Conserved cAMP signaling cascades regulate fungal development and virulence

Cletus A. D’Souza a, Joseph Heitman a,b,c,d,e,*

a Department of Genetics, 322 CARL Bldg, Duke University Medical Center, Research Drive, Durham, NC 27710, USA
b Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA
c Department of Microbiology, Duke University Medical Center, Durham, NC 27710, USA
d Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA
e The Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA

Received 20 December 2000; received in revised form 2 January 2001; accepted 13 February 2001

Abstract

Two well characterized signal transduction cascades regulating fungal development and virulence are the MAP kinase and cAMP signaling cascades. Here we review the current state of knowledge on cAMP signaling cascades in fungi. While the processes regulated by cAMP signaling in fungi are as diverse as the fungi themselves, the components involved in signal transduction are remarkably conserved. Fungal cAMP signaling cascades are also quite versatile, which is apparent from the differential regulation of similar biological processes. In this review we compare and contrast cAMP signaling pathways that regulate development in the budding yeast Saccharomyces cerevisiae, the fission yeast Schizosaccharomyces pombe, and differentiation and virulence in the human pathogen Cryptococcus neoformans and the plant pathogen Ustilago maydis. We also present examples of interaction between the cAMP and MAP kinase signaling cascades in the regulation of fungal development and virulence. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Regulatory cascade; cAMP signaling; Fungal development; Virulence

Contents

1. Introduction .......................................................... 349
2. Pseudohyphal differentiation in S. cerevisiae .................. 351
3. Sexual development in S. pombe ............................... 354
4. Mating and virulence in C. neoformans ......................... 355
5. Dimorphic transition and pathogenicity in U. maydis ....... 358
6. Conclusions and perspectives ...................................... 360

Acknowledgements ...................................................... 360

References ................................................................... 360

1. Introduction

The cellular environment plays an important role in growth and differentiation of fungi. Signal transduction cascades mediate communication between environmental signals and the cellular machinery controlling growth and differentiation. In eukaryotic cells, the secondary messenger cyclic adenosine monophosphate (cAMP) is produced in response to extracellular stimuli such as hormones and regulates a variety of physiological processes. The cellular cAMP level is dependent upon relative activities of the biosynthetic enzyme (adenyl cyclase) and the degradative enzymes (phosphodiesterases). Adenylyl cyclase regulation by heterotrimeric G-proteins is well
known [1]. Heterotrimeric G-proteins are composed of α, β, and γ subunits and are regulated by seven transmembrane receptors of the β adrenergic receptor family. Binding of an inducing ligand to the receptor triggers activation of the G-protein involving GDP-to-GTP exchange of the guanine nucleotide bound to the Gα subunit, followed by release of the Gα subunit from the Gβγ dimer [1,2,3,4]. Effectors such as adenylyl cyclase can then be stimulated by either Gα or Gβγ [5,6].

The best defined target of cAMP in mammalian cells and the budding yeast Saccharomyces cerevisiae is the cAMP-dependent protein kinase (PKA), which mediates most, if not all, physiological effects of cAMP in fungi and other multicellular eukaryotes (reviewed in [7]). When cAMP levels are low, the PKA holoenzyme is an inactive tetramer comprised of two regulatory and two catalytic subunits. Both subunits of PKA are highly conserved among fungi and other eukaryotes. Fig. 1 shows an alignment of conserved PKA catalytic subunits and Fig. 2 shows an alignment of conserved PKA regulatory subunits from a variety of fungi. When cAMP levels increase, cAMP binds to the regulatory subunits and induces a conformational change that causes dissociation of the tetramer into dimeric regulatory subunits and active monomeric catalytic subunits. Liberated catalytic subunits are enzymatically active to phosphorylate target substrates that include metabolic enzymes and transcription factors.

Components of the cAMP signaling cascade (Fig. 3), identified in a variety of fungi, have been found to be well conserved. This review is a compilation of studies on cAMP regulation of pseudohyphal differentiation in S. cerevisiae, mating and sporulation in the fission yeast Schizosaccharomyces pombe, and differentiation and virulence of the pathogenic fungi, Cryptococcus neoformans and Ustilago maydis, that have revealed useful insights on how this cascade functions in fungi.

2. Pseudohyphal differentiation in S. cerevisiae

The central role of the cAMP signaling pathway in the budding yeast S. cerevisiae is nutrient sensing and regulation of diverse biological processes including growth, metabolism, stress resistance, and entry into either meiosis or pseudohyphal differentiation. Pseudohyphal differentiation refers to the morphogenesis of S. cerevisiae diploid cells to a nutrient-scavenging filamentous form when subjected to nitrogen source starvation [8,9]. Both the cAMP and MAP kinase signaling cascades regulate pseudohyphal differentiation in parallel in S. cerevisiae [10–14]. Intracellular cAMP levels in S. cerevisiae are dependent upon the activities of adenylyl cyclase, Cyr1 [15–17], and two cAMP phosphodiesterases, Pde1 and Pde2 [18–20]. Two conditions known to increase intracellular cAMP are intracellular acidification or the addition of a rapidly fermentable sugar such as glucose to stationary phase cells (derepressed) or cells grown on non-fermentable carbon sources ([21,22], reviewed in [21,23]). The term ‘glucose-induced cAMP signaling’ refers to the rapid, transient spike in intracellular cAMP levels occurring upon glucose addition to derepressed cells. There are two models for what transpires following the transient spike in cAMP level. In one model, the cAMP level returns to slightly higher than the pre-stimulus basal level to maintain PKA activity and the fermentative metabolic state [24]. In the second model, a cAMP-independent fermentable-growth medium (FGM)-induced pathway sustains PKA activity during continued growth on glucose following the cAMP spike [21,25,26]. Elevated PKA activity results in induction of glycolytic enzymes and promotes cell proliferation, while sporulation and stationary phase processes such as gluconeogenesis, accumulation of reserve carbohydrates (trehalose and glycogen), and stress resistance are inhibited. cAMP depletion or inactivation of cAMP signaling results in cell cycle arrest in the G1 phase and permanent entry into stationary phase, indicating that the cAMP pathway signals nutrient availability to the cell cycle machinery.

Upstream components of the cAMP pathway identified in yeast include the G-protein-coupled receptor (GPCR) Gpr1 and the Gα subunit Gpa2, which stimulate cAMP production by adenylyl cyclase in response to glucose and other fermentable carbon sources (Fig. 3) [10,22,27–32].Activation of cAMP synthesis is also dependent upon uptake and hexokinase-mediated intracellular phosphorylation of glucose, suggesting that glucose is sensed extracellularly by Gpr1 and intracellularly by hexokinase [33,34]. How hexokinase activity influences cAMP synthesis remains to be established. This dual sensing mechanism may enable the cell to establish that glucose is present in the environment and that the metabolic state of the cell is supporting sufficient glucose transport for growth.

The observations that constitutively active Gpa2 mutants or exogenous cAMP induce pseudohyphal growth at nitrogen concentrations that repress differentiation of wild-type cells, and nitrogen starvation induces expression of GPRI, suggest the intriguing possibility that the Gpr1-Gpa2-regulated pathway is activated not only by carbon source abundance but also regulated by nitrogen starvation [10,31]. It was recently shown that PTC1, a phospholipase C enzyme, may function together with Gpr1 and
<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Gene</th>
<th>Mutant</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
<tr>
<td>S. cerevisiae ΔPsi1</td>
<td>S. cerevisiae</td>
<td>ΔPsi1</td>
<td>1AA abolish</td>
<td>1AA vacant</td>
</tr>
</tbody>
</table>
| S. cerevisiae ΔPsi1 | S. cerevisiae | ΔPsi1 | 1AA abol...
Gpa2 to regulate pseudohyphal growth [35]. Pck1 catalyzes the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2) into the secondary messengers inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). It remains to be determined whether the defect of a pck1 mutant in pseudohyphal growth is due to inactivation of Gpr1-Gpa2-mediated cAMP signaling. In addition to pseudohyphal growth, the Gpr1-Gpa2 pathway also regulates entry of cells into meiosis. In the presence of fermentable carbon sources or nitrogen abundance Gpa2 checks improper entry into meiosis by inhibiting the protein kinase Ime2 required for initiation of meiosis and sporulation [36].

Besides Gpa2, the G-proteins Ras1 and Ras2 also regulate cAMP production by adenylyl cyclase [37]. By stimulating cAMP production, Ras proteins signal environmental changes in nutrient availability and coordinately regulate cell cycle progression [24]. Although neither is essential, ras1 ras2 double mutant strains are inviable and overexpression of the CYR1 adenyl cyclase gene suppresses the lethal phenotype of ras1 ras2 mutant strains [37–40]. Furthermore, expression of a dominant active RAS2val19 allele results in increased cAMP levels [38]. Additionally, a single amino acid substitution in RAS2C318S prevents the cAMP pulse observed in starved wild-type cells following exposure to glucose [24]. Ras signaling in yeast is complex because Ras2 regulates filamentous growth via both the MAP kinase and cAMP signaling pathways [10,14,41].

The target of cAMP in S. cerevisiae is PKA, which consists of a regulatory subunit (Bcy1) and three catalytic subunit isoforms (Tpk1, Tpk2, and Tpk3) [42,43]. Of the three isoforms, Tpk2 positively regulates pseudohyphal growth [39]. Fig. 3. cAMP signaling pathways are highly conserved among fungi. cAMP signaling pathways in S. cerevisiae, S. pombe, C. neoformans, and U. maydis are schematically compared and contrasted. Two related receptors, Gpr1 and git3, play a conserved role in glucose sensing in S. cerevisiae and S. pombe; homologous receptors in C. neoformans and U. maydis remain to be identified. The receptors are coupled to a highly conserved G protein (Gpa2, gpa2, Gpa1, and Gpa3). The G protein is coupled to adenylyl cyclase, and cAMP regulates PKA. Targets of PKA largely remain to be identified but will likely include a variety of transcription factors. Note that cAMP functions to activate or inhibit development depending on the organism.
growth, whereas Tpk1 and Tpk3 play negative roles [11,44]. The distinct functions of the three PKA isoforms are attributable to subtle differences in the conserved PKA catalytic domains suggesting that these enzymes have different targets in S. cerevisiae [11]. Unlike the situation in many fungi, PKA is required for vegetative growth of S. cerevisiae and the three PKA isoforms are largely redundant for viability [43]. In contrast, constitutive activation of the PKA pathway in bcy1 mutants lacking the PKA regulatory subunit, or in cells expressing activated Ras2, prevent cells from entering stationary phase, accumulating glycogen, or acquiring heat-shock resistance, even death, in response to conditions (N-starvation) that promote entry into stationary phase [43,45].

For a long time following the discovery of PKA the only known targets for PKA were enzymes involved in intermediary metabolism, particularly carbohydrate metabolism. Recently identified targets of PKA include the S. cerevisiae transcription factors Msn2 and Msn4, which are key regulators of stress-responsive gene expression [46–49]. The Rim15 protein kinase is another recently identified PKA target [50] which, in addition to regulating meiotic gene expression [51], is also required for stationary phase-induced gene expression. Interestingly, mutants lacking Rim15, Msn2, Msn4, or both Msn2 and Msn4, have no defect in pseudohyphal growth [11]. Tpk2 regulation of pseudohyphal growth is not dependent on Msn2 and Msn4, or the transcription factors Ste12 and Tec1 required for MAPK cascade regulation, but is dependent on the activation of the transcriptional regulator Flo8, which in turn regulates expression of the cell surface flocculin Flo11 that is involved in cell-cell adhesion [11,44,52–54]. The transcription factor Sil1, proposed to inhibit pseudohyphal growth, is yet another PKA target as demonstrated by two-hybrid interactions with Tpk2. Moreover, sfl1 mutations suppress defects in pseudohyphal growth and Flo11 expression of tpk2 mutant strains [44]. Finally, cAMP synthesis itself is a target of the PKA pathway and, following activation, PKA participates in a negative feedback loop that inhibits cAMP synthesis [18,22,55]. The phosphodiesterase Pde1 is a good candidate for a PKA target in this negative feedback loop because it has a single PKA consensus phosphorylation site, phosphorylation in crude extracts leads to modest increases in enzyme activity, and pde1 mutations dramatically enhance cAMP production [20]. Recently, the regulator of G-protein signaling (RGS) homolog Rgs2 was identified as a novel moderator of cAMP synthesis and Rgs2 stimulates the GTPase activity of Gpa2 [56]. Rgs2 mutations enhance glucose-induced cAMP production, whereas overexpression of RGS2 attenuates this response.

In summary, nutrient sensing by the cAMP signaling pathway plays a key role in regulation of growth and differentiation in S. cerevisiae and serves as a paradigm for cAMP-mediated signal transduction in other fungi (Fig. 3). The cAMP and MAP kinase pathways coordinately regulate pseudohyphal differentiation in S. cerevisiae and crosstalk occurs at several levels. cAMP synthesis itself is tightly regulated by a negative feedback loop that limits cAMP fluctuations in S. cerevisiae. While much is known about glucose sensing by the cAMP pathway, it still remains to be established how glucose phosphorylation regulates cAMP synthesis and how nitrogen levels are sensed. Interestingly, while any one of the three PKA isoforms suffices for vegetative growth, they have distinct and opposing roles in pseudohyphal differentiation. Further insights into the unique roles of PKA isoforms may be provided by ongoing studies on nutritional regulation of cellular localization of PKA subunits [57]. In rapidly growing cells in glucose medium the Bcy1–Tpk1 complex is predominantly nuclear, whereas Tpk1 is exported to the cytoplasm in response to cAMP while Bcy1 remains nuclear. In contrast, both Bcy1 and Tpk1 are distributed in both the nucleus and cytoplasm in stationary phase cells or cells grown on a non-fermentable carbon source. Besides carbon source availability, cytoplasmic localization of Bcy1 is dependent on phosphorylation by the Yak1 kinase and a putative cytoplasmic retention factor Zds1 [58]. YAK1 expression is dependent on the transcription factors Msn2/Msn4 that are in turn negatively regulated by PKA. Subcellular targeting of PKA therefore provides an additional level of regulation of PKA-mediated signaling.

3. Sexual development in S. pombe

The fission yeast S. pombe employs cAMP signaling and PKA function to regulate sexual development involving mating, followed by meiosis and sporulation (Fig. 3). Exposure to nitrogen starvation results in decreased intracellular cAMP levels and triggers sexual development (reviewed in [59,60]). Glucose depletion augments sexual development induced by nitrogen starvation by a similar cAMP-dependent mechanism [61]. In addition to regulating sexual development, glucose monitoring also controls other PKA-inhibited processes, including gluconeogenesis, uptake of alternative carbon sources such as gluconate, and entry into stationary phase. Recently, a definitive role for the cAMP signaling pathway in glucose-induced germination of S. pombe spores was demonstrated [62]. Components of the cAMP signaling cascade were identified by employing heterologous gene probes [61,63], screening for genes conferring sterility when overexpressed [64], or by isolating git mutations (glucose insensitive transcription) that render transcription of fhp1, the gene encoding the gluconeogenic enzyme fructose-1,6-bisphosphatase, insensitive to glucose repression [65]. The cAMP cascade in S. pombe is remarkably similar to the pathway that regulates pseudohyphal differentiation in S. cerevisiae. Mutations in the ‘upstream’ genes git1, git3, git5, git7, git8 (gpa2), and git10 were suppressed by multicopy git2
(adenyllyl cyclase) indicating that these genes are required for glucose-triggered adenyllyl cyclase activation [66,67]. The G-protein α subunit gpa2/git8 of the heterotrimeric G-protein has been shown to be specifically involved in the cAMP signaling pathway in S. pombe [61,68,69]. A gpa2 null mutant mates and sporulates without nitrogen-deprivation and is unable to elevate intracellular cAMP levels in response to glucose stimulation, consistent with a defect in activation of adenyllyl cyclase [63,66,70,71]. git5 has been identified as the Gβ subunit involved in adenyllyl cyclase activation [72]. Contrary to another report [73], the Gβ subunit git5 was conclusively demonstrated not to regulate the gpa1 Gα subunit involved in pheromone sensing. The same heterotrimeric G-protein has been considered responsible for signaling both nitrogen and glucose availability, but the precise mechanism for this is still unclear [61,68].

Recently, it was reported that git3 encodes a GPCR that is distantly related to Gpr1 in S. cerevisiae [69]. Loss of git3 function results in phenotypes similar to those conferred by null mutations in other components of the cAMP signaling pathway, including constitutive fhp1 transcription, starvation-independent sexual development, repression of glucanase uptake, and delay of spore germination [61,64,69,71,72]. Consistent with a role for git3 as a glucose monitoring receptor, multicopy git3 does not suppress mutations in other upstream git genes. Additionally, git3A and pka1Δ mutations do not have an additive effect on delay in spore germination, consistent with the git3 receptor and PKA functioning in the same pathway. It has been proposed that the primary role of the git3 receptor is to activate the G-protein α subunit gpa2/git8 in response to glucose [61,69]. While this interpretation is consistent with complete suppression of a git3Δ by a constitutively activated gpa2H allele, nevertheless it is complicated by the result that git3Δ and gpa2A mutations have an additive effect on fhp1 expression. To account for this, Welton and Hoffman propose that a gpa2-independent activity of git3 plays a minor role in glucose-induced cAMP signaling [69]. The constitutively activated gpa2H allele also fully suppresses the Gβ requirement in glucose sensing suggesting that the primary role of Gβ is to couple gpa2 Gα to the git3 receptor. Other signaling components identified include cgs2/pde1, a cAMP phosphodiesterase, the git6/pka1 catalytic subunit of PKA [64,72,74] and cgs1, the regulatory subunit of PKA [75]. In addition to the fhp1 gene, the regulator of mating-specific genes, ste11, is also negatively regulated by PKA [76].

While the cAMP signaling pathway in S. pombe closely resembles that in S. cerevisiae, several differences are noteworthy. First, the S. pombe ras1 protein does not play a prominent role in adenyllyl cyclase activation, but rather functions in the pheromone sensing MAP kinase cascade [77]. Second, loss of either adenyllyl cyclase or PKA function is not lethal to S. pombe cells, although they do grow slower than wild-type cells, indicating that PKA does not primarily regulate cell viability [64,71]. Third, the S. cerevisiae Gpr1 receptor does not associate with a classical heterotrimeric G-protein, whereas the S. pombe git3 receptor does, given the involvement of the gpa2 Gα and the git5 Gβ subunits in glucose-induced activation of cAMP production [69]. Structural differences between the GPCR and Gα proteins in these yeasts may account for the differential requirement of a canonical Gβ subunit for glucose detection.

Glucose and nitrogen sensing not only influence the cAMP signaling pathway, but starvation for these nutrients, like other environmental stresses, activates a highly conserved MAP kinase cascade. The downstream target of the MAP kinase cascade, a bZIP transcriptional regulator, is required to induce fhp1 transcription [78–81]. The MAP kinase cascade also regulates processes that are negatively regulated by PKA, including sexual development, glucanase uptake, and heat-shock tolerance. Analysis of the fhp1 promoter whose transcription is coordinately regulated by the MAP kinase and cAMP signaling pathways revealed antagonistic interactions of these pathways at each of the two identified cis-acting elements, involving different mechanisms at each site [82]. fhp1 regulation by parallel signaling pathways is analogous to the regulation of FLO11 transcription by similar pathways in S. cerevisiae [11,12,83].

To summarize, S. pombe employs both the cAMP signaling and MAP kinase cascades to coordinately regulate the processes of sexual development, gluconeogenesis, and stress tolerance. When glucose is plentiful, cAMP levels and PKA activity increase, inhibiting these processes. On the other hand, when starved for glucose (or nitrogen), reduced cAMP signaling and PKA activity induce these processes. The opposing effects of cAMP on development in budding and fission yeasts clearly demonstrate the versatility of the PKA pathway. Interesting areas for future investigations include defining how the cAMP signaling pathway mediates both glucose and nitrogen sensing in S. pombe and how it regulates cell cycle progression.

4. Mating and virulence in C. neoformans

C. neoformans is an encapsulated, heterothallic, basidiomycetous yeast that is one of the most common fungal pathogens in AIDS and other immunocompromised patients (reviewed in [84]). Virulence factors that have been identified for C. neoformans include an antiphagocytic polysaccharide capsule [85–87], melanin produced in the cell wall as a probable antioxidant [88–90], production of the enzyme urease [91], the ability to grow at 37°C [86,92], and the MATα mating type locus [93]. C. neoformans exists in two mating types, MATα and MATα, and interestingly mating type is linked to virulence. When congenic strains were compared in a murine tail vein injection mod-
of systemic cryptococcal infection, MATα strains were more virulent than MATα strains [93].

Virulence, mating and filamentation in C. neoformans are regulated by environmental conditions, and specific signals that induce these processes have been identified. Melanin production can be induced by glucose limitation and requires the presence of diphenolic substrates in the growth medium (Fig. 4A) [94-97]. Abundance of the diphenolic compound dopamine and other neurotransmitters in the CNS may explain the tissue tropism of cryptococcal infections. Melanin has been recently shown to be produced in vivo in infected animals and human brain
tissue [98–101]. Formation of a polysaccharide capsule is induced by severe iron limitation (Fig. 4B) or CO₂/HCO₃⁻ exposure, both conditions found in the infected host. [85,102]. The capsule has been previously shown to protect fungal cells from phagocytosis and is now known to also enhance survival of cryptococci within macrophages [103]. Additionally, capsular antigens are shed into the circulation and may have deleterious effects on the host immune system by inducing suppressor T-cell networks [104,105], activating complement [106,107] by suppressing macrophage cytokine production [108], and inhibiting leukocyte migration into sites of infection [109]. Melanin and capsule are important for virulence, and mutants lacking either of these are either attenuated or avirulent in animal models [86,88,90,110]. In addition, virtually all clinical isolates produce both melanin and capsule [84].

Compatible C. neoformans cells mate in response to both peptide pheromones and conditions of nitrogen starvation, resulting in the production of a dikaryotic mycelium (Fig. 4C), followed by karyogamy, meiosis, and sporulation ([111,112]; Shen, W.C., Davidson, R.C., and Heitman, J., in preparation). In the absence of a mating partner and under conditions of nitrogen starvation and desiccation, some strains of the MATα mating type can undergo a process called haploid fruiting involving the formation of hyphal filaments and sporulation [113,114]. Mating and haploid fruiting both produce small spores that could serve as infectious particles.

Both the MAP kinase and cAMP signaling cascades regulate mating and pathogenicity of C. neoformans ([115,116], reviewed in [117,118]). The C. neoformans Gα subunit Gpa1 was found to be homologous in sequence to the S. cerevisiae Gα protein Gap2 involved in nutrient sensing during pseudohyphal differentiation [10,28,119]. Gpa1 plays a key role in cAMP signaling and virulence [115]. gpa1 mutant strains are unable to mate, fail to induce melanin and capsule (Fig. 4), and are considerably attenuated for virulence in both rabbit and mouse models of cryptococcal meningitis [115,116]. Melanin and capsule production in gpa1 mutant cells was restored by exogenous cAMP, suggesting that Gpa1 regulates cAMP production [115].

There is evidence to indicate that, unlike in S. cerevisiae, C. neoformans Ras1 does not primarily regulate cAMP production [120]. A rasi mutant strain exhibited no defects in melanin or capsule production, two inducible cAMP-dependent phenotypes [115]. Furthermore, the mating defect of a rasi mutant strain was only partially rescued by cAMP in contrast to full suppression of gpa1 by cAMP. Nevertheless, it appears that Ras1 may interact with the cellular machinery involved in cAMP production as suggested by the observation that the agar adherence defect of a rasi mutant strain, tested by ability of cells in a colony to withstand gentle washing, can be suppressed by exogenous cAMP. The primary role of Ras1 instead appears to be regulation of the MAP kinase pathway in C. neoformans, analogous to the role of Ras homologs in S. cerevisiae and S. pombe [14,41,77,121]. The mating defect of the rasi mutant was fully suppressed by overexpression of GPBI, the gene encoding a MAP kinase-specific G-protein β subunit, and conversely, introduction of the dominant active RAS1Q67L allele into a gbp1 mutant strain did not restore mating competence, suggesting that Ras1 functions upstream of Gbp1 in the mating signaling pathway. The C. neoformans Ras1 protein is required for growth at 37°C and therefore virulence of this pathogen [120]. The temperature-sensitive growth defect of the rasi mutant strain was not restored by either exogenous cAMP or overexpression of the GPBI [114], suggesting the presence of another, as yet unidentified, Ras-specific signaling cascade in C. neoformans. A second RAS gene, RAS2, was identified in the serotype A strain H99 (A. Alspaugh, unpublished results). Unlike rasi mutants, ras2 mutants had no defects in mating, filamentation, growth at 37°C, or virulence. However, null mutations in both RASI and RAS2 genes resulted in dramatically reduced growth rate similar to the observation of growth arrest in S. cerevisiae when both RASI and RAS2 genes are inactivated [38,39].

Unlike in S. cerevisiae, the C. neoformans PKA Pka1 is not essential for viability. However, PKA is required for both melanin and capsule production, and mating of C. neoformans [116]. Unlike gpa1 mutant strains, exogenous cAMP does not restore mating, melanin or capsule production in pka1 mutant strains, consistent with the model that Gpa1 regulates CAMP production, whereas Pka1 is the target of cAMP activation. pka1 mutant strains were also severely attenuated for virulence in two different animal models. To further analyze PKA function, the gene encoding the PKA regulatory subunit (PKRI) was identified and disrupted, both in wild-type and gpa1 mutant strains [116]. Consistent with the model that Pkr1 functions downstream of Gpa1, pkr1 mutations suppressed defects conferred by gpa1 in melanin production, capsule formation, and mating. This result also confirms that PKA mediates all of the known responses controlled by the Gpa1-regulated cAMP pathway. pkr1 mutants mate like wild-type but differ in their ability to produce capsule and melanin. pkr1 mutant cells produced larger capsules than the corresponding wild-type cells when grown in low-iron medium. Interestingly, the pkr1 gpa1 double mutant had an even larger capsule size and increased cell volume compared to the wild-type and gpa1 mutant strains. Increased capsule size in pkr1 gpa1 mutants is attributable to the elevated level of PKA activity, because high levels of exogenous cAMP are also known to enhance capsule production [115]. That pkr1 gpa1 mutant cells produced larger capsules than pkr1 single mutant cells suggests that Gpa1 may have targets in addition to the PKA pathway which inhibit capsule production. This is analogous to a similar role for the related S. cerevisiae Gα protein Gap2, which inhibits the Ime2 kinase to prevent entry into meiosis, and also positively regulates cAMP production and
pseudohyphal growth [10,28,36]. In a whole cell laccase assay, relative to the wild-type strain, pkr1 and pkr1 gpa1 double mutant strains exhibited lower levels of laccase activity as is the case when strains are exposed to high levels of exogenous cAMP, or when PKA1 is overexpressed [116]. This observation could result from an inhibitory function of elevated PKA activity in these strains.

pkr1 mutant strains were found to be hypervirulent by several measures in both rabbit and mouse models [116]. pkr1-infected mice exhibited decreased survival rates and significantly elevated fungal cell burden in the CNS relative to mice infected with wild-type. Additionally, pkr1 mutant strains were found to elaborate significantly larger capsules in the CNS of infected mice. Therefore, both larger capsule size and increased release of immunosuppressive capsular polysaccharides provide a plausible molecular mechanism for hypervirulence of pkr1 mutant strains. Such hypervirulent strains may be the cause of fungal infection in patients with no apparent defects in immune system [122–124]. Virulence was partially restored to the wild-type level in the pkr1 gpa1 double mutant strain, indicating that the virulence defect of the gpa1 mutant strain can be suppressed by constitutive activation of PKA. These findings further support the proposal that Gpa1 regulates virulence via the cAMP signaling cascade.

There is evidence indicating that crosstalk between the cAMP and MAP kinase signaling cascades may also occur in C. neoforms. One level at which this occurs may involve the G-protein Ras1. As mentioned earlier, C. neoforms Ras1 is responsible for activation of the pheromone-inducible MAP kinase cascade and also plays a secondary role in cAMP production. The transcription factor Ste12α is required for haploid fruiting, marginally functions in mating, and is required for melanin and capsule production [125–127]. Besides bearing consensus PKA phosphorylation sites, overproduction of Ste12α rescued the mating defect of a pkal mutant strain [116]. However, Ste12α overproduction did not restore melanin or capsule production in a pkal mutant strain, suggesting that Ste12α may represent one of several downstream targets of Pka1.

Overexpression of STE12α induced haploid fruiting in both wild-type and pkal mutant strains cultured on filamentation agar (FA), indicating that PKA1 is not required for haploid fruiting when STE12α is overexpressed. However, since pkal results in considerably reduced haploid fruiting in strains bearing the dominant active RAS1 allele, Pka1 appears to play secondary role in haploid fruiting ([120]; D’Souza, C, unpublished results). Additionally, when cultured in filamentation liquid medium pronounced filamentation was observed in wild-type strains overexpressing STE12α, whereas scarce filamentation was observed in pkal mutant strains overexpressing STE12α, indicating that hyphal formation under these conditions is in part dependent upon Pka1 [116]. Even after prolonged growth in liquid culture, the filaments lacked basidia or basidiospores, suggesting that other signals contribute to the induction of basidia and basidiospore production.

In summary, C. neoforms employs a highly conserved cAMP signaling pathway to regulate mating and virulence (Fig. 3). It remains to be determined how signals that induce melanin and capsule production and mating are integrated by Gpa1 into the cAMP signaling pathway. Unidentified components include the upstream GPCR and targets of PKA. The only Gβ subunit identified in C. neoforms is not coupled to Gpa1 function [114], suggesting that Gpa1 functions as a novel type of solo Gα subunit, or that unusual Gβγ partners or regulatory factors substitute for a canonical Gβγ subunit. Expected targets of PKA include transcription factors and metabolic enzymes. Ste12α is one of several candidate targets of PKA in the regulation of mating but not virulence in serotype A strains of C. neoforms. Rapid advances in the C. neoforms genome and cDNA sequencing projects should provide alternative resources to identify additional components of the cAMP cascade in C. neoforms. The conserved role of Ras proteins in regulating fungal growth and the involvement of cAMP cascade components in virulence presents these proteins as attractive targets for antifungal therapy.

5. Dimorphic transition and pathogenicity in U. maydis

The heterothallic, basidiomycete U. maydis is a maize pathogen that employs cAMP signaling to regulate dimorphic transitions during its life cycle (Fig. 3) [128–130]. Asexual haploid cells called sporidia have a yeast-like morphology and proliferate by budding. Mating between compatible partners results in cell fusion followed by a transition from the yeast form to an infectious dikaryotic mycelium. Hyphal proliferation within the plant tissue induces gall formation, followed by production of masses of diploid teliospores that germinate to initiate a new cycle. Establishment of the infectious dikaryon is regulated by two unlinked mating-type loci: a and b. The a locus encodes pheromones and pheromone receptors and controls cell–cell recognition and fusion [131]. The b locus encodes two homeodomain proteins, bE and bW, which dimerize when they are derived from different alleles to form a transcriptional regulator required for the production of stable infectious dikaryons [132–134].

Besides mating, additional factors influence cellular morphology of Ustilago species. In the presence of an abundant supply of glucose Ustilago cells exist in the sporidial form, whereas nutrient limitation, air exposure, or growth at acidic pH promotes filamentous growth in haploid cells [135–137]. Given the influence of glucose sensing on differentiation in budding and fission yeasts, it is tempting to speculate that a similar cAMP signaling mechanism may mediate glucose-regulated morphogenesis in U. maydis. Involvement of cAMP signaling in regulation of mor-
phogenesis and virulence in *U. maydis* was indicated by the constitutive filamentous growth and avirulence phenotypes of an *uac1* (*Ustilago* adenylyl cyclase) mutant [136,138]. Exogenous cAMP restores budding growth in *uac1* mutants and causes clustering of cells and multiple lateral budding even in wild-type strains, implicating cAMP regulation of both sexual development and cell cycle progression [136]. This is in contrast to *S. cerevisiae* cells in which high cAMP levels promote pseudohyphal growth. *uac1* mutants cannot activate expression of pheromone-inducible genes indicating crosstalk between the cAMP and MAP kinase signaling pathways [139].

Another indication of crosstalk between the cAMP and MAP kinase signaling pathways was the identification of several components of the MAP kinase cascade as suppressors of *uac1* [136,140–142]. This genetic screen also led to the identification of *ubc1* (*Ustilago* bypass of cyclase), which encodes the regulatory subunit of PKA [136]. *ubc1* mutants exhibit a multiple budding growth pattern similar to cells treated with high concentrations of cAMP. Additionally, mating compatible *ubc1* mutants are unable to form the dikaryotic mycelium both in vitro and in planta, and are compromised for virulence. Although colonization of the plant tissue and initial disease symptoms develop, tumor development is not initiated indicating that increased PKA activity promotes initial plant infection, whereas reduced PKA activity induces tumors [143]. Homozygous *ubc1Δ* diploids are also attenuated for virulence indicating that PKA regulates post-fusion events [143,144]. Expression of pheromone-inducible genes in *ubc1Δ* mutants is increased to levels similar to that in cells treated with exogenous cAMP, or expressing a constitutively activated adenylyl cyclase [139].

Two genes, *adr1* (aromatic dicarboximide resistance) and *uka1* (*Ustilago* kinase A), that encode isoforms of the PKA catalytic subunit have been identified [144]. Disruption of *adr1* causes constitutive filamentation in haploid cells similar to that of adenylyl cyclase mutants, and impairment of virulence in planta either in crosses or as diploid strains. No obvious phenotype was observed for the *uka1* mutant, consistent with the assignment of Adr1 as the major PKA activity in *U. maydis* cells. *adr1 uka1* double mutants were viable, suggesting that PKA activity is dispensable for growth of *U. maydis*, as is the case with many fungi with the exception of *S. cerevisiae*. *adr1 uka1* double mutants were less filamentous than *adr1* single mutants, revealing a minor role for Uka1 in filamentation. In contrast to inactivation of the cAMP cascade, moderate activation of the cAMP pathway by the constitutively active *gpa3*<sup>Q206L</sup> subunit allele or a partial loss-of-function mutation in the regulatory subunit of PKA still induces tumors, but fungal proliferation and development in these tumors is arrested [145]. Thus, precise regulation of the cAMP pathway is necessary for fungal proliferation and development but not for tumor induction in planta. Interestingly, activation of the cAMP pathway in *U. maydis* results in coating of cells with an unidentified fibrillar material much like the capsule of *C. neoformans*. However, while the *Cryptoccus* capsule is regarded as a virulence factor, it remains to be established that aberrant tumor formation caused by *gpa3*<sup>Q206L</sup> strains is linked to formation of this capsule-like material.

Evidence originally suggested that the Gpa3 Gα subunit might be directly involved in pheromone sensing in *U. maydis* [146]. This was based on the observations that *gpa3* mutants are sterile and fail to induce expression of *mfa1* and the *b* genes in response to pheromone, whereas a constitutively active *gpa3*<sup>Q206L</sup> mutant allele caused overexpression of these genes. However, the evidence for specific involvement of Gpa3 in cAMP signaling is now unambiguous [139]. First, *gpa3* mutants are avirulent and filamentous like *uac1* mutants, and exogenous cAMP restores budding growth. Second, exogenous cAMP also restores mating and pheromone gene expression in *gpa3* mutants to similar levels obtained upon pheromone stimulation. Third, the *uac1* adenylyl cyclase mutation diminishes *mfa1* gene expression in a *gpa3*<sup>Q206L</sup> mutant, indicating that Gpa3 functions upstream of adenylyl cyclase. Addition of higher amounts of cAMP results in decreased pheromone gene expression indicating that the level of PKA activity may normally be tightly regulated. Gpa3 shares a high level of sequence identity with the nutrient sensing Gα proteins of budding and fission yeasts, and *C. neoformans* (Gpa2, gpa2, Gpa1), suggesting that the *U. maydis* Gpa3 Gα protein will be coupled to a homolog of the *S. cerevisiae* Gpr1 and *S. pombe* git3 glucose receptors.

As with *gpa3*, *uac1*, and *adr1* mutants of *U. maydis*, a *fill1* mutant of its close relative *Ustilago hordei*, is also constitutively filamentous and is restored to budding growth by exogenous cAMP [147,148]. Fill1 shares a high degree of sequence identity to the Gα subunit Gp1 from the basidiomycete *C. neoformans*, required for virulence of this human pathogen [115,119]. Like cAMP, overexpression of *FILL1* also suppressed the filamentous growth of starved haploid wild-type strains, suggesting that Fill functions to activate adenylyl cyclase [148]. Interestingly, like *C. neoformans* Gpa1, Fill regulates production of a melanin-like pigment in teliospores produced on susceptible barley cultivars. cAMP and overexpression of *FILL1* transiently repressed pigment formation, whereas the cAMP phosphodiesterase inhibitor isobutyryl-3-methyl xanthine (IBMX) completely repressed pigment formation [149]. Consistent with this, pigment formation was not observed in cells expressing the constitutively active mutant allele *fill1*<sup>Q206R</sup>.

Coordinate regulation of mating and pathogenicity in *U. maydis* by the cAMP and MAP kinase signaling pathways is mediated by Prf1, an HMG-box domain transcriptional regulator and a common target of both pathways [150]. Prf1 is necessary for mating-type gene expression and pathogenicity of this fungus [151]. Analysis of the
prf1 promoter defined a cis-acting element which mediates both transcriptional activation by carbon sources, such as glucose and fructose, as well as repression by high levels of cAMP [151]. It has been proposed that divergent cAMP pathways regulate Prf1 activity and pathogenicity as observed in the rice blast fungus Magnaporthe grisea [152]. The positive effect of cAMP may be mediated by the Adr1 kinase, while transcriptional repression could be mediated by either another PKA catalytic subunit or by a PKA-independent mechanism.

In summary, the cAMP cascade regulates mating-type gene expression and pathogenicity of U. maydis (Fig. 3). Several lines of evidence indicate that signaling by the cAMP and MAP kinase pathways is coordinated, similar to the situation in S. cerevisiae, S. pombe, and C. neoformans. However, while the cAMP cascade positively regulates pseudohyphal differentiation in S. cerevisiae, it negatively influences filamentous growth in U. maydis. Crosstalk between the cAMP and MAP kinase pathways in U. maydis has been shown to be mediated by the transcriptional regulator Prf1, and divergent cAMP pathways have been proposed to regulate Prf1 activity and pathogenicity. It remains to be investigated how environmental signals regulate dimorphism of U. maydis in the absence of pheromone stimulation. Additionally, it would be interesting to identify other levels at which the cAMP pathway crosstalk with the MAP kinase pathway, and additional targets of PKA in this organism. The possible involvement of divergent cAMP pathways also opens new avenues for future studies on cAMP signaling in U. maydis. Here we have highlighted interesting parallels between the two basidiomycetes, U. maydis and C. neoformans, with respect to regulation of virulence by the cAMP cascade in these fungi. Further studies to identify new components and targets of this cascade can take advantage of similarities in cAMP signaling cascades between these fungi.

6. Conclusions and perspectives

It is apparent from the above discussion that cAMP signaling cascades are remarkably conserved in divergent fungi. Differential control of related biological processes in fungi and other eukaryotes is achieved by alterations in the mechanisms by which PKA targets are regulated. Thus, PKA either activates or inhibits transcriptional activators or repressors to achieve specificity. The wealth of information derived from studies of the cAMP signaling pathway in model yeasts like S. cerevisiae and fungi described here can now serve as reference material for excursions into, as yet uninvestigated, cAMP signaling pathways in other fungi, or even to revise current models for regulation by cAMP signaling. Superimposed on the framework of this cascade, depending on the organism, are interactions with the MAP kinase cascade. Given the complexity of signal transduction networks in cells, it will not be surprising to find similar interactions with other existing signal transduction pathways. With significant technological innovations in the areas of functional genomics and bioinformatics, it is only a matter of time before these signal transduction pathways will reveal their secrets.

Acknowledgements

We thank Carlos Gancedo for the invitation to prepare this review and Tina Jeffries for assistance with preparation of this manuscript. Our work is supported by NIAID R01 Grants AI39115 and AI42159 (to J.H.) and P01 award AI44975 from NIAID to the Duke University Mycology Research Unit. J.H. is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology and an associate investigator of the Howard Hughes Medical Institute.

References


