pheromone to a cell-type-specific receptor on cells of the opposite mating type (STE2 expressed in a cells recognized by α-factor, and STE3 expressed in α cells recognized by a factor). Recently Bennett et al., 2003 identified the α mating pheromone (α factor) of C. albicans and characterized the response it elicited in recipient a cells. The gene encoding α-factor is predicted to encode a precursor protein that processed into three identical tridecapeptides representing the mature α-factor (Fig. 1.18). C. albicans STE2 gene, which is homologous to the S. cerevisiae α-factor receptor gene (Hartig and
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MacKay, 1986; Jenness *et al.*, 1986, is required for the morphological response of a cell to α-factor. To complete the link between the tridecapeptides and mating, Bennett *et al.*, 2003 also showed that α cells (but not a cells) with the α-factor gene (MFα) deleted are deficient in mating; likewise, a cells (but not α cells) with the gene encoding the α factor the α-factor receptor deleted (STE2) are mating deficient. Conceptual translation of this gene produced a hydrophobic leader sequence, consensus Kex2 protease processing sites, and three repeats of a 13-amino-acid sequence, GFRLTNFNYFEPG. This repeats were predicted to be processed into the mature pheromone (Fig. 1.18). The overall structure of the *S. cerevisiae* MFα genes, MFα1 and MFα2 are similar with MFα gene of *C. albicans*, although there is no amino acid sequence similarity between the *S. cerevisiae* mature α-factor and the predicted *C. albicans* α-factor (Bennett *et al.*, 2003).

![Consensus Kex2p Processing Site](image)

**Fig. 1.18.** Schematic diagram of the proposed *C. albicans* α-factor pheromone precursor. The conceptually translated protein consists of a hydrophobic leader sequence, three copies of a 13 amino acid (aa) sequences and consensus Kex2p processing sites (Bennett *et al.*, 2003).

### Genes Induced by α-factor in *C. albicans* opaque a cells

<table>
<thead>
<tr>
<th>GENES</th>
<th>FUNCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM6, MCM7, PRI, PolLSa</td>
<td>DNA replication</td>
</tr>
<tr>
<td><strong>CST20</strong> (STE12 homolog),</td>
<td>MAP Kinase pathways</td>
</tr>
<tr>
<td><strong>HST7</strong> (STE7 homolog),</td>
<td></td>
</tr>
<tr>
<td><strong>CPH1</strong> (STE12 homolog)</td>
<td></td>
</tr>
<tr>
<td><strong>KAR4</strong>, <strong>KAR5</strong></td>
<td>karyogamy</td>
</tr>
<tr>
<td><strong>SST2</strong>, <strong>CPP1</strong> (MSG5</td>
<td>Pheromone adaptation</td>
</tr>
<tr>
<td>homolog)</td>
<td>a factor processing and</td>
</tr>
<tr>
<td><strong>AXL1</strong>, <strong>HST6</strong> (STE6</td>
<td>export</td>
</tr>
<tr>
<td>homolog)</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.19.** α-factor induced genes. The summary of the genes listed is those detected by microarray analysis in response of a and α opaque cells to α-factor (Bennett *et al.*, 2003).
To identify the genome-wide changes in transcription that accompanies the response to α-factor, *C. albicans* a and α opaque was monitored using microarray. In contrast to *S. cerevisiae*, pheromone treatment of *C. albicans* cells resulted in lowered expression of only very few genes. *C. albicans* transiently arrest DNA replication in response to α factor. Most of the genes induced by *C. albicans* α-factor had previously been implicated in mating in *S. cerevisiae*. But some genes are induced by α-factor in *C. albicans* but not in *S. cerevisiae* (Fig. 1.19 and Fig. 1.20). It is tempting to speculate that since *S. cerevisiae* and *C. albicans* diverge from common ancestor about 100 to 200 million years ago, structural components of the mating apparatus were adapted by *C. albicans* for use in interacting with its mammalian host.

**Fig. 1.20.** Model of the roles of switching and α pheromone induction in the expression of genes during the mating process of *C. albicans*. A key to understanding the regulation of each gene is presented in the upper right-hand corner of the figure. Genes are grouped in three categories that are colour-coded. Blue, haploid specific (HP) and mating-type-specific (MT) genes upregulated by α-pheromone in both white- and opaque-phase cells; green, genes that are activated by the α-pheromone, exclusively in the opaque phase; orange, opaque-phase-specific genes (OP), three of which are downregulated by α-pheromone (Lockhart et al., 2003).
1.11.5. Genetic and Transcriptional Circuit Regulating Mating-Type in *C. albicans* and *S. cerevisiae*

![Diagram of the mating-type circuit in *C. albicans* and *S. cerevisiae*]

**Fig. 1.21.** Transcriptional circuit regulating mating-type in *C. albicans* and *S. cerevisiae*. In *C. albicans* α1 protein activates the expression of α-specific genes and α2 protein activates the expression of a-specific genes. In a/a cells the α2 and a1 proteins act together as a repressor of the phenotypic switch from white to opaque, and of ‘haploid specific’ genes. In *S. cerevisiae* α1 protein activates the expression of α-specific genes, and α2 and a1 act together in diploid cells as a repressor of MATα1 and haploid specific genes. The a-specific genes are, however, constitutively expressed in the absence of the α2 repressor (Tsong *et al.*, 2003). Though *C. albicans* has no haploid phase, the ‘haploid specific’ term refers to preserve the analogy with *S. cerevisiae*.

In order to determine the specific roles of the *MTL*-encoded regulators (α1, α2, α1, α2) in specifying mating type, Tsong *et al* (Tsong *et al.*, 2003) tested mating behavior in a collection of diploid isogenic strains bearing all 16 possible combination of possible genes. *C. albicans* must undergo a “phenotype switch” from the white phase, the prevalent form of *C. albicans*, which is stable at 23 °C, but not 30 °C. All but four strains could efficiently switch. These four strains are the only ones that retain both α1 and α2. This experiment also shows that α1 and α2 control white-opaque switching but newly described α2 is not involved in this control. Of the twelve strains that could undergo white-opaque switching, some could mate and others could not. Efficient a-type mating (defined as the ability to mate with the α-type mating type tester) requires only α2. Likewise, efficient α-type mating requires only α1. When both α1 and α2 are present, strains mate inefficiently. This regulation differs significantly from that of *S. cerevisiae*, which lacks an HMG
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box a2. In C. albicans a-type mating requires positive regulation by a2, whereas in S. cerevisiae a type mating is in “default” behavior, and requires no input from the MAT locus. In order to prevent a-type mating in α cells, the mating by means of a2; this branch of the pathway is absent in C. albicans. On the basis of above AD Johnson’s group proposed a genetic circuit governing mating type in C. albicans (Fig. 1. 21).

To identify specific genes regulated by the C. albicans a1, a2, α1 and α2 Tsong et al performed micro array analysis on strains carrying the 16 combination of MTL genes described above. By comparing stains in white and opaque phases bearing all combinations of MTL transcriptional regulator, they were able to identify a- and α- specific genes. It was found that STE3 and MFα1 are highly induced in opaque strain relative to white strains (300- and 1000 fold respectively), but only those that carry an intact MTLα1 gene-that is, those able to mate as α cells. α1 and α2 require to repress the white-to-opaque switch in C. albicans. In absence of either components of this heterodimer, the cell can switch to the opaque phase, which is accompanied by the up- and down regulation of more than 400 genes. α-specific genes (asgs) are turned on in α-cells cells by α1, but only if the cells are in the opaque phase. In contrast to S. cerevisiae, relief of α1-α2 repression is necessary but not sufficient for mating competency, consisting a layer of transcriptional control over mating that is not present in S. cerevisiae. While a-type mating is positively regulated by a2 in C. albicans, it is negatively regulated in S. cerevisiae by α2. Obviously, it is difficult to answer the question that why a2 is missing in S. cerevisiae. AD Johnson’s group proposed a hypothetical series of events accounting for how an ancestral a2-control positive circuit branch was replaced by a α2 controlled negative circuit branch in S. cerevisiae (Tsong et al., 2003). This might occur by co-opting an abundant and/or highly adaptable regulator of transcription; in S. cerevisiae, Mcm1p has fulfilled this role (Johnson, 1995)

1. 11. 6. Cell Biology of C. albicans during mating

Both Hull et al (2000) and Magee and Magee (2000) demonstrated that after mating type-based fusion of a and α cells the cellular DNA content was increased. Even Hull et al provided evidence in a website supplement to their publication that fusion progeny were mononucleate. These observations indicated that karyogamy had occurred. Lockhart et al, 2003a however, did not observe nuclear fusion (karyogamy) in the conjugation bridge of zygotes. Continuous video microscopy, computer-assisted three-dimensional reconstruction of living cells, and fluorescence microscopy they suggested that after cellular fusion, the nuclei from original a/a and α/α cells did
not undergo karyogamy. Rather, the nuclei translocated back to the tube cell junctions through expansion of the conjugation bridge vacuole.

1.11.7. Parasexual cycle of C. albicans

Although mating has now been amply documented for C. albicans, no study has reported whether the tetraploid products formed through mating can be induced to return to the diploid state. This process could, in principle, occur via a sexual cycle (meiotic divisions) or via a parasexual cycle (reductional mitotic division. Recently Johnson’s group provided evidence for a chromosome-loss pathway which, when combined with mating, completes a parasexual cycle for C. albicans that can be readily carried out in the laboratory (Bennett and Johnson, 2003). They showed that the ploidy of a tetraploid strain of C. albicans (produced by the mating of two diploid strains) can be reduced by simple laboratory manipulations (growth on S. cerevisiae ‘pre-sporulation’ medium at 37 °C) to that of a diploid. Although the detailed mechanism is not yet known, but it is clear that loss of one or more chromosomes seems to predispose cells to lose others, with the diploid state being the final product. Growth of diploid strains of C. albicans on sorbose medium at 37°C results in the loss of one copy of chromosome 5, the chromosome containing the MTL locus (Janbon et al., 1998, 1999). The majority of diploid cells die when grown on sorbose medium, but the survivors are often monosomic for chromosome 5. It has been proposed that the level of expression of the SOU1 gene, which is essential for L-sorbose assimilation in C. albicans, is regulated by the copy number of chromosome 5 (Janbon et al., 1998). Thus chromosome loss may have evolved to provide C. albicans with an alternative pathway to meiosis and lead to the rearrangement of genetic material in this organism.

1.11.8. Mating and adaptation

Mating involves pheromone-induced conjugation tube formation, chemotropism, tube fusion, nuclear migration, and daughter cell formation from the conjugation bridge (Lockhart et al., 2003a). Unlike S. cerevisiae, a/α cells have undergone homozygosis at the MTL locus to either a/a or α/α are still not mating competent. To mate, they must switch from the white to the opaque phase (Miller and Johnson, 2002, Lockhart et al., 2003a), a phenotype transition that involves dramatic changes both in cellular architecture and gene expression (Soll, 1992; 2002; 2003). Because physiological temperature (37 °C) causes phenotype conversion of opaque cells en masse to the white phase (Srikantha and Soll, 1993), it has been suggested that mating, which requires
expression of opaque phase phenotype, may occur outside of the human body, perhaps on skin, on catheters or in non-animal reservoirs (Soll et al., 2003), where the temperature is below 37 °C and colonization can rapidly occur between multiple strains of C. albicans. Recently, this hypothesis was lent support by observations of mating on skin. Kvaal et al. (1999) had demonstrated that although white phase cells do not colonize the skin of newborn mice, opaque phase cells are highly efficient at colonizing skin. This results demonstrated that skin facilitates mating between α/α and α/α cells of C. albicans.

1.11.9. Role of SIR2 and Histone deacetylase in phenotype-switching in C. albicans

The molecular basis of phenotype switching in C. albicans is not well understood, and several possible mechanisms can be considered. C. albicans is diploid, and chromosome rearrangements occur frequently and result in changes in electrophoretic karyotypes. Altered karyotypes are associated with variant colony morphologies (Rustchenko-Bulgag et al., 1990; Ramsey et al., 1994), but it has been difficult to establish a cause and effect relationship (Janbon et al., 1998). Heritable epigenetic changes are well characterized in S. cerevisiae. For example, genes controlling mating type in budding yeast are located in chromosomal domains where chromatin adopts a specific structure (analogous in some way to heterochromatin) responsible for silencing these genes (Loo and Rine, 1994). The SIR (Silent information regulator) genes are required to establish the silence state, and mutation in these genes can affect the efficiency with which S. cerevisiae cells pass on the silent state to their daughters (Laurenson and Rine, 1992). Phenotypic switching in C. albicans could in principle be controlled using a similar mechanism (Ramsey et al., 1994).

According to this hypothesis, regulatory genes that control colony morphology would be located in chromosomal positions that exist in two alternative states: a silenced state and an active state. Transition between the two states would result from changes in the chromatin structure, and once established, the on- or off-state of chromatin would be inherited. Phenotype switching would occur when the chromatin state spontaneously changes, a characteristic of silenced domain in S. cerevisiae.

AD Johnson's group reported that the cloning and sequence of a C. albicans gene with sequence similarity to the S. cerevisiae SIR2 gene (Perez-Martin et al., 1999). They showed that deletion of the two copies of SIR2 gene in C. albicans produces a dramatic phenotype: variant colonies obtained from this sir2/sir2 mutant strain resemble the colony morphologies described by Soll and collaborators as part of phenotype switching system (Slutsky et al., 1985). An additional phenotype
of the sir2/sir2 mutant strain is high frequency of karyotypic changes, including the presence of an extra DNA band in the karyotype. But *Candida albicans* strain which is locked in the filamentous forms does not show variant colony morphologies in response to a SIR2 deletion (Perez-Martin et al., 1999).

Perez-Martin et al (1999) also demonstrated that besides the “silence information regulator” (Sir) proteins, other classes of proteins are also involved in repression or silencing of developmentally regulated genes in eukaryotes. One such class of genes, the histone deacetylases, has been demonstrated to regulate chromatin structure through selective histone deacetylase, which in turn affects chromatin folding and interactions between DNA and DNA-binding proteins (Ayer, 1999). Recently Klar et al (2001) tested whether the specific deacetylase inhibitor trichostatin A (Yoshida et al., 1995) affected the white-opaque transition. They found that the inhibitor caused a selective increase in the frequency of switching in the white-to-opaque transition, but had no effect on the frequency of switching in the opaque-to-white transition, suggesting that deacetylation through a trichostatin-sensitive deacetylase selectively suppresses switching in one direction. Srikantha et al cloned five of the major *C. albicans* histone deacetylation gene with homology of the five known histone deacetylases in *S. cerevisiae* (HDAl, RPD3, HOS1, HOS2, and HOS3) and analyzed their deduced protein sequences and expression patterns in the white-opaque transition. Among the classes of protein involved in chromatin modifications, the deacetylases have been demonstrated to function in the repression of gene loci through the selective deacetylation of histone H3 and H4 (Ayer et al., 1999; Workman and Kingston, 1998). Since TSA (trichostatin A) preferentially inhibits the major deacetylase Hda1p (Carmen et al., 1996), Srikantha et al deleted the gene (HDAl) coding for this protein in *C. albicans* strain WO-1 and found that the mutant phenotype was similar to that of TSA-treated cells (Klar et al., 2001). Even it was also found that deletion of RPD3, another histone deacetylase resulted in an increase in the frequency of switching in both the white-to-opaque and opaque-to-white direction (Fig. 1. 22). The histone deacetylase and SIR2, which was recently demonstrated to have NAD-dependent histone deacetylase activity (Imai et al., 2000), play roles in the expression of large numbers of different genes in *S. cerevisiae*. Recently, Bernstein et al (Bernstein et al., 2000) performed a bioinformatic analysis of genes up-regulated at least 1.5-fold in rpd3/rpd3, hda1/hda1, and sir2/sir mutants. They found that while the gene encoding Hdp1p plays a more prominent in regulating carbon metabolite and carbohydrate transport and utilization, RPD3 plays a role in cell cycle progression, and SIR2 plays a role in amino acid biosynthesis.
In the case of the \textit{HDA1} deletion, white-phase expression of \textit{EFG1} was down-regulated, but there was no significant effect on the white-phase specific expression of \textit{WH11} or \textit{HOS3} or opaque-phase-specific expression of \textit{OP4}, \textit{SAP1}, and \textit{SAP3}. There was also no effect on white-phase-enriched expression of \textit{MCM1}. In case of the \textit{HDA1} deletion, there was no effect on the phase-regulated expression of \textit{MCM1}. Neither deletion of \textit{HDA1} nor deletion of \textit{RPD3} affected the phase-regulated expression of the four other deacetylase. The deletion of \textit{HDA1} or \textit{RPD3} led only to down regulation, not up-regulation, of select phase specific genes is interesting, but not unique. Deletion of \textit{RPD3} in \textit{S. cerevisiae} leads to increased repression of reporter gene expression at mating type loci, telomeres, and ribosomal DNA (De Rubertis \textit{et al.}, 1996), all loci under the regulation of Sir Proteins (De Rubertis \textit{et al.}, 1996). Increased silencing genes usually suppressed by the activity of the deacetylase. So deacetylase plays distinct roles not only in the suppression of switching, but also in the activation of select phase-regulated genes, in the latter case presumably through the down-regulation of suppressor genes.
the list of components affecting morphogenesis. Given this overview of different morpho-
pathogenic determinants (listed in Table 1) and mating regulators of \textit{C. albicans}, it is not clear that
several major questions remain unanswered for all of the pathways. What is the mechanism by
which a single pathway can be activated by multiple signals?
\textit{C. albicans}, one of the first eukaryotic pathogen selected for genome sequencing, is the most
commonly encountered human fungal pathogen. The preliminary assembly for the \textit{C. albicans}
genome sequence has been released (http://www-sequence.stanford.edu/group/candida/). Very
assemble \textit{Candida} genome sequence in good agreement with available physical mapping data.
Comparative genome analysis provides important clues about the evolution of the species and its
mechanisms of pathogenesis. They showed that 64\% of the ORFs of \textit{C. albicans} have their best
match in \textit{Saccharomyces}; the remaining 14\% of ORFs found matches to other more distantly
related species than \textit{S. cerevisiae}. The genome sequence reveals a number of adaptations for
environmental sensing and response. \textit{C. albicans}' ability to pass through the diverse tract requires
to it to cope with widely varying pH environment. \textit{C. albicans} has a number of genes related to the
pH regulatory genes of \textit{Aspergillus} and encodes a small family of chloride channels with members
resembling types expressed in a variety of mammalian tissues. So the availability of a diploid
genome sequence will now take these studies to a new level. The rapidity of these sequencing
initiatives is impressive, and with the development of new functional genomic technologies
including DNA microarray, which become an indispensable tool for high-throughput gene
expression analysis, we will soon be able to analyze the whole genome responses of this fungal
pathogens. Furthermore, sensors responding to environmental changes, especially in the host during
infection, need to be identified, as well as their linkages to downstream signaling pathways.
Program specific distribution across the genome may be a general mechanism by which different
signals regulate distinct gene expression programs in response to environment. At the signaling
level, new strategies to identify nonlinear complexities in signal transductions, such as crosstalk
and feedback loops must be developed. As a result, our view and appreciation of \textit{Candida} biology
and pathogenesis will undergo something little short of a revolution in the very near future.