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cAMP signalling in pathogenic fungi: control of dimorphic switching and pathogenicity

M. Ines Borges-Walmsley and Adrian R. Walmsley

Morphological changes in pathogenic fungi often underlie the development of virulence and infection by these organisms. Our knowledge of the components of the cell signalling pathways controlling morphological switching has, to a large extent, come from studies of pseudohyphal growth of the model organism *Saccharomyces cerevisiae*, in which control is exerted via changes in the intracellular cAMP and mitogen-activated protein kinase cascades. There is evidence that pathogenic fungi also utilize these pathways to control dimorphic switching between saprobic and pathogenic forms and, as such, the elements of these pathways have potential as drug targets.

M.I. Borges-Walmsley and A.R. Walmsley are in the Divn of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK G12 8QQ.*
**tel: +44 141 330 3750,*
fax: +44 141 330 3751,
e-mail: a.walmsley@bio.gla.ac.uk

The ability of dimorphic pathogenic fungi to switch between different morphological states appears to be an important virulence determinant as mutant strains lacking this ability often have reduced virulence or are avirulent. The genes controlling morphogenesis have therefore been the focus of many investigations, as they have great potential as targets for novel antifungal drugs. Most of our knowledge of the signalling pathways involved in controlling morphological switching has been provided by studies of the switch from yeast to pseudohyphal growth in the model organism *Saccharomyces cerevisiae*. These studies have revealed that the signalling pathways are controlled by both cAMP and mitogen-activated protein kinase (MAPK) signal transduction pathways. It has become clear that pseudohyphal growth in *S. cerevisiae* is one aspect of the response to nutritional deprivation and, as such, is a response to an environmental stress. Pathogenic fungi appear to have adapted related cell signalling pathways to control morphological switching during infection. Here, we review our current knowledge of the cAMP signalling pathway in *S. cerevisiae*, drawing parallels with pathogenic fungi.

Morphological switching in *S. cerevisiae*

Diploid cells of the budding yeast *S. cerevisiae* can be induced to produce pseudohyphae under conditions of nitrogen starvation. This morphological transition entails changes in cell morphology, budding pattern and cell separation: the cells switch from an ovoid to an elongated shape; they bud apically and the buds remain attached, resulting in chains of cells that constitute the pseudohyphae. *In vitro*, they also acquire the ability to invade

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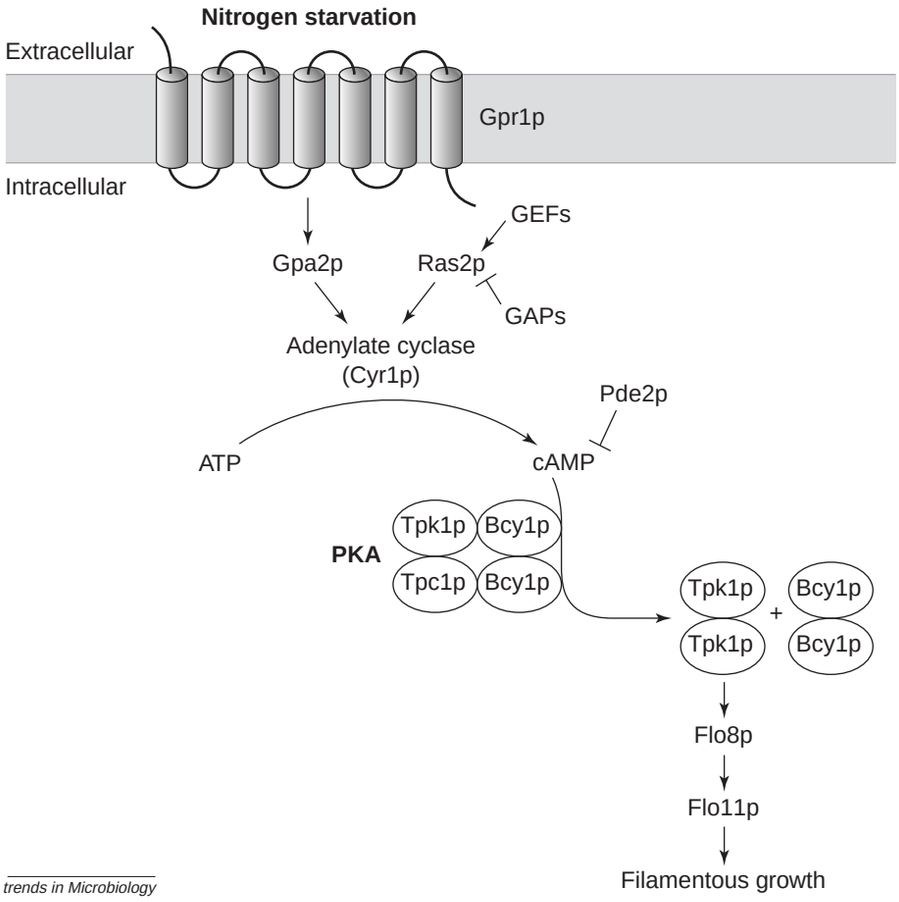

Fig. 1. A diagrammatic representation of the cAMP signalling pathway that controls filamentous growth in *Saccharomyces cerevisiae*. Adenylate cyclase (Cyr1p) can be activated via two pathways, involving the small G proteins Ras2p or Gpa2p, producing an increase in cAMP levels. The phosphodiesterase Pde2p attenuates this response by decreasing the cAMP level. cAMP binds to the regulatory subunit (Bcy1p) of protein kinase A (PKA), causing its dissociation from the catalytic subunits (Tpk). The Tpk subunits can phosphorylate target transcription factors, such as Flo8p, which in turn induce the expression of specific genes, such as cell surface flocculins, to bring about filamentous growth. The activity of Ras2p is modulated by the guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

the agar surface upon which they are growing, probably to allow better scavenging of nutrients. Haploid cells can also grow invasively but this is distinct from diploid invasive filamentous growth, because haploids are able to invade agar rich in nitrogen and diploids cannot. Although filamentation is generally regarded as a feature of diploid cells during nitrogen starvation, haploid cells of *S. cerevisiae* strains that are capable of degrading starch have been shown to form pseudohyphae under conditions of carbon starvation.

The pseudohyphal growth of *S. cerevisiae* is controlled by both MAPK (Refs 1,2) and cAMP (Refs 3,4) signal transduction pathways. Nitrogen starvation causes activation of the small G protein Ras2p, which can activate the MAPK signalling pathway and the cAMP pathway to trigger filamentous growth.

The cAMP pathway

It has become clear that the cAMP signalling pathway is a major control mechanism for morphological switching in *S. cerevisiae* (Fig. 1). Although adenylate cyclase [encoded by *cyr1* (also termed *cdc35*)] is likely to play a key role in cAMP signalling, it is difficult to demonstrate this role directly because its deletion is lethal as a basal cAMP level is essential for cell viability. However, a mutant was recently described that was able to maintain a basal cAMP level but was unable to respond to environmental cues that normally trigger elevated cAMP levels (e.g. glucose and intracellular acidification), indicating a key role for adenylate cyclase in transducing the cAMP signal[5]. The activity of adenylate cyclase is highly dependent upon the activity of Ras1p and Ras2p, and their simultaneous deletion is lethal. It is possible to activate Ras2p by replacing the second Gly with Val in the GXGXXG motif, where X is any amino acid (e.g. G19V)[1]. This motif is highly conserved in the G-protein superfamily and the Gly-to-Val mutation reduces GTPase activity, stabilizing the GTP-bound active form. This activated form of Ras2p causes an elevation in the cAMP level and stimulates filamentous growth[1]. The activity of Ras2p is controlled by the GEFs (guanine nucleotide exchange factors) Cdc25p and Sdc25p, and the GAPs (GTPase-activating proteins) Ira1p and Ira2p (Ref. 6). The deletion of the genes encoding Cdc25p, but not those encoding Sdc25p, is lethal, whereas deletion of the genes encoding Ira1p and Ira2p causes overactivation of the cAMP pathway, and *ira1Δ/ira1Δ* strains are hyperfilamentous.

The induction of filamentous growth by elevated cAMP is not only dependent upon activation of adenylate cyclase by Ras2p but is also dependent upon activation of another small G protein, Gpa2p (Refs 7,8). A *gpa2Δ/gpa2Δ* strain was found to be defective in pseudohyphal growth on nitrogen-limiting media. When activated Gpa2p, for example, Gpa2p (G132V) was introduced into diploid cells on a galactose-inducible episomal plasmid, it enhanced pseudohyphal growth upon induction by galactose but not glucose, on both nitrogen-limiting and rich media. Epistasis analysis and reporter-gene studies have indicated that Gpa2p acts independently of the MAPK pathway. Furthermore, pseudohyphal growth is severely hindered in a *ras2Δ gpa2Δ* double-deletion strain, but not in the single-deletion strains, and a *ras2Δ gpa2Δ* mutation can be complemented by the

addition of exogenous cAMP. Conversely, activated mutants of Gpa2p and Ras2p both stimulate pseudohyphal growth.

In an attempt to identify other components of the cAMP pathway, particularly those upstream of Ras2p and Gpa2p, two-hybrid screens have been undertaken. In the case of Ras2p, only the aforementioned GEFs and GAPs have been cloned and no clear upstream activator has been identified. Recently, however, the Hsp70p heat shock protein Ssa1p was shown to bind to Cdc25p, possibly to maintain the level of active Cdc25p under stressful conditions (see below)[9]. By contrast, using Gpa2p in a two-hybrid screen, a G-protein-coupled receptor, Gpr1p, has been cloned[10]. A *gpr1Δ/gpr1Δ* strain is defective in pseudohyphal growth and a *gpr1Δ ras2Δ* strain displays a severe growth defect. This defect can be suppressed by activated Gpa2p, for example Gpa2p (R273A), indicating that Gpr1p acts upstream of Gpa2p. Rgs2p, a member of the recently identified family of regulators of heterotrimeric G-protein signalling (RGS), has been shown to inhibit Gpa2p by enhancing its GTPase activity[11]. Rgs2p functions as a negative regulator of glucose-induced cAMP signalling.

Ras2p and Gpa2p activate adenylate cyclase (Cyr1p), probably by direct interaction. The consequent elevation in the cellular cAMP level activates one or more cAMP-dependent protein kinases. Yeast protein kinase A (PKA) plays a major role in cAMP signalling. In resting yeast cells, PKA is an inactive tetramer comprising two catalytic subunits, encoded by the *tpk* genes, and two regulatory subunits, encoded by the *bcy1* gene[6]. There are three *tpk* genes that encode proteins with a highly conserved carboxy-terminal domain of ~300 residues and a shorter, distinct amino-terminal domain. The binding of cAMP to the regulatory subunits causes dissociation of the active catalytic subunits. Disruption of *bcy1*, producing an active cAMP-independent PKA, has been shown to induce pseudohyphal growth. Strains deficient in all three of the Tpk proteins are inviable, but those containing any one of the Tpk proteins are viable. Deletion and overexpression experiments have revealed distinct roles for each Tpk protein[12,13]: Tpk2p exerts positive control over filamentation, whereas Tpk1p and Tpk3p exert a negative effect.

Although *tpk2* expression is induced by nitrogen starvation, differences in the *tpk1–3* gene promoters and transcriptional regulation do not account for their different functions. This difference in role appears attributable to sequence differences in the carboxy-terminal domain of the Tpk proteins rather than to differences in the unique amino-terminal domain. The PKA-activating *bcy1* mutation can suppress the filamentation defect in *gpa2Δ/gpa2Δ* and *gpr1Δ/gpr1Δ* strains, and the *tpk2* deletion completely suppresses filamentation, as a result of the expression of activated Gpa2p [e.g. Gpa2p (G132V)], indicating that Tpk2p functions downstream of Gpr1p and Gpa2p. The *tpk2* deletion partially suppresses filamentation by allowing the expression of activated Ras2p [e.g. Ras2p (G19V)], consistent with Ras2p signalling occuring via both the cAMP/PKA and MAPK pathways. Interestingly, there is some evidence to point to a role for Bmh1p and Bmh2p (so-called 14-3-3 proteins after their pattern of movement on 2-D gels, also known to be involved in the MAPK pathway) in the Ras2p–Cyr1p–PKA pathway: *bmh1Δ bmh2Δ* double-mutant strains accumulate abnormally high levels of glycogen, a phenotype of strains deficient in PKA activity[5]. Furthermore, Ras2p (G19V) or overexpression of Tpk1p can suppress this phenotype.

Comparative analysis of the cAMP and MAPK signalling pathways indicates dual pathway control is necessary to control distinct aspects of pseudohyphal development: the cAMP/PKA pathway regulates unipolar budding, whereas the MAPK pathway regulates cell elongation[13]. Invasive growth is under the control of both the MAPK and cAMP/PKA pathways.

Following activation of the cAMP signalling pathway, cAMP is hydrolyzed by low- and high-affinity phosphodiesterases (PDEs), encoded by *pde1* and *pde2*, respectively, returning it to the basal level[6]. cAMP stimulates filamentation in *pde* deletion strains, even on nitrogen-rich medium. The overexpression of *pde2* inhibits filamentation, even in a strain expressing Ras2p (G19V). The activity of PKA is subject to strong feedback inhibition by cAMP synthesis, which apparently plays a role in curtailing signalling events. The cAMP level is higher in a Ras2p (G19V)-expressing *pde1Δ pde2Δ* strain than a Ras2p (G19V)-expressing strain, indicating that PDEs are able to prevent hyper-accumulation of cAMP. In a strain with low PKA activity, the PDEs are unable to prevent cAMP hyper-accumulation, indicating that PKA stimulates PDE activity[14].

Pathway activation

Although the cAMP and MAPK pathways have been studied in detail, expanding our knowledge of the central components of these signalling pathways, we know little of the stimuli or agonists that trigger activation of each pathway. Only two conditions are known to strongly stimulate cAMP accumulation *in vivo* in yeast cells: (1) the addition of glucose to stationary-phase cells growing on a non-fermentable carbon source triggers a rapid transient spike in the cAMP level, and (2) conditions that lead to intracellular acidification, such as the addition of protonophores at low extracellular pH, cause a higher and long-lasting increase in the cAMP level[6]. There is some controversy regarding the role of Ras2p in mediating these effects. There is evidence for and against Ras2p responding to glucose stimulation. For example, Ras2p (C318S)-expressing strains, which can maintain a basal cAMP level, fail to elicit a glucose-stimulated transient increase in cAMP. These strains also exhibit a delay in glucose-stimulated phosphorylation and turnover of the PKA substrate fructose-1,6-bisphosphate. These findings are consistent with the involvement of Ras2p in the glucose-stimulated increase in cAMP in glucose-starved cells[15]. By contrast, there is apparently no increase in the ratio of GTP:GDP

on the Ras proteins following glucose stimulation, suggesting that glucose does not stimulate Ras. However, intracellular acidification does increase the ratio of GTP:GDP on the Ras proteins, indicating a role for the Ras proteins in signalling the change in intracellular pH (Ref. 16). Additionally, in response to intracellular acidification, a strain in which both *cdc25* and *sdc25* are deleted shows the same increase in Ras-bound GTP:GDP and the same level of cAMP as the wild type, indicating that these GEFs are not involved in this signalling process. (This lethal deletion of *cdc25* and *sdc25* can be overcome by overexpression of *tpk1*.) By contrast, there is no increase in the Ras-bound GTP:GDP in response to intracellular acidification in an *ira1Δ/ira2Δ* strain, indicating a role for these GAPs in signalling the change in intracellular pH. Glucose-stimulated cAMP signalling is largely absent in *gpa2Δ* and *gpr1Δ* strains, suggesting that Gpr1p acts as a receptor for glucose[17]. Pde1p has a specific role in downregulating the glucose- and acidification-induced stimulation of cAMP production, whereas Pde2p specifically controls the basal cAMP level in the cell[14]. Activation of Pde1p apparently occurs following phosphorylation of a Ser residue (Ser252) by PKA.

Nitrogen starvation

Clearly, nitrogen starvation triggers pseudohyphal growth and activates the Ras2p and Gpa2p pathways, stimulating cAMP production, and these G proteins are necessary for filamentation. Furthermore, the expression of Gpa2p and Gpr1p is induced by nitrogen starvation. However, a direct link between nitrogen sensing, cAMP and filamentation is yet to be established. The first priority in establishing such a link is to identify a receptor for nitrogen that can activate Ras2p or Gpa2p (or induce intracellular acidification). One such receptor could be the ammonium permease, Mep2p, which detects ammonium and activates the cAMP signalling pathway[18]. A *mep2Δ/mep2Δ* strain is defective in pseudohyphal growth but filamentation can be induced by the addition of exogenous cAMP. This defect can also be suppressed by constitutive activation of the cAMP pathway [e.g. by expressing Ras2p (G19V) or Gpa2p (G132V)] but not the MAPK pathway[19]. Two-hybrid screens have failed to identify a direct interaction between Mep2p and Gpa2p and therefore the mechanism of signal transduction between these proteins remains unknown. Although pseudohyphal growth is induced by general nitrogen starvation, regardless of the nitrogen source, Mep2p only senses ammonium, indicating that there must be other nitrogen receptors. Pseudohyphal development can also be triggered by activation of the high osmolarity glycerol (HOG) pathway[20]. Deletion of *sho1*, which encodes an integral membrane protein osmosensor, causes a defect in pseudohyphal growth. Deletion of *hog1*, which encodes a MAPK on the HOG pathway, causes hyper-pseudohyphal growth. In *Schizosaccharomyces pombe*, the pathway that responds to osmotic stress also responds to nutrient limitation[3]. A testable hypothesis is that Sho1p, or related protein receptors, can respond to nutritional cues to activate Ras2p or Gpa2p in *S. cerevisiae*.

Carbon and nitrogen sensing

The fact that Gpr1p is apparently a carbon sensor that not only activates the cAMP pathway but is also necessary for the stimulation of pseudohyphal growth under conditions of nitrogen starvation is intriguing. One proposal is that Gpr1p serves as a dual sensor of carbon abundance and nitrogen starvation: the expression of Gpr1p is induced by low nitrogen concentrations and the carbon ligand is detected by direct interaction with the receptor, regulating cAMP production[21]. Thus, both nitrogen and carbon levels will affect the response of the signalling pathway. However, the interaction of ligands with Gpr1p has not been demonstrated directly. An alternative hypothesis is that Gpr1p can sense both carbon and nitrogen, activating the cAMP pathway to trigger filamentous growth under conditions of either nitrogen or carbon starvation. The fact that carbon starvation can stimulate pseudohyphal growth in some strains of *S. cerevisiae* is consistent with this hypothesis.

There is a connection between carbon and nitrogen signalling. Diploid cells have two mutually exclusive fates upon nutrient starvation: pseudohyphal growth or sporulation. Nitrogen starvation stimulates pseudohyphal growth, but if the cells are also starved of carbon then meiosis is triggered and the cells sporulate. Both responses can be considered defensive but pseudohyphal growth is a response to the less severe conditions of nitrogen starvation, when the cells are perhaps able to forage for new nitrogen sources by producing filaments, whereas sporulation occurs in response to the potentially lethal conditions posed by both nitrogen and carbon starvation. Recent studies have revealed a role for Gpa2p in switching between these responses by interacting with the protein kinase Ime2p to inhibit sporulation[22]. The transcriptional activator Ime1p induces the expression of Ime2p upon exhaustion of nutrients. Ime2p is responsible for triggering sporulation, but in the presence of glucose it is only expressed at low levels, which might be insufficient to trigger sporulation. Upon nutrient starvation, both Ime2p and Gpa2p expression is induced. However, sporulation is prevented in the presence of nitrogen, because this triggers Gpa2p activation, by binding GTP, and activated Gpa2p can bind to the carboxy-terminal regulatory domain of Ime2p causing inhibition.

Downstream signalling targets

Our knowledge of the downstream targets of the cAMP/PKA and MAPK pathways that result in pseudohyphal growth is also rudimentary. Recently, it has become clear that these pathways converge on the promoter of the *flo11* (*muc1*) gene[23]. Flo11p (Muc1p) is a cell surface flocculin – a class of yeast Ser/Thr-rich glycosylphosphatidylinositol-anchored cell wall proteins that have a role in the calcium-dependent process of cell–cell adhesion known as flocculation[24].

Its deletion indicates it has a critical role in invasive and pseudohyphal growth in response to nitrogen starvation. The *flo11* gene has an unusually large promoter, containing several activation and repression elements. Furthermore, *flo11* expression is also induced by cAMP, apparently via the transcription factor Flo8p, for which there is also a binding site in the *flo11* promoter. The deletion of *ira1*, which leads to a higher level of active Ras2p (i.e. Ras2p with bound GTP) and an elevated cAMP level, induces *flo11* transcription; this mutant strain is hyper-invasive. Strains lacking both Flo8p and Ira1p have reduced levels of Flo11p and are non-invasive, whereas strains lacking Ira1p and Ste12p (a transcription factor from the MAPK signalling pathway) have wild-type levels of Flo11p and are invasive. Strains lacking Tpk2p and Tpk3p show a marked decrease and increase in Flo11p levels, respectively, consistent with the role of Tpk2p and Tpk3p in stimulating and inhibiting pseudohyphal growth, respectively. These findings suggest that pseudohyphal growth can be activated by signal transduction down a Ras2p–Cyr1p–cAMP–Tpk2p–Flo8p–Flo11p pathway[13,23,24]. However, recent studies have revealed that *flo11* transcription is also regulated by the transcriptional activators Mss10p (also known as Phd2p and Msn1p) and Mss11p (which has homology to Flo8p)[25]. The overexpression of *MSS10* and *MSS11* induces invasive growth and their deletion represses invasive growth. The overexpression of *MSS11* alone induces filamentation in both diploid and haploid strains. Epistasis analysis indicates that these transcription factors act in a linear pathway downstream of Mep2p and Ras2p. The position of Mss11p downstream of Mss10p might be due to an interaction between the two proteins. Additionally, there is evidence to suggest independent functions for Ste12p and Tec1p (transcription factors which interact in the MAPK signalling pathway, in Flo11p expression), which might result from an interaction of Ste12p with Mss11p. A two-hybrid screen using *tpk2* as 'bait' identified another transcription factor, Sfl1p, which downregulates *flo11* transcription[12]. Consistent with this role, strains lacking Sfl1p are hyper-invasive and hyper-filamentous. Epistasis and phenotypic analyses indicate that Sfl1p acts downstream of Tpk2p and upstream of Flo11p.

Another PKA target implicated in filamentation is the protein kinase Sok2p, which also regulates the expression of genes involved in cell proliferation and metabolism[1,3]. It has high sequence similarity in the DNA-binding region to another transcription factor, Phd1p, which also regulates pseudohyphal growth but is not activated by PKA (Refs 1,3). A *sok2Δ*/*sok2Δ* strain and a Phd1p-overexpressing strain are hyper-filamentous, whereas a Sok2p-overexpressing strain and a *phd1Δ*/*phd1Δ* strain have a wild-type phenotype, indicating that Sok2p represses and Phd1p stimulates pseudohyphal growth, respectively. The downstream genes regulated by Sok2p and Phd1p that bring about the change in morphology have yet to be found. *S. cerevisiae* strains containing mutations that diminish PKA activity have increased thermotolerance and hyper-accumulate glycogen, whereas increased PKA activity produces the opposite effects. A *sok2Δ*/*sok2Δ* strain has increased thermotolerance and glycogen accumulation. Furthermore, Sok2p represses the expression of Gac1p (the regulatory subunit of the protein phosphatase that regulates the activity of glycogen synthase) and Ssa3p (a heat shock protein). The Ser/Thr kinase Elm1p also acts downstream of PKA to repress filamentous growth[26,27]. *elm1Δ* strains that are also auxotrophic for tryptophan have a phenotype similar to strains with increased PKA activity (e.g. sensitivity to heat shock and low levels of storage carbohydrates), suggesting that Elm1p might process intracellular signals such as tryptophan levels.

Pseudohyphal cells have a unique cell cycle in which the G1 delay before S phase is almost eliminated and the G2 phase is extended. Elements controlling the cell cycle, such as cyclins and the kinases that they regulate, could have a role in controlling pseudohyphal growth. Activation of the Ras2p–cAMP pathway stimulates expression of the G1 cyclins Cln1p and Cln2p, and increases Cln3p protein levels and Cln3p–Cdc28p kinase activity[28]. Moreover, deletion of Cln1p and Cln2p represses filamentation, whereas deletion of Cln3p enhances filamentation[29]. The daughter-cell-specific zinc finger transcription factor Ash1p stimulates pseudohyphal growth[30]. The cAMP pathway might regulate Ash1p because it acts downstream of Ras2p but is independent of the MAPK pathway.

Morphological switching in pathogenic fungi

The virulence of a number of plant and human pathogenic fungi is associated with morphological changes, which are regulated by cAMP. Recent molecular studies have identified components of the cAMP pathway, which appear to play analogous roles to those involved in controlling filamentation in *S. cerevisiae*.

Plant fungal pathogens

Ustilago maydis

One of the best studied examples is the plant pathogen *Ustilago maydis*, the causative agent of corn smut disease. This phytopathogenic fungus can adopt a sporidial, or yeast-like, non-pathogenic form and a pathogenic filamentous form. *In vitro*, haploid cells must mate to form a dikaryon before filamentous growth can occur; *in planta*, haploid cells can form filaments in response to nutrient starvation or acidic pH. This morphological transition is believed to be controlled by cAMP, as a knockout mutation in *uac1*, which encodes adenylate cyclase, induces filamentous growth in haploid cells[3,4]. Furthermore, this filamentous phenotype can revert to a budding phenotype in the presence of exogenous cAMP. This control contrasts with that in *S. cerevisiae*: low cAMP levels stimulate the transition from the sporidial to the filamentous form of *U. maydis*, whereas high cAMP levels stimulate pseudohyphal growth in *S. cerevisiae*.

U. maydis has at least four G-protein-encoding genes (*gpa1–4*)[31]. The sequential disruption of these

genes has identified Gpa3p as a potential upstream component of the filamentous growth pathway. *gpa3Δ* strains have a distorted morphology and are filamentous, resembling *uac1Δ* and *adr1Δ* strains, suggesting that these genes act in the same pathway. Moderate concentrations of cAMP can reverse the defect in cell morphology and suppress filamentous growth; higher cAMP concentrations induce multiple budding, similar to that seen in *ubc1Δ* strains[32]. Epistasis analysis indicates that Gpa3p acts upstream of adenylate cyclase (Uac1p). By analogy, a constitutively filamentous mutant of *Ustilago hordei* has been identified and has been shown to have a defective G protein (encoded by *fil1*)[33]. As in *U. maydis*, the filamentous growth of this *fil1* mutant can be suppressed by the addition of exogenous cAMP.

Filamentation in *U. maydis* is controlled by PKA. The *U. maydis* PKA has two catalytic subunits, encoded by *adr1* and *uka1*, and a regulatory subunit, encoded by *ubc1* (Refs 34,35). The *ubc1* gene was cloned as a suppressor of the filamentous growth of a *uac1Δ* strain. *ubc1Δ* strains cannot form dikaryotic mycelium and have a defect in bud-site selection and cytokinesis, resulting in so-called multiple budding. These results indicate that elevated PKA activity inhibits filamentation. Interestingly, *in planta*, mating compatible *ubc1* haploid strains can form mycelium but do not form tumours. The deletion of *adr1* and *uka1* suggests different roles for these proteins: *uka1Δ* strains have no detectable defects in mating, morphogenesis or pathogenicity, whereas *adr1Δ* strains are filamentous but have reduced pathogenicity.

A second protein kinase, *ukc1*, which belongs to a subfamily of Ser/Thr kinase genes related to, but distinct from, the cAMP-dependent protein kinases, has been identified and has been shown to have a role in morphological switching[36]. In contrast to the *adr1* deletion, disruption of *ukc1* in a diploid strain causes a defect in aerial-filament formation and the strain has reduced virulence, suggesting that Ukc1p regulates the morphological switch that is required for pathogenicity.

Magnaporthe grisea

The plant pathogen *Magnaporthe grisea*, which is the cause of rice blast disease, also undergoes morphological changes that are triggered by activation of a cAMP signalling pathway. Conidia on the hydrophobic surface of the plant develop into a specialized structure, the appressorium, which uses turgor pressure to aid in penetration of the host cell. The recognition of surfaces that are susceptible to infection involves a thigmotrophic response in which the developing germ tube can detect and penetrate breaks in surfaces. This response is mediated in part by hydrophobin proteins; disruption of the hydrophobin-encoding gene *mpg1* results in decreased pathogenicity[3,4]. The addition of cAMP to conidia on non-inducive hydrophillic surfaces stimulates appressorium formation. The *M. grisea* genes encoding adenylate cyclase, (*mac1*; Ref. 37) and the catalytic (*cpkA*; Ref. 38) and regulatory (*sum1*; Ref. 39) subunits of PKA have been cloned. Deletion of *mac1* prevents appressorium formation, a defect that is reverted in *sum1Δ* strains or by the addition of exogenous cAMP. A G-protein-encoding gene, *magB*, has been identified in *M. grisea*, and strains in which this gene is disrupted have a defect in appresorium formation and reduced pathogenicity, which can be reverted by the addition of exogenous cAMP (Ref. 40).

Pathway crosstalk

Whether there is crosstalk between the pathogenicity and mating response pathways, owing to a sharing of protein components, is controversial. There is some evidence to suggest that these pathways might share a G protein. In *U. maydis*, there is evidence to suggest that Gpa3p is also involved in the pheromone signal transduction pathway: *gpa3*Δ mutants are sterile, non-pathogenic, have a morphology similar to a *uac1Δ* strain and they lack pheromone-induced gene expression. In a strain carrying activated Gpa3p (R206L), the expression of pheromone genes is uncoupled from the pheromone, occurring at an elevated level similar to that in pheromone-stimulated cells[35]. However, pheromone gene expression is dependent upon Uac1p (and cAMP), which might act by indirect modulation (via cAMP changes) rather than acting as an upstream gene in the pheromone response pathway. Indeed, elevated cAMP levels do not render the cells mating competent. In addition, Gpa3p has homology to a subfamily of Gpa proteins (including Gpa2p from *S. cerevisiae*), which are involved in cAMP signalling[35]. Epistasis analyses place Gpa3p upstream of Uac1p, where it probably plays a role in transducing the signal from an as-yet-unidentified nutrient-sensing receptor.

In *M. grisea*, the formation of appresorium can be inhibited by α factor, an *S. cerevisiae* pheromone[41]. This inhibition is overcome by disruption of *sum1* and by the addition of exogenous cAMP, suggesting that *S. cerevisiae* α factor interferes with the cAMP signalling pathway in *M. grisea*. This could occur if the α factor acts as an *M. grisea* pheromone, to either inhibit binding of *M. grisea* pheromones to the MAT1-2 receptor, which triggers an increase in cAMP, or by triggering a reduction in cAMP. However, the pheromone genes from *M. grisea* have recently been identified, revealing that both are homologous to α-factor from *S. cerevisiae* and other fungal pheromones that are all lipid modified, arguing against the α-factor acting as an analogue of *M. grisea* pheromones[42].

Human fungal pathogens

Candida albicans

In response to environmental cues such as serum and increasing temperature, *C. albicans* undergoes a morphological transition from a budding form to a filamentous form (e.g. pseudohyphal and hyphal). This transition is important for the development of pathogenicity because a strain in which the transcription factors Cph1p and Efg1p are simultaneously

deleted 'locks' the fungi in the yeast form and this mutant is avirulent[43,44]. Cph1p is a component of a MAPK signalling pathway that clearly controls filamentous growth in *C. albicans*, whereas Efg1p is proposed to be a component of a putative cAMP signalling pathway[43]. Indeed, the fact that exogenous cAMP and PDE inhibitors induce filamentation in *C. albicans* implies that morphogenesis is under the control of a cAMP signalling pathway. The formation of hyphae can occur in response to a small molecule (i.e. MW <1 kDa) present in serum and this response is mediated by Ras1p (Ref. 45). A *ras1Δ* strain is viable but cannot form hyphae in response to serum; whereas a strain expressing active Ras1p (G19V) has enhanced hyphal growth. This behaviour is consistent with Ras1p transmitting a signal to both the MAPK and cAMP signalling pathways. The *C. albicans* genome-sequencing project has identified genes (such as *gpa*, *cyr*, *tpk* and *pde*) that are indicative of a cAMP signalling pathway, but the role of their encoded products remains to be elucidated.

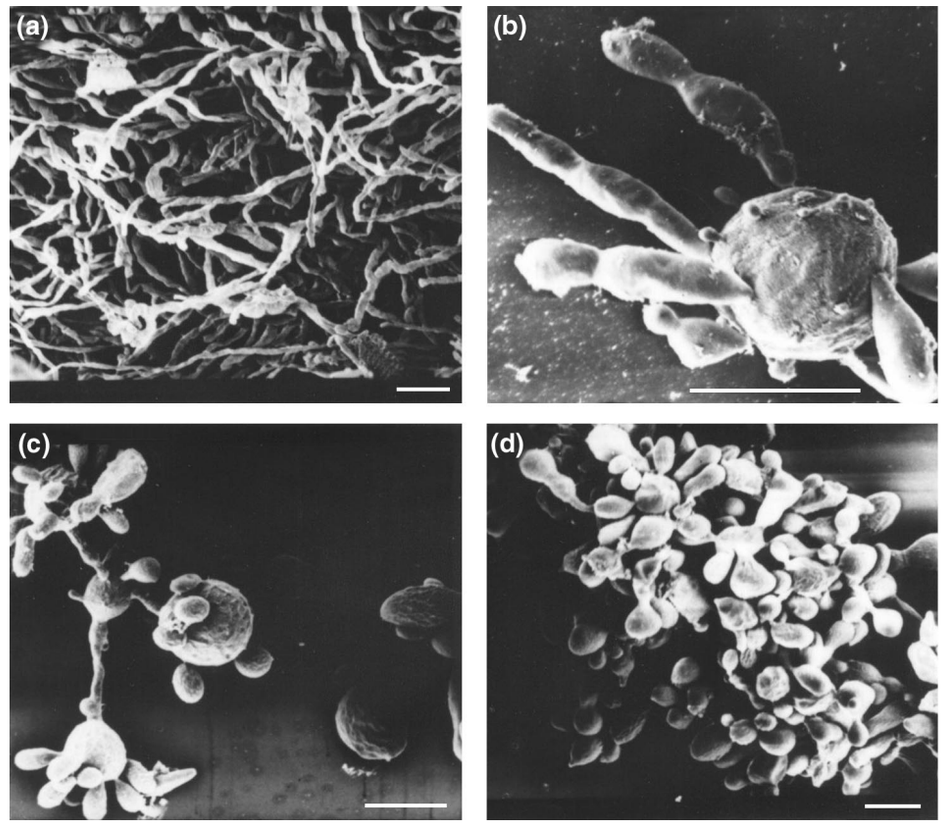

Fig. 2. Electron-micrographs showing the temperature-induced morphological transition of the human pathogenic fungus *Paracoccidioides brasiliensis*. The four panels show **(a)** mycelium at 26°C, **(b)** mycelium grown at 36°C for 11 days, **(c)** mycelium grown at 36°C for 15 days and **(d)** yeast grown at 36°C. Notice the transition from filamentous to budding growth in shifting the temperature from 26°C to 36°C. Scale bars = 10μm. Reprinted, with permission, from Ref. 47.

Paracoccidioides brasiliensis and Histoplasma capsulatum

An increase in cAMP has differential effects upon the human fungal pathogens *Paracoccidioides brasiliensis* and *Histoplasma capsulatum*, supporting the pathogenic yeast form and non-pathogenic mycelial form, respectively[46]. Perhaps *H. capsulatum* responds to decreasing levels of cAMP as does *U. maydis*. It will be of considerable interest to determine whether, and how, these fungi utilize elements of the cAMP and MAPK signalling pathways to control the transition to the pathogenic yeast form.

An interesting feature of *P. brasiliensis* and *H. capsulatum* is that temperature changes are sufficient to induce the morphological transition between the mycelial (26°C) and yeast (37°C) forms, suggesting that the temperature change is detected by one of the signalling genes (Fig. 2). Interestingly, activation of the cAMP pathway (e.g. PKA) causes an increase in the heat-shock sensitivity of *S. cerevisiae* cells. Similarly, the deletion of *pka1*, which encodes the catalytic subunit of PKA, increases the thermotolerance of *S. pombe*. If the activation of the cAMP pathway in *P. brasiliensis* or *H. capsulatum* led to an increase in the heat-shock sensitivity this would be detrimental to cells undergoing the mycelium-to-yeast transition, which occurs over several days. Is this difference in behaviour attributable to differences in the thermotolerances of *S. cerevisiae*/*S. pombe* and *P. brasiliensis*/*H. capsulatum* or have the latter fungi developed strategies for protecting these gene products during the morphological transition? Recent studies have revealed that the activity of the cAMP pathway in *S. cerevisiae* depends upon the amount of Cdc25p, and the cellular content of Cdc25p in *S. cerevisiae* is controlled by the Hsp70p protein Ssa1p. In contrast to most heat shock proteins, which are only transiently expressed, we have shown that a *P. brasiliensis hsp70* gene is induced during the 21-day mycelial-to-yeast transition[47]. An interesting hypothesis is that Pb*hsp70* detects and stabilizes components of the cAMP signalling pathway to elicit the temperature-induced morphological transition. In other words, is activation of the stress response, caused by the increase in temperature that human pathogenic fungi experience upon entry into the host, the detection system used to trigger morphogenesis? The stress response in *S. cerevisiae* provides both inputs and outputs to the cAMP pathway controlling filamentation. For example, activation of the HOG pathway can lead to an increase

Questions for the future

- What are the signals or stimuli sensed by pathogenic fungi and how are these signals detected?
- Have pathogenic fungi adapted pheromone and nutrient signalling pathways to control the morphological changes that underlie pathogenicity?
- Are the pheromone and filamentation pathways integrated in pathogenic fungi and does the pheromone receptor have potential as a target for oligopeptide drugs that disturb the signalling process?
- Do signalling pathways similar to those controlling filamentation in *S. cerevisiae* and *C. albicans* control the formation of pathogenic yeast in *P. brasiliensis* and *H. capsulatum*?
- What are the downstream enzymes that bring about the changes in cellular morphology in pathogenic fungi and how are these enzymes modulated by cell signalling pathways?

in PKA activity, which can repress the stress responsive transcription factors Msn2p and Msn4p (Ref. 48).

Cryptococcus neoformans

Although morphological switching in *Cryptococcus neoformans* does not appear critical to its pathogenicity, the production of virulence factors is under cAMP control[46]. A Gα protein, Gpa1p, has been identified that is required for mating and the induction of two well established virulence factors – melanin synthesis in response to glucose starvation and capsule production in response to iron limitation. Exogenous cAMP suppresses the *gpa1*Δ mutant phenotypes, restoring mating, and capsule and melanin production. Moreover, the virulence of a *gpa1*Δ strain is significantly attenuated in an animal model of cryptococcal meningitis.

Interestingly, the morphogenesis and virulence of *C. neoformans* is linked to the mating type, with the *MAT*α type the most virulent and clinically predominant. *MATα* strains differentiate in response to nitrogen starvation or pheromone secretion by *MATa* cells, forming filaments and spores by haploid fruiting. Most clinical isolates are haploid and diploid strains were thought to be unstable, occurring only transiently. However, stable, self-fertile diploid strains have recently been identified[49]. An interesting feature of these diploid strains is that they are thermally dimorphic, growing as yeast cells in nitrogen-rich medium at 37°C but forming filaments and sporulating at 24°C. Presumably upstream nitrogen- and pheromone-sensing components of the signalling pathway are no longer required for morphological switching in these diploid strains. Have such sensors become obsolete in triggering morphological switching in other thermally dimorphic human pathogenic fungi as a result of the formation of stable diploid strains with two mating loci? In this respect, it is interesting to note that *C. albicans*, an asexual diploid fungus, has recently been reported to have two mating type loci[50].

Conclusions

In summary, the cAMP pathway is used to transduce signals in many fungi that trigger morphological changes. In pathogenic fungi these morphological changes are frequently associated with an increase in virulence. We still know little about how the cAMP pathway is triggered, or the target enzymes that produce the changes in cell morphology. However, it seems likely that pathogenic fungi have adapted both nutrient and stress response systems to trigger morphological changes that allow them to better adapt for life in the host. The identification of unique sensory and effector proteins that regulate morphological changes in pathogenic fungi will aid in the design of novel drug therapies for combating the growing threat posed by fungal pathogens.

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Triggers and targets of cAMP signalling

In their recent article[1], Borges-Walmsley and Walmsley presented a timely review of the role of cAMP signalling in the morphogenesis and pathogenicity of fungi. This area of investigation has been expanding rapidly as more fungal pathogens are analysed and additional phenomena are found to be regulated by cAMP (a recent example is the analysis of conidial differentiation in the powdery mildew fungus *Erysiphe graminis*[2]). In their review, Borges-Walmsley and Walmsley described the connection between cAMP and morphogenesis (pseudohyphal growth) in *Saccharomyces cerevisiae* and used this information as a springboard to discuss the information currently available for pathogens. In general, the information from *S. cerevisiae* has proven to be a valuable guide for examining signalling and other phenomena (such as mating-type regulation[3]) in pathogenic fungi.

Signalling in pathogenic fungi has received considerable attention because of the possible involvement of cAMP in the fungal perception of the host and in the morphological changes associated with infection. In light of this, my goal in this letter is to focus attention on the aspects of these signalling pathways that have been difficult to uncover, even in *S. cerevisiae*. As highlighted by Borges-Walmsley and Walmsley, these are the triggers that activate cAMP signalling and the downstream targets of the pathway.

To date, the emphasis for pathogenic fungi has been on identifying the components of the cAMP pathway and defining their roles in morphogenesis and virulence. The upstream signals that influence the pathway are not well defined, although there are some tantalizing clues about the features of the host that fungi perceive during infection. These include specific host factors (e.g. serum[4]), nutrient starvation (e.g. nitrogen limitation) and physical features (e.g. surfaces and temperature). Interestingly, surface sensing appears to play an important role for both animal[5] and plant pathogens[6,7], suggesting that insight could come from comparing mechanisms between these two groups.

The work to describe the molecular connections that link protein kinase A to changes in gene expression and other downstream functions in pathogenic fungi has only just begun. A useful framework has been established for *S. cerevisiae* with the description of the transcription factors Flo8p and Sfl1p, and the downstream target Flo11p (Ref. 1). However, a comparable view must be obtained for pathogens in order to make connections with the genes that control important aspects of disease such as infection-structure formation[6], virulence factor expression[8] and sporulation[6,9]. Borges-Walmsley and Walmsley also make the case for focusing on stress genes that potentially mediate thermotolerance and temperature-regulated dimorphism in animal pathogens such as *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*.

A ray of hope for speeding up the characterization of signalling in fungal pathogens is provided by the genome sequencing projects that are underway for many of these organisms. These efforts will identify candidate components of signalling pathways, and facilitate the application of functional genomic methods[10] [such as microarrays and serial analysis of gene expression (SAGE)] to uncover signalling targets. This work could provide additional opportunities to define the triggers involved in cAMP signalling, and hopefully lead to new strategies to control fungal diseases.

James W. Kronstad
Biotechnology Laboratory,
University of British Columbia,
Vancouver, BC, Canada V6T 1Z3

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Response from Borges-Walmsley and Walmsley

We concur with Dr Kronstad's comments on the importance of identifying the triggers and targets of the cAMP pathway that controls morphological switching in pathogenic fungi. However, we would also like to highlight two other areas that warrant attention if we are to understand fully the mechanism(s) of morphological switching in pathogenic fungi.

An interesting aspect of cell signalling in pathogenic fungi that is yet to be determined is whether cAMP and mitogen-activated protein kinase (MAPK) pathways similar to those found in *Saccharomyces cerevisiae* can reversibly control the mycelium–yeast transition. In several dimorphic pathogenic fungi (e.g. *Paracoccidioides brasiliensis*), it has been shown that the unicellular or yeast-like form is pathogenic. Increasing and decreasing cAMP levels are used in *S. cerevisiae* and *Ustilago maydis*, respectively, to control morphological switching, suggesting that cAMP levels can be used to favour one form over the other[1]. In the MAPK pathways of both *S. cerevisiae* and *Candida albicans*, there are checkpoints where filamentation can be induced or repressed. In *S. cerevisiae*, the phosphorylation state of the MAPK Kss1p determines whether

filamentation occurs[2,3], and Cek1p probably plays an analogous role in *C. albicans*[4]. Moreover, the phosphatase Cpp1p represses filamentation, possibly by removing phosphate from Cek1p (Ref. 5). Is a phosphatase a key element in stimulating yeast growth (by repressing filamentous growth) in fungi, such as *P. brasiliensis*, that have a pathogenic yeast form? We not only need to identify the components of the signalling pathways but also address their interactions, with a view to understanding how these pathways can be modulated to elicit different effects.

Morphogenesis appears to be regulated by several cell signalling pathways: are these pathways integrated into a network to control different aspects of the morphological changes, implying a need to control subtle changes in cell morphology, or is there a requirement for functional redundancy in the pathways controlling switching? In *S. cerevisiae*, several signalling pathways control morphological switching. The filamentation MAPK and cAMP pathways have been shown to control cell elongation and unipolar budding, respectively, suggesting that different pathways control distinct aspects of the morphological changes[6]. The protein kinase C (PKC) pathway also regulates morphological switching, possibly leading to crosstalk between the PKC and filamentation MAPK pathways. For example, the protein Spa2p appears to play a role as a scaffolding protein that sequesters Ste11p and Ste7p during the filamentation response, but it can also bind the cell polarity proteins Pea2p and Bud6p, and the PKC pathway proteins Mkk1p and Mkk2p (Ref. 7). The PKC pathway probably plays a role in controlling aspects of the morphological change in pathogenic fungi. Morphological switching involves changes in the cell wall composition of many pathogenic fungi: for example, in *P. brasiliensis* there is a change from β-glucan to α-glucan and the small G protein Rho1p, which controls the activity of β-glucan synthase, is a component of the PKC pathway[8]. Pseudohyphal development in *S. cerevisiae* and *C. albicans* can also be triggered by activation of the high osmolarity glycerol (HOG) pathway[9]. Recent work on *Candida* has revealed a further level of control by components (e.g. sensory histidine kinases and response regulators) of 'two-component' cell signalling systems[10,11].

In our opinion, the future goals are not only determining the triggers and targets of the different pathways controlling filamentation in dimorphic pathogenic fungi, but also unravelling the complexity afforded by the integration of several different pathways in controlling morphological switching.

M. Ines Borges-Walmsley and Adrian R. Walmsley
Divn of Infection and Immunity,
Institute of Biomedical and Life Sciences,
University of Glasgow,
Glasgow, UK G12 8QQ

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Response from Kronstad

I agree with Drs Borges-Walmsley and Walmsley that it will be interesting to discover the intricacies of morphological control that arise from the interplay between the cAMP and mitogen-activated protein kinase (MAPK) pathways. Of course, we will need to know a lot more about the details of the pathways in pathogenic fungi before we can begin to appreciate more subtle aspects such as pathway crosstalk and signal attenuation. The point of my letter was to emphasize the idea that we do not know much about upstream signals and downstream targets of the pathways in pathogenic fungi. These aspects of signalling in pathogens are particularly interesting because they are likely to define critical features of the fungal interaction with the host during infection.

James W. Kronstad