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The cAMP/Protein Kinase A Pathway and Virulence in *Cryptococcus neoformans*

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The basidiomycete fungus *Cryptococcus neoformans* is an important pathogen of immunocompromised people. The ability of the fungus to sense its environment is critical for proliferation and the generation of infectious propagules, as well as for adaptation to the mammalian host during infection. The conserved cAMP/protein kinase A pathway makes an important contribution to sensing, as demonstrated by the phenotypes of mutants with pathway defects. These phenotypes include loss of the ability to mate and to elaborate the key virulence factors capsule and melanin. This review summarizes recent work that reveals new targets of the pathway, new phenotypic consequences of signaling defects, and a more detailed understanding of connections with other aspects of cryptococcal biology including iron regulation, pH sensing, and stress.

KEYWORDS : ***Cryptococcus neoformans*****, Signal transduction, Mating, Virulence**

Signal transduction pathways provide organisms with the ability to respond to changing environmental conditions. One of these pathways, the cAMP/protein kinase A (PKA) pathway, has been characterized in some detail in the model fungus *Saccharomyces cerevisiae*, as well as in several saprophytic and pathogenic species [1-7]. This review focuses on the cAMP/PKA pathway in the basidiomycete pathogen *Cryptococcus neoformans*. This fungus is the frequent cause of meningoencephalitis in immunocompromised people such as acquired immunodeficiency syndrome (AIDS) patients [8-12]. Many AIDS patients acquire cryptococcosis and the resulting mortality contributes to the fact that AIDS is one of the leading causes of death from infectious diseases worldwide [8-11]. In fact, a recent analysis indicates that there are ~1 million cases of cryptococcal meningoencephalitis each year leading to ~600,000 deaths [8]. Highly active antiretroviral therapy has reduced the impact of *C. neoformans* in developed countries, but the incidence of cryptococcosis is still high among people with human immunodeficiency virus (HIV)/AIDS in sub-Saharan Africa [9, 12]. Cryptococcosis also occurs in immunocompetent people as demonstrated by a startling emergence of infections caused by *Cryptococcus gattii* in western North America [13-18].

The cAMP/PKA pathway is important in the virulence of *C. neoformans* in animal hosts and therefore has received considerable attention. Virulence in *C. neoformans* depends on three main attributes: a polysaccharide capsule,

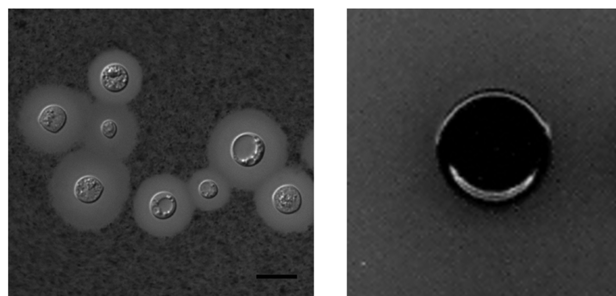

Fig. 1. Capsule and melanin production by *Cryptococcus neoformans*. The photograph on the left shows cells in the presence of an india ink stain to reveal the polysaccharide capsule surrounding the cells. The photograph on the right shows a single colony of *C. neoformans* (~4 mm in diameter) after growth on medium containing the laccase substrate L-3,4-dihydroxyphenylalanine (L-DOPA).

deposition of the antioxidant melanin in the cell wall, and the ability to proliferate at 37°C (Fig. 1) [10, 19-26]. The capsule is the major virulence factor and acapsular mutants are non-pathogenic [24-26]. Remarkably, the cAMP/PKA pathway regulates capsule size, melanin formation, and virulence, as well as additional traits such as mating [3, 4, 27-30]. The characterized pathway components are illustrated in Fig. 2 [27-40] and include upstream functions such as a candidate G-protein coupled receptor (Gpr4), a Gα protein

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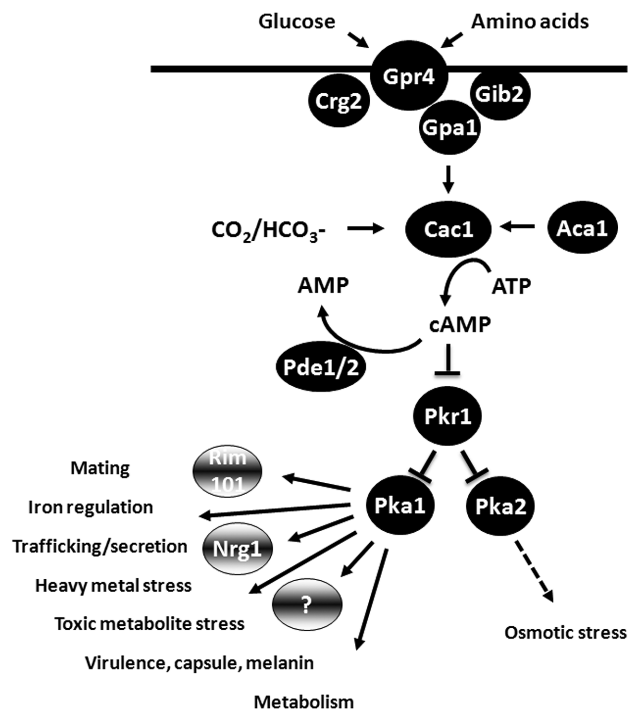

Fig. 2. Model for the components and downstream targets of the cAMP/protein kinase A (PKA) pathway in *Cryptococcus neoformans*. The upstream components of the proposed pathway are associated with the plasma membrane (horizontal line) and respond to extracellular signals such as glucose and amino acids [27, 31-33]. The proteins include the G-protein coupled receptor Gpr4, a Gbeta-like/RACK1 homolog Gib2, a regulator of G-protein signaling Crg2 and the G-protein alpha subunit Gpa1 [27, 31-33]. These components influence the activity of adenylyl cyclase (Cac1), as does the Aca1 protein and CO_2/HCO_3-, to ultimately influence cAMP levels [30, 34, 35-37]. Phosphodiesterases (Pde1/2) can also act to reduce cAMP levels and dampen signaling [38]. The levels of this second messenger influence the activity of PKA by binding the regulatory subunit (Pkr1) and causing its dissociation from the catalytic subunits (Pka1 and Pka2) [28, 29]. Processes that are discussed in the text and influenced by the pathway and by Pka1 and Pka2 are shown below the catalytic subunits. Some of these processes are regulated by the transcription factors Rim101 and Nrg1, although direct connections have not been established in all cases [39, 40]. This situation is reflected by the arrows from Pka1 and by the possibility of other regulatory factors indicated by the protein with a question mark.

(Gpa1), a Gbeta-like/RACK1 homologue (Gib2), and a regulators of G-protein signaling (RGS) protein (Crg2) [27-34, 41-43]. These proteins along with CO_2/HCO_3- (regulated by carbonic anhydrase [Can2]) influence the activity of adenylyl cyclase (Cac1) and the production of cAMP [35-37]. In addition to CO_2, the upstream components mediate the response of adenylyl cyclase to glucose and amino acids (e.g., methionine) [31]. The Ras1 protein also regulates mating and invasive growth via the cAMP/PKA pathway [44]. The level of cAMP influences the dissociation of the catalytic (Pka1, Pka2) and regulatory (Pkr1) subunits of PKA leading to activation of Pka1 and downstream signaling [28]. The phosphodiesterase (Pde1) is activated by PKA and functions to control intracellular cAMP levels [38].

The importance of the cAMP/PKA pathway in the pathogenesis of *C. neoformans* is illustrated by the finding that mutants defective in pathway components have altered virulence in a mouse model of cryptococcosis. For example, *gpa1*, *cac1*, and *pka1* mutants show reduced capsule and melanin formation, sterility, and reduced virulence [27-30]. In contrast, loss of *PKR1* causes an enlarged capsule and hypervirulence [28]. The underlying mechanisms for these intriguing phenotypes are clearly important for understanding connections between environmental sensing and the ability of *C. neoformans* to cause disease. This review focuses on recent studies that provide additional insights into the involvement of the cAMP/PKA pathway in morphogenesis, nutrient acquisition, and stress. This work expands our understanding of the integration of cAMP signaling with the properties of *C. neoformans* that enable it to cause disease in mammalian hosts. Several previous reviews have described the cAMP/PKA pathway in *C. neoformans* and in other fungi [1, 3, 4, 45]. Recent reviews have also appeared that provide additional information on the pathogenesis of *C. neoformans* [20, 46-48].

Regulation of Virulence, Morphogenesis, and Secretion

Insights into the downstream targets of the cAMP/PKA pathway that influence specific phenotypes have been obtained by transcriptional profiling using mutants defective in pathway components. Initially, a microarray study was performed to compare the transcriptomes of a wild-type strain and a *gpa1* mutant [49]. This study revealed that the pathway regulates the transcript levels for several genes required for capsule production as well as the *LAC1* and *LAC2* genes encoding the laccases that synthesize melanin. This analysis also identified the gene encoding a predicted transcription factor, Nrg1, as being regulated by Gpa1 and the cAMP/PKA pathway. A subsequent detailed study of Nrg1 revealed that the protein, which contains a C_2H_2 zinc finger domain, plays a role in capsule formation, mating, and virulence [39]. A comparison of the transcriptomes for the *nrg1* mutant and a wild-type strain also showed that the transcription factor regulates genes encoding enzymes related to the cell wall, carbohydrate metabolism, and substrate oxidation, as well as transporters, cell cycle proteins, signaling components, and miscellaneous proteins. Overall, these studies provide a partial view of

the network of functions regulated by cAMP/PKA signaling and begin to explain the activities underlying the phenotypes of pathway mutants.

It is likely that the cAMP/PKA pathway influences the expression of virulence functions such as the capsule via multiple downstream functions. In addition to the capsule-related genes identified in the transcriptional analysis of the *gpa1* and *nrg1* mutants, transcriptional profiling by serial analysis of gene expression (SAGE) revealed connections with the secretory pathway [50]. Specially, a SAGE experiment was performed to compare the transcriptomes for the wild-type strain, the *pka1* mutant (small capsule), and *pkr1* mutant (large capsule) [50]. Three findings from the analysis revealed connections to the trafficking of capsule polysaccharide via the secretory pathway. First, the transcripts for some components of the secretory pathway, including orthologs of the Sec24 (endoplasmic reticulum [ER] to golgi trafficking) and Ypt31/32 (intra- and post-Golgi vesicle formation) proteins of *S. cerevisiae*, were elevated in the *pka1* mutant. This pattern was also observed for the transcripts of several exported capsule-associated mannoproteins, as well as some stress-related proteins. Second, the transcript levels for other secretory components, including the exocyst protein Sec15, were reduced in the *pka1* mutant. This observation raises the possibility that loss of PKA leads to a smaller capsule because of reduced exocytosis. The third finding from the SAGE analysis was that several secretory pathway components, including two orthologs of the *S. cerevisiae* SNAREs, had elevated transcripts in the *pkr1* mutant. One could hypothesize that specific SNAREs may function at keys steps in the secretory pathway, such as ER to Golgi trafficking or in the post-Golgi-endosomal pathway, to facilitate vesicle trafficking for capsule export.

The importance of exocytosis for capsule formation has been demonstrated by the use of a temperature sensitive (ts) mutant for the exocyst GTPase Sec4/Rab8 (designated Sav1) [51]. The mutant was found to accumulate intracellular vesicles at the restrictive temperature and immunoelectron microscopy with anti-capsule antibody revealed that the intracellular vesicles contained capsule polysaccharide. This study therefore provides support for intracellular synthesis of the polysaccharide and secretion via exocytosis. Another recent study examined the role of the exocyst protein, Sec6, in exosome formation by constructing RNAi mutants [52]. These mutants showed a partial attenuation of virulence and were defective for melanin production as well as the export of urease and soluble capsule polysaccharide.

Overall, the observed influence of defects in the catalytic and regulatory subunits of PKA on the expression of secretory pathway components, as identified by the SAGE study, may partially explain the difference in capsule size between the *pka1* and *pkr1* mutants [50]. However, it is not yet clear how PKA might exert an influence on expression of secretory pathway components. Regulation could influence capsule elaboration at multiple levels (that include gene expression, protein and RNA degradation, and protein localization) and via several mechanisms including regulation of polysaccharide synthesis, export, degradation, and attachment to the cell wall. It is also possible that the transcriptional influence of the PKA mutations is the result of an indirect effect on the secretory pathway, e.g., via the unfolded protein response [53].

The functional significance of the observed PKA regulation of one of the exported mannoproteins has been tested as a follow-up to the SAGE analysis [50]. Specifically, the PKA-regulated gene *OVA1* was deleted and the resulting mutant had an enlarged capsule. This suggests that Ova1 is a negative regulator of capsule size. The increase in capsule size for an *ova1*, *pka1* mutant relative to a *pka1* mutant was consistent with this conclusion. Ova1 is a putative phosphotidylethanolamine binding protein that may interact with phospholipids during secretion. Interestingly, the examination of Ova1 revealed a connection between capsule production and lithium sensitivity [50]. Lithium treatment in *S. cerevisiae* results in inositol depletion and a shift in the phosphatidylinositol/phosphotidylcholine ratio that influences secretory vesicle formation [54, 55]. Many secretory pathway mutants in *S. cerevisiae* show elevated inositol excretion, and transcriptional profiling by SAGE and microarrays also indicated that PKA regulates of inositol metabolism and transport in *C. neoformans* [39, 50]. The influence of lithium was tested on *ova1*, *pka1*, and *ova1*, *pka1* mutants; growth of the *pka1* mutant was reduced in the presence of lithium [50]. Loss of Ova1 exacerbated the lithium sensitivity of the *pka1* mutant, and lithium treatment also reduced capsule size in wild-type and mutant strains. Overall, these studies demonstrate the value of the transcriptional profiling experiments in identifying downstream functions that contribute to capsule formation.

The finding that the putative phospholipid-binding protein Ova1 influences capsule size may be relevant to recent observations that phospholipids influence capsule enlargement during the interaction of *C. neoformans* with amoebae and macrophages [56]. Specifically, Chrisman *et al.* [56] observed capsule enlargement upon incubation of *C. neoformans* with the amoeba *Acanthamoeba castellanii* and subsequently found that extracts containing polar lipids were stimulatory. The ability of *C. neoformans* cells to produce phospholipase B, a known virulence factor, contributed to capsule enlargement. Subsequent testing of purified phospholipids revealed that phosphatidylcholine and derivatives were capable of triggering capsule enlargement. In the context of a possible role for phospholipid binding proteins, Chrisman *et al.* [56] found that proteinase K treatment of amoeba extracts resulted in

an increase in capsule-inducing activity. Therefore, cryptococcal proteins may mediate the response to phospholipids leading to speculation that oxysterol binding proteins might be involved in binding phospholipids [56].

The influence of amoeba extracts on *C. neoformans* morphogenesis also extends beyond the capsule to the formation of an enlarged cell phenotype of the fungus [56]. The formation of enlarged cells (called giant or titan cells) is a fascinating morphological phenomenon in *C. neoformans* and two groups have recently characterized these cells after purification from the lungs of infected mice [57, 58]. In the lung, the giant cells were found as a subpopulation of enlarged cells of 20~100 μm within the larger population of yeast cells of 4~10 μm, and this unusual cell type has been observed previously in clinical samples of infected humans [59, 60]. The large cells are uninucleate and polyploid suggesting that endoreplication of the genome is occurring in the cells. The cells are also resistant to phagocytosis and may therefore contribute to fungal evasion of the host immune response. In the context of this review, the characterization of the giant cells revealed that their formation requires cAMP/PKA signaling because a mutant lacking adenylyl cyclase (encoded by *CAC1*) failed to produce giant cells in mice and in culture [57]. More recently, Okagaki *et al.* [61] demonstrated that the Ste3**a** pheromone receptor from mating type **a** cells and another G-protein-couple receptor, Gpr5, both contribute to the induction of cell enlargement during infection. These receptors appear to signal through the cAMP/PKA pathway via the G-protein Gpa1. A role for Gpa1 was determined by infecting mice with a mating type **a** strain expressing a constitutively active allele of the *GPA1* gene (GPA1^{Q284L}) and demonstrating a two-fold increase in large cells compared to the wild-type strain. Interestingly, expression of the constitutively active allele in a strain of the a mating type did not influence the formation of giant cells. An interaction between Gpa1 and the Ste3**a** pheromone receptor was also demonstrated and, taken together, these results indicate that signaling from both Gpr5 and Ste3**a** impinge on Gpa1 to induce giant cell formation [61]. As described further below, the PKA-regulated transcription factor Rim101 plays a critical role in the signaling downstream of Gpa1 to control cell enlargement.

The signals that trigger giant cell formation include the phospholipid phosphatidylcholine, as demonstrated by Chrisman *et al.* [56] in their analysis of capsule enlargement during interactions between *C. neoformans* and amoeba. Interestingly, Okagaki *et al.* [61] found that phosphatidylcholine did not signal via the Gpr5 receptor to cause cell enlargement. Similarly, amino acids such as methionine that are known to influence cAMP signaling through a related receptor, Gpr4, did not stimulate giant cell formation [31]. It is clear that additional experimentation is needed to identify the extracellular factors and conditions that trigger cAMP/PKA signaling. One possibility is that other lipid-related signals are important because fatty acids, for example, trigger a morphological response in another basidiomycete pathogen, *Ustilago maydis*. In this case, several fatty acids of different chain length promote a switch from yeast-like to filamentous growth [62, 63].

Connections between PKA, Iron Acquisition, and pH Sensing

In addition to the regulation of morphogenesis, the cAMP/PKA signaling pathway is interconnected with functions that mediate iron acquisition and pH sensing in *C. neoformans* [40, 64-66]. The connection with iron was detected during a detailed characterization of iron regulation of gene expression and virulence [64]. In this study, the major regulator of iron uptake and homeostasis, Cir1, was identified and found to be a GATA-type zinc finger protein [64]. Remarkably, iron influences the transcript levels for ~700 genes in *C. neoformans* and practically all of this transcriptional response is mediated by Cir1. In fact, a *cir1* deletion mutant is practically blind to changes in iron levels. In addition, Cir1 regulates all of the known virulence factors including melanin and capsule formation. The capsule observation is consistent with the finding that loss of Cir1 activity influences the transcript levels of cAMP pathway components including, most notably, the G-protein coupled receptor Gpr4. Cir1 positively regulates the expression of Gpr4 under both low and high iron conditions. Given that Gpr4 also regulates capsule size via the cAMP/PKA pathway, as described above, the regulation by Cir1 is consistent with the long-standing observation that iron levels influence capsule production.

Cir1 also regulates all of the iron uptake functions including the *CFT1* and *CFO1* genes that encode the iron permease and ferroxidase components, respectively, of the high affinity reductive uptake system [64, 65]. In addition, Cir1 regulates some of the transporters that allow *C. neoformans* to exploit small molecule iron chelators (siderophores) produced by other microbes [64]. One of these, Sit1, has been characterized in some detail and found to encode a transporter for the siderophore ferroxamine [67]. Interestingly, the expression of these iron uptake functions is also regulated by cAMP/PKA signaling [50, 64, 65, 67]. For example, SAGE analysis of the mutants defective in subunits of PKA revealed reduced expression of the high affinity iron uptake components relative to the wild-type strain [50]. In contrast, PKA positively regulates transcript levels for the *SIT1* gene [67]. This pattern of regulation is similar to that observed for the Arn3 siderophore transporter in *S. cerevisiae*. In this case, the cAMP pathway and the *TPK2* gene encoding a catalytic subunit of PKA negatively regulated *ARN3*

transcript levels [68]. Additionally, the cAMP/PKA-regulated transcription factor, Nrg1, also regulates the *SIT1* gene in *C. neoformans* [39]. The influence of cAMP/PKA signaling on iron acquisition in *C. neoformans* extends beyond transcriptional control because loss of PKA activity changes the localization of the ferroxidase protein, Cfo1 [65]. In particular, a Cfo1-GFP fusion protein was found to localize to a perinuclear location in a *pka1* mutant, and this location may represent the endoplasmic reticulum. The fusion protein was localized to the plasma membrane in the wild-type strain, as expected. These observations are consistent with a role for cAMP/PKA signaling in protein trafficking, as described earlier [50].

The connections between iron and cAMP/PKA signaling extend to the pH-responsive transcription factor, Rim101 [40]. As mentioned above, this factor is an important regulator of giant cell formation and O'Meara *et al.* [40] have shown that it is also required for capsule attachment to the cell surface. The protein has a potential PKA phosphorylation site at serine 773, and the mutation of this site to alanine resulted in both nuclear and cytoplasmic localization of a Gfp-Rim101 protein, in contrast to the nuclear pattern of the wild-type fusion protein. The nuclear and cytoplasmic location was also observed upon introduction of the wild-type protein into a *pka1* deletion mutant. Interestingly, the allele with the mutation at serine 773 also failed to complement the capsule defect in a *rim101* deletion mutant. This deletion mutant also showed growth defects at alkaline pH and upon iron limitation, although it also had increased virulence relative to the wild-type strain. Transcriptional profiling revealed that Rim101 regulates the transcript levels for genes related to capsule production, the cell wall and both iron and copper homeostasis [40].

The cAMP/PKA Pathway and Stress

Connections between cAMP/PKA signaling and the stress response are well established in fungi. For example, the pathway is important in the ability of *S. cerevisiae* to respond to a variety of stresses including nutrient starvation, heat shock, DNA damage, osmotic stress, and oxidative stress [69, 70]. Part of the contribution of the cAMP/PKA pathway occurs through phosphorylation of the transcription factor Msn2 to influence its nuclear localization. The role of the cAMP/PKA pathway in the stress response of *C. neoformans* has been examined in some detail and compared with the involvement of the Ras signaling pathway [71]. The Ras pathway is of interest for comparison because the Ras1 and Ras2 proteins directly activate adenylyl cyclase in *S. cerevisiae* to influence PKA activity [1]. However, in *C. neoformans*, interactions of the Ras proteins with adenylyl cyclase have not been demonstrated. Ras1 appears to be the major Ras protein and it regulates thermotolerance and the actin cytoskeleton through a guanine nucleotide exchange factor Cdc24 and the Rho-like GTPase Cdc42 [72]. Ras1 also regulates invasive growth and mating through the cAMP/PKA pathway.

To compare the downstream networks controlled by the Ras and cAMP/PKA pathways, transcriptome analysis was employed with cells of the following mutants grown in rich medium: *ras1Δ*, *gpa1Δ*, *cac1Δ*, *aca1Δ*, and *pka1Δ pka2Δ* [71]. This analysis revealed that the transcriptional influence of a defect in the *RAS1* gene was distinct from the influence of the cAMP/PKA pathway mutants. In particular, 400 genes were differentially expressed in the *ras1Δ* mutant versus the wild-type strain, while 132 genes showed differences for the other mutants. All of the mutants in the cAMP/PKA pathway showed similar patterns with each other, although the *gpa1Δ* and *aca1Δ* mutants showed minor differences that suggested additional signaling activities. Interestingly, the *cac1Δ* mutant showed a nearly identical pattern to the *pka1Δ pka2Δ* mutant indicating that the Pka1 and Pka2 catalytic subunits are the key downstream kinases for signaling via adenylyl cyclase.

A careful examination of the Ras- and cAMP-dependent genes revealed a significant fraction that were regulated by environmental stress conditions such as oxidative or osmotic stress, or treatment with the antifungal drug fludioxonil [71]. Follow up genetic analyses revealed that the Ras pathway regulates the response to osmotic stress and is involved in maintaining cell wall integrity. The Pka2 protein was also involved in the osmotic stress response under glucose starvation and deletion of the *PKA1* gene in the *pka2Δ* mutant background restored a wild-type level of sensitivity. This interesting result suggests that the two PKA catalytic subunits may have antagonistic roles upon glucose starvation. Similar experiments revealed that Ras1, the cAMP pathway, and another stress response pathway (the Hog1 pathway) control the response to oxidative stress independently. A test of the mutants on the polyene antifungal drug, amphotericin B, also indicated that mutants in both the Ras and cAMP/PKA pathways had increased susceptibility. In the case of the cAMP/PKA-regulated genes, the susceptibility could be traced, at least in part, to the regulation of two genes encoding related Hsp12 proteins (Hsp12 and Hsp112). That is, deletion of these genes increased susceptibility. Overall, this extensive transcriptional and genetic analysis provides a detailed view of the involvement of Ras and cAMP signaling in the response to environmental stress and drug treatment [71]. The information will be useful in developing new strategies to treat fungal infections.

Conclusions and Future Directions

The molecular details of cAMP/PKA signaling in *C. neoformans* are emerging through the application of

transcriptional profiling techniques and the further examination of pathway mutants for novel phenotypes. The transcriptional profiling approach has been particularly fruitful in revealing connections with transcription factors and other proteins that function in the regulation of virulence factor expression, the secretory pathway, the stress response, and the iron and pH regulatory networks. Remarkably, phenotypic studies identified additional regulators of capsule formation, such as the Ova1 protein, and established a link between cAMP/PKA signaling and the formation of enlarged cells. In addition to continuing the approaches that have been successful to date, it is clear that new tools and systems biology approaches are needed to develop a detailed molecular understanding of cAMP/PKA signaling. In this context, an important goal is to identify all of the direct targets of PKA phosphorylation. These targets undoubtedly include transcription factors, metabolic enzymes, and other proteins that may be components of the secretory pathway. One key approach and immediate goal will be to define the phosphoproteome of *C. neoformans* through further exploitation of mutants with defects in pathway components. A detailed understanding of the pathway and its targets will likely contribute to the development of strategies to treat cryptococcosis.

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