Adaptation of Cryptococcus neoformans to Mammalian Hosts: Integrated Regulation of Metabolism and Virulence

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The basidiomycete fungus Cryptococcus neoformans infects humans via inhalation of desiccated yeast cells or spores from the environment. In the absence of effective immune containment, the initial pulmonary infection often spreads to the central nervous system to result in meningoencephalitis. The fungus must therefore make the transition from the environment to different mammalian niches that include the intracellular locale of phagocytic cells and extracellular sites in the lung, bloodstream, and central nervous system. Recent studies provide insights into mechanisms of adaptation during this transition that include the expression of antiphagocytic functions, the remodeling of central carbon metabolism, the expression of specific nutrient acquisition systems, and the response to hypoxia. Specific transcription factors regulate these functions as well as the expression of one or more of the major known virulence factors of C. neoformans. Therefore, virulence factor expression is to a large extent embedded in the regulation of a variety of functions needed for growth in mammalian hosts. In this regard, the complex integration of these processes is reminiscent of the master regulators of virulence in bacterial pathogens.

Cryptococcus neoformans is a frequent cause of fungal meningoencephalitis in immunocompromised people such as HIV/AIDS patients (21, 112). Highly active antiretroviral therapy has reduced the impact of C. neoformans, but the incidence of cryptococcosis is still high among the >25 million people with HIV/AIDS in sub-Saharan Africa (11, 12, 42, 56). In fact, recent estimates indicate that there are one million cases of cryptococcal meningitis globally per year in AIDS patients, leading to approximately 625,000 deaths; the majority of these deaths occur in Africa (110). Remarkably, the related species Cryptococcus gattii has recently emerged as the agent of cryptococcosis in immunocompetent people in western North America (9, 10, 17, 33, 43, 71, 72, 94, 95).

C. neoformans and C. gattii share three main virulence attributes that contribute to their ability to cause disease: the production of a polysaccharide capsule, the ability to grow at 37°C, and the deposition of melanin in the cell wall (20, 77, 78, 91, 141). The capsule is the major virulence trait, and it provides protection from phagocytosis and has immunomodulatory properties (36, 132, 133, 138). Capsule-reactive antisera have also been used to classify isolates into serotypes A, D, and AD for C. neoformans and B and C for C. gattii (36, 132). Specific enzymes also contribute to virulence, including phospholipase B, proteases, urease, and superoxide dismutase (31, 77, 105, 126). Cryptococcal cells appear to have a specific delivery system for moving some virulence-related material to the cell surface via vesicles that have been termed “virulence factor delivery bags” (22). These extracellular vesicles, which contain capsule polysaccharide and a variety of proteins, influence the activity of phagocytic cells, the initial line of host defense upon cryptococcal infection (104). C. neoformans also undergoes a specific morphological response to the host pulmonary environment in that a proportion of the fungal cells enlarge into giant or titan cells that can be up to 10 times larger in diameter (30 to 100 μm) than typical yeast cells (103, 139). This cell enlargement is a dramatic reflection of the broader fungal response to specific conditions in the host environment that include defense molecules, physical aspects of temperature, oxygen, and CO2, and the availability of specific macro- and micronutrients (e.g., glucose and iron).

In this review, we focus on recent studies to characterize C. neoformans adaptation and proliferation in the mammalian host environment with an emphasis on conditions relevant to disease such as iron limitation and hypoxia. An emerging theme is the coordinate regulation by several regulatory proteins of functions for adaptation, nutrient acquisition, and the elaboration of virulence factors. The activities of these proteins are illustrated in Fig. 1 and summarized in Table 1. This situation is reminiscent of well-characterized examples of similar integration in bacterial pathogens, as illustrated by the Fur regulator that controls virulence and iron regulation in many bacterial pathogens, the DtxR regulator that controls iron metabolism and toxin production in Corynebacterium diphtheriae, and the PrfA protein that regulates the saprophytic to pathogenic switch in Listeria monocytogenes (19, 34, 130). For considerations of other topics on C. neoformans and C. gattii, readers are referred to several recent reviews (7, 15, 16, 75, 77, 78, 86, 91). In addition, the recent book Cryptococcus: from Human Pathogen to Model Yeast contains a wealth of information on C. neoformans, C. gattii, and cryptococcosis (49).

ADAPTATION TO GROWTH IN PHAGOCYTIC CELLS AND HOST TISSUES

Gene expression in response to phagocytosis and humoral immunity. The lung is generally considered the initial site of infection by cryptococcal spores or yeast cells. In this environment, Feldmesser et al. showed that alveolar macrophages rapidly take...
up cryptococcal cells and that the fungus persists in intracellular and extracellular locations as infection progresses (40). The proportion of phagocytosed yeast cells is higher at an early stage of infection and shifts toward more extracellular cells after 24 h. It has also recently been shown that \textit{C. neoformans} can escape from phagocytic cells and move between phagocytes by a nonlytic expulsion process (4, 5, 92, 93). The adaptation of \textit{C. neoformans} to the intracellular environment of phagocytic cells has been examined by gene expression profiling. Fan et al. identified a group of 525 differentially regulated genes in \textit{C. neoformans} cells after phagocytosis by a murine macrophage-like cell line (39). The upregulated genes included predicted membrane transporters for hexoses, amino acids, iron, ammonium, nicotinic acid, and phosphate. Additional elevated transcripts encoded fatty acid transporters, autophagy proteins, peroxisome transport functions, and enzymes for lipid metabolism. The phagocytosed fungal cells also appeared to be mounting a stress response because genes encoding oxidative stress functions such as flavohemoglobin (Fhb1) were upregulated. Other factors that were more highly expressed upon phagocytosis included components of the cyclic AMP (cAMP) signal transduction pathway and known virulence factors. Many of the genes with lower expression in phagocytic cells encoded translation initiation and elongation factors and rRNA processing proteins. Similar patterns of gene expression were also found using serial analysis of gene expression (SAGE) to characterize \textit{C. neoformans} transcript levels upon phagocytosis (G. Hu and J. Kronstad, unpublished results). Overall, the internalized fungal cells appear to experience a nutrient-limited and stressful environment. Similar but more limited results were obtained in a study to characterize and compare the expression of \textit{C. neoformans} and \textit{C. gattii} genes during intracellular growth in rat peritoneal macrophages by representational difference analysis (RDA) (45).

The adaptive immune system also plays a role in controlling cryptococcal disease (132, 133). In fact, a recent innovative study revealed that binding of host antibodies to the cell surface of \textit{C. neoformans} not only facilitates humoral immunity, but also has direct antimicrobial effects and modulates fungal gene expression (96). The binding of monoclonal antibodies of different isotypes and protective efficacies to \textit{C. neoformans} is associated with changes in the expression of genes for a number of different processes. For example, the entire fatty acid biosynthetic pathway was coordinately upregulated and ergosterol synthesis was also increased upon antibody binding. Furthermore, a variety of genes encoding protein translation functions were downregulated, while various genes involved in metabolism, secretion, and cell wall synthesis also showed changes in expression. These changes were dependent on the type of antibody and the cellular location of the epitope. Overall, these studies open the door to considerations of the influence of the humoral response on pathogen gene expression that should take place in parallel with the influence of innate immunity and phagocytosis.

**Gene expression during early pulmonary infection.** In the context of the interaction of \textit{C. neoformans} with lung tissue and phagocytic cells, transcriptional profiling by SAGE of cryptococcal cells recovered from a murine pulmonary infection revealed a general remodeling of central carbon metabolism. That is, cells recovered from mouse lungs at 8 and 24 h after initial infection showed elevated expression levels of transcripts encoding enzymes for the glyoxylate pathway, gluconeogenesis, \(\beta\)-oxidation, and amino acid biosynthesis (55). This expression pattern suggests that glucose-limited microenvironments exist in the host.
| Regulatory factor | Mutant phenotypes (virulence factors) | Virulence in mice | Other phenotypes | Influence on gene expression | Reference(s) |
|-------------------|---------------------------------------|-------------------|------------------|-------------------------------|--------------|
| Cir1              | Capsule defect, poor growth at host temp, enhanced melanization | Avirulent | Increased sensitivity to high iron, FK506, calcium, SDS, and amphotericin B and reduced sensitivity to miconazole | Regulation of functions for iron acquisition, cell wall and membrane biosynthesis, and signaling pathways (cAMP, Ca, MAPK) and regulation of transcription factors | 66 |
| Cuf1              | Melanin defect | Attenuated | Growth defect upon copper or iron limitation | Regulation of genes involved in copper homeostasis | 35, 62, 63, 86, 134 |
| Gat1              | Capsule defect, enhanced growth at high temp, enhanced melanization | Slight hypervirulence or wild-type virulence | Essential for growth on some nitrogen sources (e.g., uric acid, urea, creatinine) | Regulation of nitrogen metabolism | 73, 84 |
| Gat201            | Reduced capsule size, increased melanization, enhanced phagocytosis | Avirulent | Reduced lung infectivity | Regulation (upon Gat201 overexpression) of carbohydrate-related functions, including sugar transporters, glycol hydrolyases, and glycol transferases, and regulation of transcription factors | 29, 88 |
| Gcn5              | Reduced capsule size and defect in capsule attachment, delayed growth at host temp | Avirulent | Increased sensitivity to FKS06 and oxidative stress | Regulation of functions for cell wall biosynthesis and oxidative stress as well as transcription factors, signaling components, ribosomal proteins, and mitochondrial proteins | 106 |
| Nrg1              | Capsule defect | Attenuated | Cell aggregation, mating defect, increased susceptibility to elevated salt and sorbitol stress and SDS | Regulation of genes for carbohydrate metabolism, sugar and siderophore transport, and oxidation/oxidative stress | 32 |
| Rim101            | Capsule defect | Hypervirulent | Growth defects at alkaline pH, high salt, low iron | Regulation of metal homeostasis, cell wall proteins, and capsule-related genes | 107 |
| Sre1 (serotype A) | Subtle capsule and melanin defects | Attenuated | Sensitive to hypoxia | Regulation of functions related to stress, carbohydrate uptake and metabolism, respiration, sterol and heme biosynthesis, and fatty acid metabolism | 30 |
| Sre1 (serotype D) | Increased laccase activity, but no defects in capsule or melanin | Avirulent | Sensitive to hypoxia, growth defect on low-iron medium | Regulation of sterol biosynthesis, iron and copper transport, stress | 24 |
| Tup1 (serotype A) | Enlarged capsule, reduced melanin | Attenuated | Reduced mating, poor growth on low-iron and low-copper media, increased sensitivity to SDS and flucytosine and resistance to fluconazole | Regulation of iron and copper genes and capsule-related genes | 83 |
| Vad1              | Melanin defect | Avirulent | Reduced growth on glucose medium with caffeine, on glycerol or lactate as carbon source, and on media with high salt or sorbitol | Regulation of Notl transcriptional regulator, laccases, phosphoenolpyruvate carboxykinase, elongation factor Tu1, and a mannoprotein | 109 |

* MAPK, mitogen-activated protein kinase.
and that alternative carbon sources such as acetate, lactate, fatty acids, or amino acids may be exploited for proliferation. The elevated expression of acetyl coenzyme A (acetyl-CoA) synthetase, pyruvate decarboxylase, aldehyde dehydrogenase, and acetate transporters was of particular interest because of the connections of these functions with acetate, one of the most abundant metabolites found in C. neoformans-infected tissue (51, 55). Subsequent deletion of the highly expressed ACS1 gene encoding acetyl-CoA synthetase yielded a mutant with a growth defect on acetate and slightly attenuated virulence (55). However, the acs1 mutant still caused disease, indicating that acetate is not a critical carbon source in vivo, a result consistent with the analysis of glyoxylate cycle mutants in C. neoformans (58, 120). This result also indicates that other pathways for acetyl-CoA production may be more important in vivo, and recent studies of mutants defective in ATP-citrate lyase or β-oxidation enzymes support this conclusion (E. J. Griffiths, M. Kretschmer and J. Kronstad, unpublished results). Transcripts for transporters (e.g., for monosaccharides, iron, copper and acetate) and for stress response proteins were also elevated in cells from lung tissue, thus further indicating a nutrient-limited and hostile host environment (55).

**Genetic analysis of lung infectivity.** In a genetic approach to examine adaptation to the murine lung environment, Liu et al. employed signature-tagged mutagenesis (STM) to screen deletion mutants for decreased or increased infectivity (88). Of 1,100 mutants, 164 had decreased infectivity and 33 showed an increase. Parallel screens of the 164 mutants with reduced infectivity for in vitro virulence phenotypes identified 85 mutants with defects in capsule production, melanization, and/or growth at 37°C. Of these, 33 novel genes were found to influence melanin production, and five novel genes regulated capsule formation. Another 40 mutants were identified that demonstrated defects in lung infectivity without altered growth, melanin synthesis, or capsule production (88). Interestingly, this analysis yielded few genes related to metabolism, perhaps due to the selection criteria used for the construction of the deletion set. Of 1,500 genes initially targeted for deletion, most (~900) were selected because they did not have obvious homologs in the well-characterized fungus *Saccharomyces cerevisiae*. The others represented a wide variety of processes. For example, one encoded an ortholog of the Snf1 AMP-activated kinase that regulates alternative carbon source utilization in *S. cerevisiae*. The deletion mutant for this gene showed substantially reduced infectivity. Similarly, Hu et al. found that deletion of SNF1 in *C. neoformans* caused reduced melanin production, severely attenuated virulence, and poor persistence in brain tissue, as well as growth defects on acetate, ethanol, and sucrose at 37°C (55). In a separate study, the application of STM using *Agrobacterium*-mediated transformation also yielded a mutant with a virulence defect but no obvious changes in known virulence factors (57). This mutant showed sensitivity to elevated pH and was defective in the ENA1 gene encoding a predicted sodium or potassium P-type ATPase.

In the STM study of Liu et al., one mutant with severely reduced infectivity had a defect in the GATA transcription factor, Gat201, and also showed impaired induction of capsule formation (88). The mutant exhibited a weaker capsule defect compared with the well-characterized mutants in the *CAP* genes that are required for capsule formation, but was more readily phagocytosed than cap mutants. As mentioned earlier, the capsule plays an important role in the ability of *C. neoformans* to avoid phagocytosis and killing. Interestingly, *gat201 cap* double mutants showed a greater level of phagocytosis than single cap mutants. This result suggests that Gat201 controls a capsule-independent antiphagocytic mechanism. Microarray profiling of a strain overexpressing *GAT201* revealed increased transcript levels for genes encoding carbohydrate regulation, including sugar transporters (40 genes), glycosyl hydrolases (eight genes), and glycosyl transferases (five genes). This pattern of expression is consistent with enhanced nutritional requirements that may be necessary, in part, for capsule formation (88). The profile suggests that Gat201 exerts at least a portion of its influence by activating the expression of factors involved with host interactions and that the transcription factor may link the regulation of nutritional functions and an antiphagocytic mechanism. Finally, an interesting set of eight mutants showed increases in infectivity, and the corresponding genes encoded transcription factors and signaling pathway components, as well as proteins of unknown function. Overall, the STM approach provided a wealth of novel virulence functions for further analysis.

More recent work on the transcriptional influence of Gat201 identified ~1,000 genes that showed statistically significant differential expression in a *gat201* strain (29). Of these, chromatin immunoprecipitation with microarray technology (ChIP-chip) experiments showed that Gat201 directly bound 126 genes and controlled expression of 62. In a macrophage uptake screen performed to enrich for the downstream effectors involved in Gat201-dependent macrophage evasion, mutants with mutations in two of the 62 genes were found to have a marked increase in macrophage uptake—a gene coding for a Barwin-like protein 1 (Blp1) and a gene coding for a transcription factor (Gat204)—indicating a direct role of these effectors in phagocytosis evasion. Blp1 was responsible for approximately a 2-fold increase in uptake by macrophages compared to the wild-type strain, and Gat204 was responsible for an 11-fold increase in macrophage uptake. However, a 31- to 37-fold increase in association was observed in a *blp1 gat204* double mutant, indicating a synergistic mechanism. Furthermore, of the small subset of 62 Gat201 effectors, seven of the directly regulated genes are predicted or described transcription factors such as Liv3 and Cir1 (66, 88). Interestingly, the copper transporter Ctr2 was also found to be important for antiphagocytic properties of *C. neoformans*, independently confirming the reported link between Ctr2 and macrophage phagocytosis (28) (see below). Overall, these studies reveal that Gat201 integrates virulence and metabolism, as illustrated in Fig. 1 and summarized in Table 1.

**Genetic analysis of survival in the CNS.** As mentioned above, *C. neoformans* cells generally disseminate to the central nervous system (CNS) following the initial pulmonary infection. Lee and coworkers recently examined the ability of *C. neoformans* to survive in cerebrospinal fluid (CSF), a unique stress environment for the pathogen that is known to provoke a remodeling of gene expression (81, 128). Using the mutant library of 1,201 individual strains constructed by Liu et al., 13 mutants were found to have survival defects in CSF at 37°C (81, 88). Mutants with deletions in the ENA1, *RUB1*, and *PIK1* genes encoding a cation ATPase transporter (described above), an NEDD8 ubiquitin-like protein, and a phosphatidylinositol 4-kinase, respectively, were examined in more detail. These mutants were defective for survival in human CSF, showed decreased intracellular survival in macrophages, and had attenuated survival in a rabbit meningitis model. In general, the diversity of functions that influence survival highlight the
challenge of building a comprehensive view of adaptation to the complex environment of the CNS.

**Glucose sensing, glycolysis, gluconeogenesis, and the glyoxylate cycle.** The ability of *C. neoformans* to sense glucose availability and respond with appropriate metabolic remodeling may be critical for initial host colonization. This idea is supported by recent evidence that expression of antiphagocytic protein 1 (App1) is dependent upon glucose availability (136). App1 is not only upregulated under low-glucose conditions, but also in bronchoalveolar lavage fluid, serum, and CSF. Importantly, this protein inhibits macrophage phagocytosis in a dose-dependent and complement-mediated manner and contributes to the virulence of *C. neoformans* (90). Utilization of glucose as a carbon source appears to be a critical component of *C. neoformans* virulence, although its requirement is ancillary for persistence in the host. Price et al. examined the importance of glycolysis and showed that the hexose kinase I and II (*hxk1Δ* and *hxk2Δ*, respectively) mutants and a pyruvate kinase (*pyk1Δ*) mutant were impaired in glucose utilization and showed severe attenuation of virulence in the murine inhalation model (116). These mutants also exhibited decreased persistence in rabbit CSF but survived in the lungs of mice in an inhalation model of cryptococcosis. For gluconeogenesis, it was also found that a *pck1Δ* mutant with a defect in phosphoenolpyruvate carboxykinase had wild-type persistence and viability in the rabbit CSF model of cryptococcosis (116). The mutant had previously been shown to have severely attenuated virulence in a murine inhalation model of infection (109). The *PCK1* gene was initially identified in a study to characterize the role of the DEAD-box RNA helicase Vad1, a regulator of multiple virulence-associated genes (Fig. 1; Table 1) (109). Loss of Vad1 resulted in the upregulation of a global repressor of transcription, NotI, as well as the downregulation of the additional virulence determinants, *PCK1*, *TUF1*, and *MPF3*, involved in gluconeogenesis, mitochondrial protein synthesis, and cell wall integrity, respectively (109). Along with the virulence defect of the *pck1Δ* mutant, a reduction in virulence in a mouse model was also observed for *TUF1* and *MPF3* mutants (109). Overall, these studies illustrate the importance of carbon metabolism and suggest that a concerted effort is needed to characterize addition regulatory factors.

In *C. neoformans*, the genes encoding glyoxylate cycle enzymes (isocitrate lyase and malate synthase) are dispensable for virulence, despite their upregulation during infection (55, 58, 120). The elevated expression of the glyoxylate pathway and gluconeogenesis genes during cryptococcal interactions with host tissue and phagocytic cells is similar to the regulation observed in *Candida albicans*. In this pathogenic fungus, phagocytosis and growth in the host result in an apparent shift in carbon utilization away from glucose and upregulation of transcripts for enzymes in the glyoxylate cycle, β-oxidation, and gluconeogenesis (8, 18, 89, 114, 115, 118, 125, 140). *C. albicans* mutants with defects in these processes are attenuated for virulence to various levels, in contrast to the lack of involvement in the virulence of *C. neoformans*. Conversely, a defect in peroxisomal β-oxidation attenuates virulence in *C. neoformans* (M. Kretschmer and J. Kronstad, unpublished results), but the process is not required for virulence in *C. albicans* (114). The reduced virulence of a *fox2Δ* mutant lacking the Mfe multifunctional enzyme for β-oxidation in *C. albicans* may be due to a defect in the glyoxylate cycle (115).

The glyoxylate pathway and β-oxidation are both associated with peroxisome function. In *C. neoformans*, peroxisomal *PEX* mutations (*pex1Δ* and *pex6Δ*) impair protein localization to peroxisomes, but peroxisome function is not required for virulence in either mouse or insect models of infection. Similarly, the peroxisome mutations did not influence the expression of virulence factors such as capsule, melanin, or growth at 37°C (58). However, in contrast to other fungi, *C. neoformans* has an unusual requirement for peroxisome function for efficient growth in the presence of monosaccharides, such as glucose, mannose, and fructose, that require hexokinase activity for entry into glycolysis (58).

In general, considerable additional work on carbon utilization during colonization of different mammalian niches is needed to fully understand the pathogenicity of *C. neoformans*. Also, there is intriguing evidence for glucose and other carbon sources as signals to regulate virulence factor elaboration. For example, glucose represses melanization in *C. neoformans* (141), and growth in mannitol results in increased capsule volume relative to growth in glucose (46). Interestingly, treatment of *C. neoformans*-infected mice with mannitol also resulted in reduced fungal dissemination to the brain (46). Lipids may also be important signals that are processed by *C. neoformans* to influence the immune response and contribute to virulence (54, 137). Along these lines, phospholipids derived from macrophages and amoebae, particularly phosphatidylycholine, were recently shown to trigger capsular enlargement in vivo (27).

**VIRULENCE AND NITROGEN METABOLITE REPRESSION**

Although carbon metabolism has received more attention, two recent studies examined nitrogen metabolite repression in *C. neoformans* and identified its importance in both virulence and the regulation of virulence factors, such as melanin, capsule, and growth at 37°C (73, 84). *C. neoformans* utilizes a limited number of nitrogen sources relative to well-characterized fungi such as *Neurospora crassa* and *Aspergillus nidulans* and generally can use ammonium, amino acids, and purines, but not nitrite or nitrate. The fungus can also use the nitrogen sources creatine, urea, and uric acid, which are abundant in pigeon guano, a common environmental niche. In addition, Lee et al. demonstrated that nitrogen metabolite repression occurs in *C. neoformans*, whereby ammonium is the preferred nitrogen source that represses the expression of catabolic functions for secondary sources (84). Interestingly, the regulatory influence of nitrogen extends to the regulation of capsule and melanin production, as well as growth at 37°C in *C. neoformans*. Specifically, capsule size is generally small for cells grown on ammonium, glutamate, proline, and alanine, but much larger on asparagine, urea, uric acid, or creatine. In some cases, these nitrogen sources had the inverse influence on melanin formation. For example, the highest melanin production was seen with ammonium, and lower melanization occurred on creatine and uric acid. The GATA transcription factor, Gat1, mediates nitrogen metabolite repression and also positively regulates capsule formation (73, 84). In contrast, Gat1 showed negative regulation of both melanin production and growth at elevated temperature. In the study by Kmetzsch et al., loss of Gat1 did not influence virulence in a mouse inhalation model of cryptococcosis when a high inoculum was used (73); however, Lee et al. employed a lower dose and reported a modest increase in virulence for the mutant (84). Overall, these studies highlight Gat1 as a transcription factor that, like the GATA factor Gat201, regulates both metabolic functions and virulence factor elaboration (Fig. 1; Table 1).
ADAPTATION TO IRON LIMITATION

Iron uptake mechanisms. Mammalian hosts sequester and withhold iron as a defense mechanism, thereby presenting a hostile nutritional environment for invading pathogens such as *C. neoformans* (64, 123). The majority of mammalian iron is locked in iron-binding proteins, such as transferrin, lactoferrin, and ferritin, and in heme-containing proteins, such as hemoglobin. Therefore, successful pathogens must have competitive iron acquisition and uptake systems. For example, pathogenic bacteria acquire iron from host proteins and heme via the elaboration of siderophores (secreted iron-binding small molecules), hemophores (heme-binding proteins), and dedicated transport systems (23, 76, 119). Similarly, *C. neoformans* and other fungal pathogens also utilize iron from host proteins and heme, and the ability to acquire iron is important for fungal virulence (2, 3, 41, 52, 67, 68, 74, 117, 122, 124, 135).

The different iron uptake systems in fungi include ferric reductases, high-affinity ferroxidase/permease complexes, siderophore biosynthetic functions, and specific uptake systems for siderophores. As in other fungi, *C. neoformans* possesses cell surface reductases that reduce ferric iron to its ferrous state, and the fungus exports reductants such as 3-hydroxyanthranilic acid (61, 100, 101). Furthermore, melanin is also implicated in ferric iron reduction (60). The high-affinity ferroxidase (Fet3)/permease (Ftr1) complex has been characterized in several fungi, including *S. cerevisiae* and *C. albicans* (3, 70, 74, 79, 113, 117, 124, 127). Briefly, ferrous iron is first oxidized by the ferroxidase Fet3, before transport into the cytosol as ferric ion by the permease Ftr1. In *C. neoformans*, the Fet3 and Ftr1 orthologs Cfo1 and Cft1 are essential for ferric iron uptake, iron acquisition from transferrin, and full virulence in mice (67, 68). However, they are not required for iron acquisition from heme or the siderophore ferrioxamine. Interestingly, the reductive iron uptake function encoded by *CFT1* and *CFO1* is also required for *C. neoformans* dissemination to the brains of infected mice (67, 68).

Most pathogenic microbes also produce siderophores and the corresponding transporters for uptake, or they scavenge siderophores from other microbes. Most fungi internalize siderophile-bound iron either via transporters of the ARN/SIT subfamily of the major facilitator superfamily or via the reductive uptake system (Fet3/Ftr1) (47, 113). Although *C. neoformans* doesn’t produce siderophores, Sit1 has been characterized as the specific transporter for uptake, or they scavenge siderophores. As in other fungi, the high-affinity iron permease/ferroxidase system (*CFT1/CFO1*) (67, 68). Subsequently, the *Cryptococcus* iron regulatory protein Cir1, which governs the global transcriptional response to different environmental iron levels, was identified via similarity to other iron regulatory proteins in fungi (66). Specifically, Cir1 is a GATA-type zinc finger protein with similarity to the iron regulators Urb1, SRE, Fep1, Sfu1, SREB, and Sre1 in Ustilago maydis, Neurospora crassa, Schizosaccharomyces pombe, *C. albicans*, Histoplasma capsulatum, and Blastomyces dermatitidis, respectively (6, 26, 44, 48, 80, 111). Comparisons of transcriptomes between a *C. neoformans* cir1 mutant and the wild-type strain revealed a large number of genes that are differentially regulated by Cir1, including 2,311 genes under the iron-limited condition and 1,623 genes upon iron repletion. Moreover, only a few genes were differentially expressed in the cir1 mutants, upon comparison between iron-limited and iron-replete conditions, suggesting that Cir1 might play a sensory role for iron levels (65, 66). Cir1 was also shown to play both positive and negative regulatory roles in *C. neoformans*. For example, Cir1 positively regulated genes for siderophore transport and negatively regulated genes for reductive iron uptake and melanin synthesis. Remarkably, loss of Cir1 attenuated virulence and influenced the elaboration of all of the major virulence factors, including derepression of melanin production and defects in capsule production and growth at 37°C. Therefore, Cir1 represents an iron-responsive transcription factor that integrates micronutrient (iron) acquisition with the expression of virulence factors and virulence (65, 66). It is notable that Cir1 shares positive regulation of capsule formation and negative control of melanin production with the two other GATA-type transcription factors, Gat1 and Gat201 (Fig. 1; Table 1).

Other transcriptional regulators involved in the regulation of iron acquisition functions have been identified in *C. neoformans*, including Tup1, Nrg1, Rim101, and HapX (32, 69, 83, 107). The global regulator Tup1 was found to influence iron uptake functions, and this protein is known to function with iron regulatory transcription factors in other fungi (82). This is consistent with the observation that a *tup1* deletion mutant shows similar phenotypes and influences on gene expression as the *cir1* mutant (Fig. 1; Table 1). The transcription factor Nrg1, which is thought to be a downstream target of the cAMP pathway, also regulated the transcript level of the siderophore transporter Sit1, as well as transcripts for functions involved in cell wall biosynthesis and carbohydrate metabolism (32) (Fig. 1; Table 1). More recently, O’Meara et al. identified the pH-responsive transcription factor Rim101 as a positive regulator of many of the same iron-related functions that are regulated by Cir1 (107). These include the high-affinity uptake system encoded by *CFT1* and *CFO1*, putative siderophore transporters and *SIT1*, and the highly iron-responsive gene *CIG1* (85, 129). In addition, Rim101 positively regulated the *CTR4* gene encoding a copper transporter and also regulated the expression of some of the genes necessary for capsule elaboration, including *UGD1* (UDP-glucose dehydrogenase), *CMT1* (mannosyltransferase), and *PNM* (phosphomannomutase). Interestingly, the rim101 mutant produced capsule polysaccharide but failed to properly attach it to the cell wall, a phenotype consistent with Rim101 regulation of cell wall biosynthetic functions. Despite the capsule defect, the rim101 mutant was slightly more virulent than the wild-type strain in a mouse inhalation model and was also better at surviving macrophage killing. Overall, Rim101 emerges as an important pH-responsive factor that regulates metal homeostasis, capsule production, and cell wall formation (107). Similar
to the rim101 mutant, a defect in capsule attachment also results from deletion of the GCN5 gene encoding a histone acetyltransferase (106). In this case, however, the mutant was unable to cause disease in mice (Fig. 1; Table 1).

The Hap proteins, Hap3, Hap5, and HapX, also regulate iron homeostasis in C. neoformans (69). These proteins and their homologs have been shown to contribute to iron regulation in S. cerevisiae, S. pombe, and A. nidulans (53, 97), and the transcriptome analysis of the cir1 mutant revealed that the HAP genes are also regulated by Cir1 in C. neoformans (66). HapX positively regulates a subset of the genes encoding putative siderophore transporters in C. neoformans and a hapX mutant showed a minor virulence defect, although the mutant produced wild-type levels of capsule and melanin. It has been suggested that HapX may be particularly important as a regulator of acquisition mechanisms for environmental rather than host iron sources (69).

**COPPER UPTAKE AND VIRULENCE**

The role of copper in the virulence of C. neoformans has been investigated through characterization of the copper regulatory transcription factor Cuf1 and copper transporters (28, 35, 62, 63, 87, 134). The CUF1 gene was initially identified as a quantitative trait locus on chromosome 7 that influenced variation in melanin formation (87). Deletion of the gene, which was designated MAC1 at the time, reduced melanin production, filamentation, and growth at high temperature. The mutant also showed growth sensitivity to both low- and high-copper conditions. Subsequently, CUF1 was also found in a collection of insertion mutants defective in melanin, and loss of Cuf1 interfered with copper-dependent functions, including loading of the metal on laccase (hence the melanin defect) (134). In this study, characterization of the cuf1Δ mutants revealed a growth defect under copper- or iron-limiting conditions, no defects in capsule or urease production, and attenuated virulence in a mouse model of cryptococcosis (35, 62, 63, 134) (Fig. 1; Table 1). The virulence analysis suggested that copper may be limiting in the CNS because the cuf1Δ mutant showed poor dissemination to the brain but no growth defect in lung tissue. Interestingly, Cuf1 has the dual role of regulating both copper acquisition (e.g., transporters) and detoxification (e.g., metallothionein) functions (35). In S. cerevisiae, these functions are each regulated by separate transcription factors, Mac1 and Ace1 (121).

Two functional copper transporters,Ctr1 and Ctr4, are present in C. neoformans (28, 35, 134). Ctr4 was initially characterized because its promoter was developed as a tool for regulated gene expression (108). More recently, a fungal strain carrying a Ctr4-green fluorescent protein (GFP) fusion was employed to demonstrate copper limitation after phagocytosis and in mouse brain tissue (134). In a separate study, CTR4 was shown to complement the defect in copper transport of an S. cerevisiae cuf1Δ ctr3Δ mutant, although deletion of the gene did not impair growth on copper-deficient medium (35). The CTR1 gene also complemented the yeast mutant, but deletion of this gene in C. neoformans caused a severe growth defect under the copper-deficient condition. The CTR1 sequence (gene CNAG_07701) was characterized previously and designated CTR2 (28). Based on phylogenetic evidence and functional studies, Ding et al. renamed the gene CTR1 (35). The gene is required for evasion of phagocytosis, and a deletion mutant has defects in capsule and melanin production and shows sensitivity to copper starvation (28). Similarly, rim101Δ, rim20Δ, and vps25Δ mutants have melanin and capsule defects, sensitivity to copper limitation, and increased phagocytosis (28). Thus, copper acquisition appears to be linked to capsule biogenesis and inhibition of phagocytosis.

**ADAPTATION TO HYPOXIA**

C. neoformans cells grow optimally in culture at atmospheric oxygen concentrations (21%), although levels in the human brain and near sites of inflammation or infection are significantly lower (99, 102). Therefore, C. neoformans must sense oxygen and adapt to hypoxia to colonize the brain and other tissues. Recently, homologs of the sterol regulatory element binding protein (SREBP) and the multispan membrane protein SREBP cleavage activating protein (SCAP) have been identified in C. neoformans (named Sre1 and Scp1, respectively) (24, 30). Sre1 is responsible for regulating genes involved in the transcriptional response to hypoxia, and activation of the protein requires Scp1 and processing by the Site-2 protease Stp1 in response to sterol depletion and hypoxic conditions (14, 24, 30, 131).

Characterization of the SREBP pathway in C. neoformans revealed its importance for regulating sterol synthesis, sensing oxygen, and mediating virulence in mice (13, 14, 24, 25, 30, 37, 59, 82). For example, Chang et al. characterized the pathway in a serotype D strain and used microarray analysis to demonstrate that Sre1 regulates genes for ergosterol biosynthetic enzymes, iron and copper uptake, stress-related functions, and various transport and metabolic functions under the low-oxygen condition (1%) (24). They also found that mutations in SRE1 and SCP1 genes resulted in reduced growth under low-oxygen and limiting iron conditions, although no defects were seen in capsule or melanin production (Table 1). The contribution of Sre1 to virulence was interesting because the sre1Δ mutant failed to cause disease (up to 60 days) when injected into mice via the tail vein. However, both the wild-type and mutant strains disseminated to the brain with equal efficiency at 5 h after injection, but the sre1Δ mutant failed to proliferate in this tissue in comparison to the wild-type strain at 2 days. In parallel, characterization of sre1Δ, stp1Δ, and scp1Δ mutants in a serotype A strain revealed a hypoxia-sensitive phenotype for all three mutants, as well as subtle defects in capsule and melanin production for the sre1Δ mutant (30). The mutants also showed attenuated virulence in a mouse intranasal infection model. The sre1Δ mutants in C. neoformans are hypersensitive to inhibitors of sterol biosynthesis, such as theazole antifungal drugs (e.g., fluconazole) (24, 30). In addition, disruption of the Sre1 pathway converts the activity of azoles drugs from fungistatic to fungicidal, thus raising the possibility of a synergistic effect between inhibitors of fungal SREBPs and current antifungal treatments (14). Overall, Sre1 represents an additional transcription factor that integrates metabolic activities for sterol biosynthesis, oxygen sensing, and metal uptake (among other functions) with an influence on virulence (Fig. 1; Table 1).

**CONCLUSIONS**

Transcriptional profiling and genetic studies are providing insights into C. neoformans adaptation to key features of the mammalian host environment, including glucose and nitrogen availability, iron and copper limitation, pH, and hypoxia. An outline of the nutritional requirements for fungal proliferation during disease is slowly emerging, along with the realization that many of the regulatory factors identified so far control metabolic and other
functions (e.g., the response to stress) as well as the elaboration of virulence factors. This integration of virulence with other functions is similar to the activities of some transcription factors in bacterial pathogens and is particularly marked for the transcription factors Gat201, Cir1, and Gat1. It is striking that these three GATA factors have a reciprocal influence on capsule formation and melanization, a finding that reinforces the complex regulation of these factors (Fig. 1). Certainly many more regulators remain to be characterized, particularly in the context of other aspects of adaptation, such as the fungal response to glucose limitation, host defense, and stress. Systematic deletion approaches will undoubtedly provide a more comprehensive view of the transcription factors that influence virulence. Additionally, there is considerable information available on the role of signal transduction in host adaptation, and these pathways go hand in hand with transcriptional regulation. For example, the transcription factor (Sp1) regulates virulence factor expression as part of the protein kinase C/cell wall integrity pathway (1). A key area of investigation will be to further dissect tissue-specific nutritional requirements to understand (and hopefully block) the striking neutropotism of C. neoformans. Overall, a detailed understanding of the requirements for fungal proliferation will contribute to strategies for pathogen control, including the discovery of relevant targets for antifungal therapy.

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