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The *Cryptococcus neoformans* Capsule: a Sword and a Shield

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INTRODUCTION

Prior to the widespread emergence of human immunodeficiency virus (HIV) infection, disease due to the opportunistic fungus *Cryptococcus neoformans* was uncommon. However, over the past several decades, this fungal pathogen has caused life-threatening disease in millions of patients worldwide. Recent epidemiological data from the World Health Organization suggest that over 1 million cases of cryptococcal infection occur each year among HIV-infected patients in sub-Saharan Africa, resulting in more than 600,000 annual deaths (161). Additionally, *Cryptococcus* species have caused recent infectious disease outbreaks in the Pacific Northwest regions of Canada and the United States. These trends emphasize the importance of understanding the basic biology of this fungus, especially the ways in which it has adapted to cause human disease.

C. neoformans lives primarily in the environment in a yeast-like form. Spores or small yeast cells are inhaled, resulting in primary pulmonary infection. Seroepidemiology studies indicate that the majority of people in areas where the fungus is endemic are exposed to it at a young age; however, in immunocompetent hosts, *C. neoformans* infections are minimally symptomatic and rapidly cleared (76). Serious disease occurs in the absence of intact cell-mediated immunity, such as in patients with advanced AIDS or organ transplant recipients receiving immunosuppressive therapies. In these immunocompromised hosts, *C. neoformans* can disseminate from the lungs and cross the blood-brain barrier, frequently resulting in meningoencephalitis, a central nervous system (CNS) infection that is fatal if it is not treated.

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Supplemental material for this article may be found at http://cmr.asm.org/.

doi:10.1128/CMR.00001-12

In both the environment and the infected host, *C. neoformans* produces a characteristic polysaccharide capsule. Investigators have speculated that this capsule may protect the fungus from environmental desiccation and/or natural predators, such as nematodes or amoebae (39, 70, 150, 191, 192, 226). In the host, the capsule serves many protective functions, including reducing host immune responses by downregulating inflammatory cytokines, depleting complement components, and inhibiting the antigen-presenting capacity of monocytes (174, 200, 201). The capsule can also act as a shield on the cell wall to regulate phagocytosis by macrophages (50, 160). Once inside macrophages, capsule serves as a sink for reactive oxygen species generated by the host, thus providing effective antioxidant defenses (224).

The *C. neoformans* capsule is also familiar to clinicians. Its characteristic appearance around the yeast cell is the basis for rapid microbiological identification in clinical samples such as cerebrospinal fluid (CSF). Recognition of encapsulated yeast cells in histopathological material, which are clearly visualized by mucicarmine staining, is sufficient to diagnose *C. neoformans* infections, even in the absence of culture data. Additionally, the capsular polysaccharide is the basis for very sensitive and specific diagnostic assays for cryptococcal infections.

There is considerable evidence that the capsule plays a central role in allowing *C. neoformans* to survive within the host and to cause disease. Unencapsulated *C. neoformans* cells are rarely observed in clinical samples. Moreover, specific mutations resulting in capsule defects typically result in a dramatic attenuation of *C. neoformans* virulence. Therefore, similar to bacterial capsules, the *C. neoformans* capsule is considered the most important virulence-associated factor of this organism. However, the chemical structure and organization of this fungal capsule are quite distinct from those of bacterial capsules.

In addition to having a unique chemical composition, the *C. neoformans* capsule is highly regulated in terms of its relative size and complexity. This regulation is important for the survival of *C. neoformans* in the host. When incubated under rich and permissive laboratory growth conditions, this fungus produces a small ring of capsule on the cell surface. However, *C. neoformans* dramatically induces capsule in response to host-specific conditions. In fact, many *in vitro* approximations of human host conditions have been used to induce capsule, including tissue culture media, 5% CO_2, low iron, and human physiological pH (pH 7) (11, 199, 225).

Some aspects of *C. neoformans* capsule regulation occur at the level of transcription. For example, incubation in the presence of the transcriptional inhibitor actinomycin D completely inhibits encapsulation without immediately affecting viability (77). However, many interacting and complementary signaling pathways likely regulate the complex biology of the capsule. The *C. neoformans* transcriptional programs triggered by these host environmental conditions have been investigated in an effort to understand the networks involved in the induction of capsule in response to host conditions. This review focuses on the different regulation programs that respond to specific host environmental cues to induce encapsulation. We attempt to critically review and synthesize the current information on the regulation of *C. neoformans* capsule synthesis, export, and assembly. Additionally, we suggest that fungal cell wall remodeling is an underexplored component of appropriate encapsulation within the host.

BIOLOGY OF THE CRYPTOCOCCAL CAPSULE

Capsule Structure

The *C. neoformans* capsule is composed of complex polysaccharides that are synthesized within the cell, transported across the cell wall through vesicles, and then attached noncovalently to the cell surface, where they can assemble into long polymers. Biochemical analyses of capsule by various chromatographic techniques and mass spectrometry demonstrated that it is composed primarily of glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal). Nuclear magnetic resonance (NMR) was used to examine the precise structures of these components. GXM is composed of *O*-acetylated α-1,3-linked mannose residues with xylosyl and glucuronyl side groups (118). The approximate weight-averaged mass of GXM is between 1,700 and 7,000 kDa, and it makes up approximately 90% of the *C. neoformans* polysaccharide capsule (140). In contrast, GXMGal is an α-1,6-linked galactose polymer with mannose, xylose, and glucuronic acid modifications (83).

Dynamic changes in capsule were demonstrated initially by alterations in antibody binding and later by more detailed and direct biophysical measurements of capsule structure (71, 72, 190). For example, the number and order of each of the modified residues in the capsule polymers can vary, leading to the antigenic heterogeneity used in diagnostics and serotyping (141). Analysis of the radius of gyration of the polysaccharide fibrils demonstrated complex branching of the polysaccharide polymer, which can result in further structural heterogeneity (45). Mass spectrometry and NMR analysis demonstrated that some of the structural differences detected by variable antibody binding can be caused by glucuronic acid positional effects (141). Importantly, the overall structure of the capsule can also vary in different host environments (36, 53, 118). For example, *C. neoformans* recovered from different organs during murine infections demonstrates variable binding to anticapsule antibodies (64). Additionally, experimental infection of *Galleria mellonella* wax moth larvae results in capsules with increased density compared to those of identical strains grown *in vitro*, as measured by the penetrance of antibody binding (70). The changes in capsule structure, size, and density are potentially a mechanism for escape or evasion from the immune system, demonstrating that capsule is a dynamic structure that is highly regulated by the cell in response to specific environmental cues.

Capsule Synthesis

The capsular polysaccharide is made from simple sugars that are modified and assembled into more complex structures. Investigators have studied the initial biochemical processes involved in the synthesis of the capsular monomers and the addition of the subunits to the elongating capsule polymer. Using bacterial capsule synthesis as a model, the Doering lab was able to determine (via homology) some of the enzymes required for capsule synthesis in *C. neoformans.* This work was complemented and supported by genetic screens for capsule mutants performed by the Janbon lab. Although some of the genes and biochemical intermediates of capsule are known, there are many steps that have not been elucidated completely.

Capsule monomers. The capsular polysaccharide is made by polymerization of simple sugars into an elongating carbohydrate backbone. These initial steps depend upon carbohydrate metabo-

lism to allow for a sufficient supply of the starting sugars. Moreover, the addition of different carbon sources to the growth medium can result in alterations in capsule composition (80). The base components of the capsule are UDP-glucuronic acid, UDP-galactose, UDP-xylose, and GDP-mannose. UDP-glucuronic acid is made from the conversion of UDP-glucose to UDP-glucuronic acid via the membrane-localized Ugd1 UDP-glucose dehydrogenase (78, 96, 146). The Uxs1 decarboxylase then converts UDP-glucuronic acid to UDP-xylose (19). UDP-galactose, which is required for GXMGal, is created from UDP-glucose by the Uge1 epimerase (148). GDP-mannose is synthesized via a phosphomannose isomerase, a phosphomannomutase, and a GDP-mannose pyrophosphorylase. Currently, only the phosphomannose isomerase, Man1, has been examined in *C. neoformans* (214). Putative genes for potential phosphomannomutases have been identified in the genome, but their direct action on the production of GDP-mannose has not been defined in detail.

Modification of capsule monomers. The base monomers of both GXM and GXMGal are then combined and modified with specific side chain moieties that are important for the assembly, branching, and overall structure of the fibrils. One modification of the GXM and GXMGal monomers is xylosylation. This process is mediated by the Cxt1 β-1,2-xylosyltransferase (28, 113, 114). This enzyme transfers xylose to α-1,3-dimannoside to create Xyl-β-1,2-Man-α-1,3-Man. In a *cxt1*Δ mutant strain, the cell has reduced xylose on GXM monomers and a complete lack of xylose on the GXMGal monomers; this strain is subsequently attenuated for virulence (113). The Cap10, Cap1, Cap4, and Cap5 proteins have homology to Cxt1, and these enzymes may be involved in the addition of β-1,3-linked xylose to capsule (114). Due to the amount of branching and the observed phenotypic switching of strains, it is likely that these proteins are regulated specifically to alter the overall capsular structure.

Another modification is the addition of activated mannose groups to the carbohydrate backbone. This addition occurs within an organelle, and transport of GDP-mannose is mediated by the Gmt1 GDP-mannose transporter (48). Mannosylation of the backbone is performed by α-1,3-mannosyltransferases, most likely Cmt1 and Cap59 (57, 187).

Further modification of GXM and GXMGal comes through *O*-acetylation, and this is performed by the Cas1 glycosyltransferase (98). The *O*-acetylation occurs on the mannose and glucuronylated mannose residues, and the antigenicity of the capsule in *cas1*Δ mutant strains is drastically altered (98, 118). The Cap64-like proteins Cas3, Cas31, Cas32, Cas33, Cas34, and Cas35 may be involved in assembling the monomers or adding modifiers. These proteins were identified in a screen for mutants involved in capsule structure (145). However, only Cap64 is required for the production of visible capsule around the cell (33).

Pbx1 and Pbx2 are parallel β-helix proteins that potentially act as a complex to regulate the incorporation of glucose residues into the backbone (133). Mutations in these proteins do not prevent encapsulation, but the mutant capsule is easily detached from the cell by sonication. This fragile capsule contains GXM with aberrant glucose molecules. However, the role of normal glucose incorporation in GXM is still unclear.

Finally, the capsule contains hyaluronic acid (HA), which is important for crossing the blood-brain barrier (102). Cells lacking HA have a slightly decreased capsule diameter and a defect in cell wall ultrastructure, although the cause-and-effect relationship is not clear (31, 102). Recent work revealed that the Cps1 protein is responsible for the synthesis of HA, although the timing, amount, and induction of HA are still under examination (102). Most interestingly, the presence of HA on the cell surface may actually facilitate fungal cell entry into the CNS by facilitated transport across the blood-brain barrier (101, 102). The genes involved in capsule biosynthesis are presented in Table 1.

Location of capsule synthesis. Capsule nucleotide sugar donors are synthesized in the cytoplasm, and the backbone and modifiers are assembled near the cell wall, in organelles, before transport across the cell wall (220). After transport across the cell wall, the polymers grow in length when cells are placed under inducing conditions (151, 166, 221). Currently, the mechanism by which the polymers extend is unknown, although there is consensus that the size is mediated at the level of individual polysaccharide molecules (68, 221). One hypothesis is that the capsular fibrils have inherent properties that promote self-assembly via divalent cations (140). Recent work demonstrated that the new capsular material can be incorporated at the edge of the capsule, distally from the cell, with some intercalation of new material throughout the structure (227). The long fibrils can then act as a scaffold, allowing for the formation of a dense capsule structure near the cell (68). However, both antibody and complement binding, used to determine the position of the newly incorporated capsule, can affect the capsular structure, making it difficult to determine the normal process of capsular enlargement (56, 65, 139). Identifying the position of the new capsule has implications for the processes involved in extending the length of the polymer.

The polysaccharide capsule is visualized most easily when it is maintained at the cell surface. However, it is clear that some polysaccharide is secreted and not maintained around the cell. Recently, there has been interest in exploring the differences between this exopolysaccharide and the surface-attached polysaccharides (67, 79). Analysis of capsular material, either shed into the medium or removed from the cell by various chemical treatments, demonstrated that although the composition was consistent between the two preparations, the ratios of the components varied significantly between the soluble and the attached polysaccharides (67). It is currently unclear whether different biosynthesis processes create these two types of polysaccharide.

Capsule Secretion

Due to the large size of the capsular polysaccharide, this polymer must be actively transported across the cell wall. Initial reports demonstrated the presence of vesicles potentially carrying capsular polysaccharides after the cryptococcal cells were ingested by macrophages (178, 197, 220). In the past few years, several microscopic, biochemical, and genetic studies have verified the vesicular transport of capsule. Analysis of excreted vesicles demonstrated the presence of virulence-associated components, including capsule (154, 177). Quick-freeze deep etching revealed the accumulation of particles/vesicles in the outer region of the cell wall. The number of particles was greater *in vivo* than *in vitro*, which Sakaguchi et al. attributed to an increase in the secretion of vesicles containing capsular precursors (179). Treatment with inhibitors of vesicle transport, such as brefeldin A, nocodazole, monensin, and *N*-ethylmaleimide, decreased the amount of capsule (92).

TABLE 1 Genes potentially involved in capsule biosynthesis

CNAG ID	Gene product annotation	Capsule phenotype of mutant	Domain(s)	Reference(s)
CNAG_00124	Cas32	Alteration in carbohydrate ratios, hypocapsular when combined with *cas3*Δ mutant	Signal peptide, transmembrane domain	145
CNAG_00596	Utr2		Signal peptide, transmembrane domain	Homology to chitin transglycolase
CNAG_00600	Capsular associated protein		Signal peptide	Homology to glycosyltransferase
CNAG_00697	Uge1	Larger capsule but no GXMGal	Transmembrane domain	148, 149
CNAG_00701	Cas31	Decreased capsule, alteration in carbohydrate ratios	Signal peptide, transmembrane domain	145
CNAG_00721	Cap59	Decreased capsule	Signal peptide domain	32, 56, 69
CNAG_00744	α-1,6-Mannosyltransferase		Transmembrane domain	Homology to CMT (1)
CNAG_00746	Cas35	Decreased capsule	SGNH superfamily	145
CNAG_00926	Glycolipid mannosyltransferase			Homology to mannosyltransferases
CNAG_00996	Pmt4	Decreased capsule size	11 transmembrane domains	213
CNAG_01156	Cap2		Transmembrane domain	Homology to Cap (10)
CNAG_01172	Pbx1	Dry colony morphology, defect in capsule integrity	Signal peptide domain	133
CNAG_01283	Cap5		Transmembrane domain	Homology to Cap (10)
CNAG_01654	Cas34	Decreased capsule size	Signal peptide, transmembrane domain	145
CNAG_02036	Cas4	Altered reactivity against GXM antibodies	9 transmembrane domains, transporter domains	147
CNAG_02581	Cas33	Decreased capsule size	Transmembrane domain, SGNH superfamily	145
CNAG_02797	Cpl1	Decreased capsule	Signal peptide, transmembrane domain	132
CNAG_02885	Capsule-associated protein		Transmembrane domain	Homology to Cas (35)
CNAG_03096	Uge1	Defective GXMGal production, larger capsule size	Glucose epimerase	148, 149
CNAG_03158	Cmt1	Decreased capsule size	Transmembrane domain	187
CNAG_03322	Uxs1	Capsule is missing xylose	Epimerase domain	118, 147
CNAG_03438	Hxt1	Increased capsule size	Signal peptide, 10 transmembrane domains, sugar transporter	38
CNAG_03644	Cas3	Decreased capsule when combined with *cas31*Δ, *cas32*Δ, or *cas33*Δ mutants; defect in O-acetylation	Transmembrane domain, signal peptide	145
CNAG_03695	Cas41		8 transmembrane domains, transporter domains	Homology to Cas (4)
CNAG_03735	Cap4		Transmembrane domain, signal peptide	Homology to Cap (1)
CNAG_04312	Man1	Defect in capsule production	Phosphomannose isomerase	214
CNAG_04320	Cps1	Slight defect in capsule	Glycosyltransferase, 3 transmembrane domains	31, 102
CNAG_04969	Ugd1			78, 146
CNAG_05139	Ugt1	Increased capsule size		149
CNAG_05148	Cap3		Transmembrane domain, xylosyltransferase	Homology to Cxt (1) and Cap (10)
CNAG_05562	Pbx2	Dry colony morphology, defect in capsule integrity	Pectin lyase-like domain	133
CNAG_06016	Cap6			Homology to Cmt (1) and Cap (59)
CNAG_06813	Cap1		Signal peptide, transmembrane domain	Homology to Cap (10)
CNAG_07554	Capsule-associated protein		Signal peptide, transmembrane domain	Homology to Cap (10)
CNAG_07937	Cas1	Defect in capsule O-acetylation, reactivity to GXM antibodies		147

Mutations in the secretory pathway (Sec4/Sav1 and Sec6) also resulted in decreased capsule on the cell surface (159, 220). Because Sec4 is involved in post-Golgi secretion events, the Golgi apparatus was implicated in capsule secretion (220). Additionally, Arf1, an ADP-ribosylating factor involved in vesicle formation and intracellular trafficking via the Golgi apparatus, is involved in capsule secretion (204). Recently, a Golgi reassembly and stack protein (GRASP) was shown to be required for capsule secretion (115). *grasp*Δ mutants had defects in capsule size and consequent increases in phagocytosis rates and decreases in virulence. Kmetzsch et al. (115) suggested that the defect in capsule size in the *grasp*Δ mutant may have been a product of decreased polysaccharide secretion.

Appropriate vesicle physiology is also required for capsule induction around the cell. Vph1, a V-type ATPase that is required for vesicle acidification, is important for capsule transport. Without Vph1, cells demonstrate a dramatically reduced capsule. Treatment with bafilomycin A1, which prevents vesicle acidification, also represses capsule (62).

However, the mechanism by which the capsule is packaged and released from the vesicles to then attach to the surface of the cell is currently unknown. It is also possible that there is a difference in the secretion of exopolysaccharide and attached polysaccharide (67).

Attachment

After secretion, the capsule must be maintained around the cell. The cell wall appears to be the major determinant of capsular attachment, whether it is through direct linkages between wall components and capsular material or through providing a scaf-

fold for proteins that then mediate the attachment. The cell wall is a dynamic material, with continuous remodeling required for budding, growth, and mating. Investigators studying other fungal species have demonstrated changes in cell wall composition in response to the host, and this process is being explored in *C. neoformans* as well (16, 24, 144, 152, 226). Additionally, the cell wall has been of particular interest due to the resistance of *C. neoformans* to the echinocandin class of antifungal agents, which inhibit cell wall β-glucan synthesis (136). The effects of cell wall composition and remodeling on capsule attachment have not been explored fully, but there are hints from transcriptional profiling that changes in the cell wall are required for encapsulation within the host.

The *C. neoformans* cell wall is composed of β-1,3 and β-1,6 glucan, α-1,3 glucan, chitin, and chitosan, in addition to mannoproteins and other glycosylphosphatidylinositol (GPI)-anchored proteins (1, 14–16, 18, 75, 126). Although these components are extensively cross-linked, there are still overall striations or layers that can be visualized through electron microscopy and quick-freeze deep etching (18, 173, 179). The inner layer is composed primarily of β-glucans and chitin, and the outer layer contains α-glucan and β-glucan (173). Unlike those of other fungi, the *C. neoformans* cell wall has more β-1,6 glucan than β-1,3 glucan; however, the β-1,3 glucan synthase, Fks1, is essential, indicating the importance of this conserved cell wall component (75, 196). Table 2 includes all cell wall genes that have a demonstrated effect on capsule attachment and some genes putatively involved in cell wall biogenesis.

Cell wall glucans. Recently, the Skn1/Kre6 family of potential β-1,6 glucan synthases was examined in detail, and Gilbert et al. demonstrated that Kre5 and both Kre6 and Skn1 are required for maintenance of normal capsular architecture, as determined by dextran penetrance and India ink staining (75). However, a more dramatic phenotype was observed when the gene encoding the α-1,3 glucan synthase, *AGS1*, was mutated. In the *ags1*Δ strain, there was no capsular attachment, but apparently normal capsular material was shed into the medium, where it could attach to other acapsular cells (172, 173, 186). Our recent work demonstrates that α-glucan is induced on the cell wall under capsule-inducing conditions (unpublished data). *Histoplasma capsulatum*, another opportunistic pathogen, induces α-1,3 glucan to hide immunogenic cell wall components from recognition by the host (137, 171). Therefore, the α-glucan in *C. neoformans* may be involved in avoiding immune recognition in two ways. First, it is required for attaching capsule, and second, it may shield the immunogenic β-glucans and chitin molecules from the host immune system.

Chitin and chitosan. Chitin and chitosan make up approximately 10% of the *C. neoformans* cell wall in a *cap67*Δ mutant strain (74, 97). In the *C. neoformans* genome, there are 8 genes for chitin synthesis, 3 for chitin synthase regulators, 4 for chitin deacetylases, and 5 for chitinases, making the role of a single gene difficult to determine (14, 16, 18). However, substantial work by the Lodge lab has elucidated the roles of many of these components.

In *Saccharomyces cerevisiae*, a chitin synthase gene is transported to the membrane through the Golgi secretory pathway. During cell stress, chitin accumulates in the cell wall, and the overall increase in chitin can also be regulated by increases in the levels of chitin precursors (UDP-GlcNAc) (25). The regulation of chitin accumulation in *C. neoformans* is similar, with accumulation during cell stress. Unlike the case in *S. cerevisiae*, the levels of chitosan in *C. neoformans* are three to five times higher than the levels of chitin, and the ratio of chitin to chitosan changes with cell density (18). Banks et al. (18) also determined that, during vegetative growth, the Chs3 protein produces chitin that is subsequently converted to chitosan. Additionally, they demonstrated that Chs3 activity is regulated by Csr2. Accordingly, in *chs3*Δ and *csr2*Δ mutant strains, the levels of chitin are increased and the levels of chitosan are decreased.

To further examine the regulation and synthesis of chitosan, Baker et al. created triple and quadruple mutants of the four chitin deacetylase genes (14). In *cda1*Δ *cda2*Δ double mutants and the *chs3*Δ single mutant, decreased chitosan levels correlated with increased chitin levels and increased capsule size (14). One hypothesis is that chitosan normally masks capsule attachment sites, preventing encapsulation of the cell. Chito-oligomers can interfere with capsular assembly *in vitro*, so decreased chitosan may allow for better capsule assembly (66). However, chitin-like structures can be incorporated into the capsular material, and this can result in increased shear resistance and cross-linking (226). Additionally, chitosan-deficient strains grow slowly, especially under *in vivo* conditions. This slower growth may allow for increased capsule size, as suggested by Zaragoza et al. (15, 223).

Cell wall proteins. Proteins that are embedded in the cell wall carbohydrates are also likely to be important for capsule attachment, potentially acting as anchors for the polysaccharide fibrils. The two most highly studied mannoproteins in *C. neoformans* are MP98 and MP88, both of which have GPI anchors (93, 126). These proteins were first identified as highly immunogenic molecules, capable of stimulating a robust T-cell response. The MP98 protein is a chitin deacetylase, and it may play a role in chitosan levels in the cell wall (14).

Phospholipase B1 (Plb1) is another GPI-anchored protein in *C. neoformans.* Plb1 is covalently bound to β-1,6 glucan and is involved in the maintenance of cell wall integrity (183). Although the diameter of the capsule of *plb1*Δ mutants is similar to that for the wild type, transmission electron microscopy (TEM) established that the capsule density is decreased in the mutant. Recent work has suggested an association between Plb1 activity and titan cell formation, and this is discussed later in this review. Plb1 may be necessary to cleave certain host phospholipids to allow for activation of specific signaling pathways (39). Plb1 is secreted using the same vesicle-dependent pathway as that used by the capsule monomers.

REGULATION OF CAPSULE INDUCTION IN SPECIFIC ENVIRONMENTS

An important facet of the *C. neoformans* surface capsule is that it is induced upon entry into the host. The cell must be able to sense the external environment and respond appropriately, especially because the capsule is an important factor in survival within the host. The degree of encapsulation corresponds with survival under many host-specific conditions.

There are a number of external signals that are able to induce capsule in *C. neoformans.* Each condition can induce specific capsule phenotypes, ranging from the size of the induced capsule to the antigenic variability of the capsule. The various capsule phenotypes in different organs suggest that the ability of *C. neoformans* to dynamically alter its capsule is physiologically relevant

TABLE 2 Genes potentially involved in capsule attachment and cell wall remodeling

CNAG ID	Gene product annotation	Capsule phenotype of mutant	Domain(s)	Reference(s)
CNAG_00373	Glucan 1,3-β-glucosidase		Transglycosidase family	
CNAG_00546	Chs4		5 transmembrane domains	18
CNAG_00897	Skn1	Increased capsule diameter and altered appearance when combined with *kre6*Δ mutant		75
CNAG_00914	Kre6	Increased capsule diameter and altered appearance when combined with *skn1*Δ mutant		75
CNAG_00939	Putative glucan 1,3-β-glucosidase			
CNAG_01230	Cda2	Increased capsule when combined with *cda1*Δ mutant		14
CNAG_01239	Cda3	Increased capsule when combined with *cda1*Δ mutant		14
CNAG_01941	Putative β-1,3 glucan biosynthesis-related protein			Homology to glucan synthesis and regulation proteins
CNAG_02217	Chs7		7 transmembrane domains	18
CNAG_02225	Cellulase		Signal peptide	
CNAG_02283	Glucan 1,4-α-glucosidase		Signal peptide	
CNAG_02351	Chi4	No change		16
CNAG_02598	Chi21	No change		16
CNAG_02850	α-1,3 Glucosidase		Signal peptide	Homology to Agn (1)
CNAG_02860	Endo-1,3(4)-β-glucanase		Signal peptide	
CNAG_03099	Chs1		6 transmembrane domains	18
CNAG_03120	Ags1	Decrease in capsule attachment		172, 173
CNAG_03326	Chs2		7 transmembrane domains	18
CNAG_03412	Chi2	No change		16
CNAG_03648	Kre5	Increased capsule diameter, altered appearance		75
CNAG_04033	α-1,4-Glucosidase		Signal peptide, transmembrane domain	
CNAG_04245	Chi22	No change		16
CNAG_05581	Chs3		6 transmembrane domains	18
CNAG_05663	Scw1		RNA binding domain	
CNAG_05799	Cda1	Increased capsule when combined with *cda2*Δ and *cda3*Δ mutants		14
CNAG_05815	Kre64		Transmembrane domain	75
CNAG_05818	Chs5		6 transmembrane domains	18
CNAG_06031	Kre63		Homology to Skn1	75
CNAG_06336	Glucan 1,3-β-glucosidase protein		Transmembrane domain	
CNAG_06411	α-1,3-Glucanase		Signal peptide	Homology to Agn (1)
CNAG_06487	Chs6		5 transmembrane domains	18
CNAG_06508	Fks1		16 transmembrane domains	196
CNAG_06659	Hex1		Signal peptide	16
CNAG_06678	Csr1		Sel1 repeats	18
CNAG_06726	Csr3		Sel1 repeats	18
CNAG_06832	Kre62		Transmembrane domain	75
CNAG_06835	Kre61		Transmembrane domain	75
CNAG_07499	Chs8		6 transmembrane domains	18
CNAG_07636	Csr2		Sel1 repeats	18
CNAG_07736	Glucan endo-1,3-α-glucosidase		WSC domains	Homology to Agn (1)

(141). Figure 1 demonstrates the production of capsule in a wild-type strain under commonly used *in vitro* capsule-inducing conditions, including low-iron medium (LIM), Dulbecco's modified Eagle's medium at 37°C with 5% CO_2 (DMEM), and 10% Sabouraud's medium buffered to pH 7.3. However, the induction of capsule around the cell does not appear to be due solely to the induction of the various biosynthetic genes. The next sections discuss the signaling pathways that regulate capsule and the transcriptional outputs that result in capsule induction (see Table S2 in the supplemental material) (44, 119, 194).

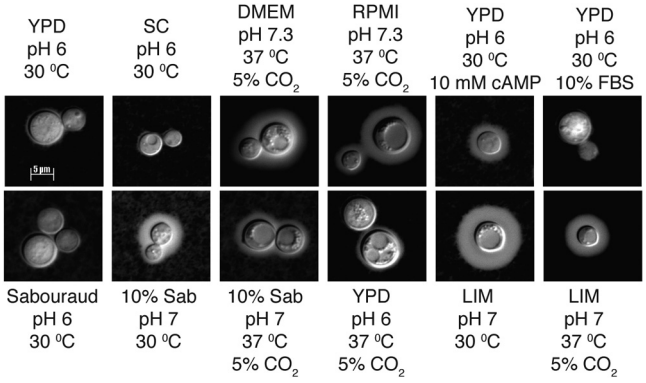

FIG 1 Different inducing conditions result in various degrees of encapsulation in the wild-type strain. Cells were incubated for 48 h in the specified media. Capsule was visualized by counterstaining with India ink. SC, synthetic complete medium; FBS, fetal bovine serum; Sab, Sabouraud medium.

SIGNAL TRANSDUCTION PATHWAYS THAT INDUCE CAPSULE

Low Iron

Iron binding and sequestration of iron are among the most basic mechanisms of protection against invading microorganisms (94, 104, 198, 210–212). The host sequesters iron in hemoglobin, transferrin, lactoferrin, and ferritin, preventing microbes from accessing this essential nutrient (211). To adapt to this low-iron environment, many microorganisms use iron transporters and siderophores to facilitate the uptake and scavenging of iron from the environment (94, 169, 195). Some fungi are unable to synthesize their own siderophores, but they may also acquire iron via siderophores produced from other species. As an opportunistic human pathogen, *C. neoformans* must also adapt to these low-iron conditions. In addition to increasing active transport of iron and uptake of siderophores, *C. neoformans* responds to low iron by inducing a large amount of surface capsule (199). Low-iron conditions alone are able to induce larger capsules than those induced by most of the other known *in vitro* capsule-inducing conditions (Fig. 1) (123, 199). Currently, there are two main signaling pathways that regulate adaptation to low iron, although the specific sensors and downstream outputs are still being defined. Figure 2 demonstrates the current knowledge of the iron-regulated transcriptional network.

One of the major regulators of adaptation to low iron is the Cir1 transcription factor. Cir1 is a repressive GATA-type transcription factor with homology to the iron-regulating Fep1, Sfu1, and Urbs1 transcription factors in other fungi (163, 164, 203). Additional levels of iron regulation come from the HapX, Hap5, and Hap3 CCAAT-binding factors, which act in concert with the Cir1 transcription factor in *C. neoformans* and with other iron-regulating factors in other fungi (106). The CCAAT-binding complex is able to repress iron-dependent processes under low-iron conditions, and in *C. neoformans*, this complex also induces processes that increase iron uptake (7, 100, 106). Interestingly, only *hap3*Δ and *hap5*Δ mutants have a defect in capsule; a *hapX*Δ mutant, despite having a larger effect on iron-related transcription, does not exhibit a capsule defect (106). Encoded upstream of Cir1 is the Gat201 transcription factor, which directly transcriptionally regulates the expression of Cir1 (40).

Sensing or maintaining iron homeostasis is important for multiple host-specific phenotypes. The importance of iron regulation in adaptation to the host is exemplified by the phenotypes of a *cir1*Δ mutant strain. *cir1*Δ mutants have a defect in capsule induction, along with temperature sensitivity and dysregulation of melanin production, all of which are important adaptations to the host (108). The abundance of the Cir1 protein increases under high-iron conditions, as expected for a transcription factor that responds to high iron by increasing iron uptake by the cell (105).

Further evidence for low-iron induction of capsule comes from two mutant strains. Cig1 is involved in iron uptake, and mutations in this gene result in increased induction of surface capsule under iron-replete conditions (108). In the JEC21 strain, mutation of the Cft1 iron uptake gene also results in increased capsule (131). In these mutants, it is likely that cells cannot accurately maintain iron homeostasis because they are unable to import iron under normal conditions. By mutating these iron uptake processes, the cells are effectively experiencing a low-iron state, which results in capsule induction.

To determine the processes induced by low iron that are involved in regulating capsule, the Kronstad group performed serial analysis of gene expression (SAGE) and microarray analyses to assess global transcription patterns under low-iron and high-iron conditions. Surprisingly, in response to incubation in LIM, most of the known capsule biosynthesis genes discussed previously were not significantly differentially regulated. Although the transcription of Cap60 was increased 2-fold in LIM at 6 h in the B3501 strain, Cap59, Cap10, and Cap64 were not expressed differentially (131). In the H99 strain after 6 h in LIM, the only capsule biosynthesis genes that were differentially transcribed were a Cap64-like gene and *UXS1* (108). The major biological process induced by low iron appeared to be cell wall and membrane synthesis, as revealed by both microarray and SAGE analyses of expression (106). These transcriptional profiles suggest that cell wall attachment may be the main capsule-associated phenotype regulated under low-iron conditions.

To determine how the Cir1 transcription factor regulates these processes, Jung et al. examined the transcriptional profile of the *cir1*Δ mutant under both high- and low-iron conditions. From these experiments, they determined that Cir1 also regulates many cell wall integrity processes under low-iron conditions, in addition to regulating the expression of the capsule biosynthetic enzymes (108). These results were supported by data from Chun et al., who examined the transcriptional profile of a *gat201*Δ strain under tissue culture conditions (40).

Another important pathway for responding to low iron is the Pka1-cyclic AMP (cAMP) pathway; mutants in this conserved signaling cascade are unable to induce capsule in response to low-iron conditions (3–5, 59, 92, 131, 170). Figure 2 illustrates the known elements of the highly conserved cAMP-protein kinase A (PKA) pathway. External signals are sensed by G-protein-coupled receptors (GPCRs) that then activate a heterotrimeric G protein and cause dissociation of the Gα subunit (Gpa1) from the Gβγ subunits (Gib2, Gpg1, and Gpg2) (3, 127, 158, 218). Activated Gpa1 then signals through the adenylyl cyclase Cac1, which acts to convert ATP to cAMP (5). Although the cAMP and Ras pathways are separate in *C. neoformans*, the *C. neoformans* Cac1 protein still interacts with a CAP/Srv2 homologue (Aca1) to regulate cAMP levels (9). Cac1 also responds directly to intracellular carbon dioxide, a process mediated by the Can2 carbonic anhydrase (see

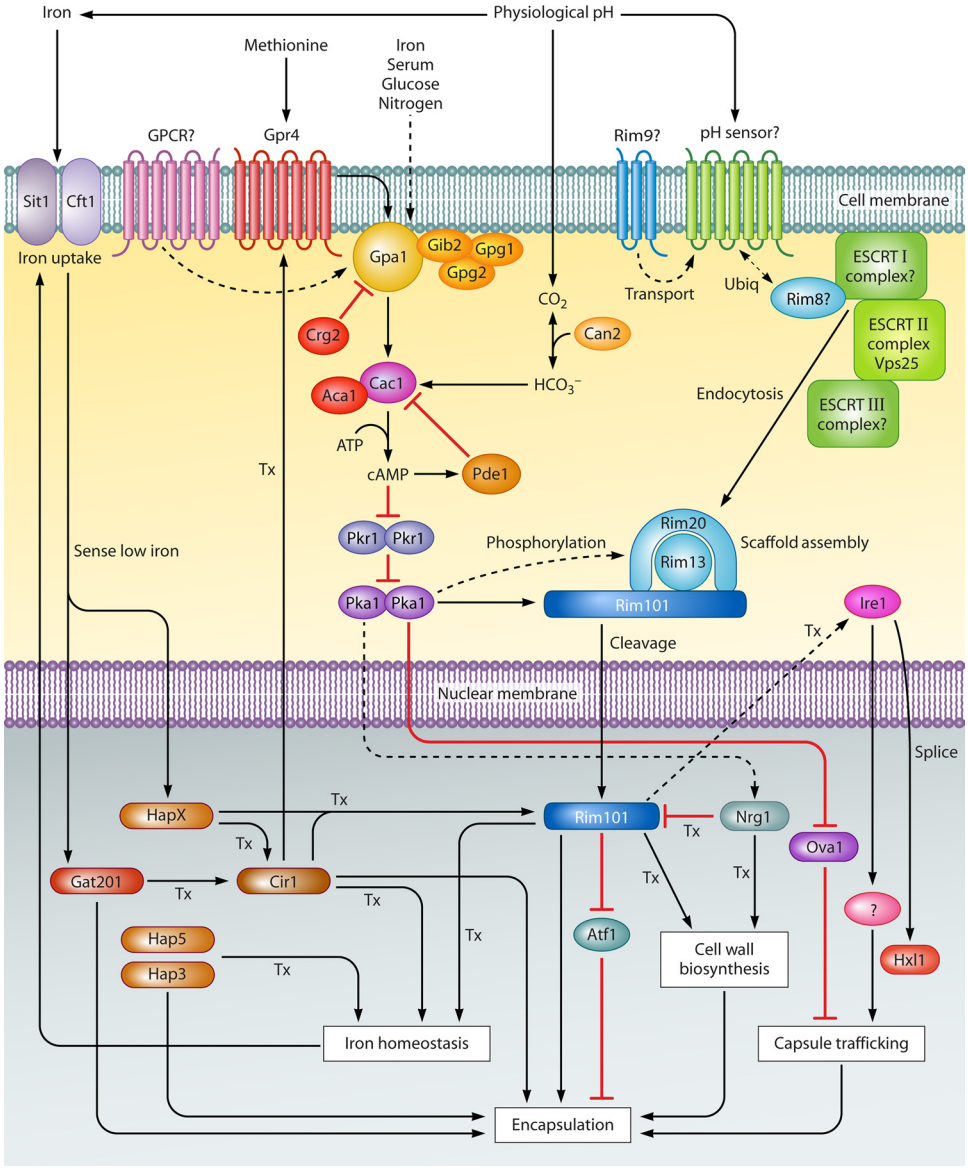

FIG 2 *C. neoformans* signal transduction networks that respond to iron, glucose, physiological CO_2, and host pH signals. The dashed lines indicate connections that are established primarily by transcriptional data or homology; these processes require further study to determine the nature of the interaction.

below) (11, 143). Production of cAMP causes release of the two regulatory subunits (Pkr1) from the protein kinase A active subunits (Pka1) (59). Pka1 is then free to phosphorylate a number of downstream targets, including the Rim101 transcription factor, to allow for cellular adaptation to the environmental conditions that initially activated the cascade (59, 91, 156). The Ova1 mannoprotein is negatively regulated by Pka1, and the *ova1*Δ mutant has increased capsule (92). Due to its homology with phosphatidylethanolamine-binding proteins (PEBPs), Ova1 was implicated in the regulation of capsule trafficking. PEBPs have also been implicated in mitogen-activated protein kinse (MAPK) signaling, potentially connecting Ova1 with other signaling cascades.

The pathway also has a number of negative-feedback elements, including the Crg2 regulator of G-protein signaling, which interacts directly with Gpa1 to limit cAMP production (180, 219). Additionally, the Pde1 phosphodiesterase negatively regulates the pathway by degrading intracellular cAMP (90). The basic structure of this signaling cascade is highly conserved among eukaryotes. However, the specific activating stimuli and downstream effectors in *C. neoformans* allow this pathogenic fungus to use the cAMP-PKA pathway to respond to numerous conditions relevant to the host.

The connections between *C. neoformans* cAMP signaling and low iron were first examined in the context of a *gpa1*Δ mutant. This mutant displays a striking defect in capsule under low-iron conditions, and this defect is rescued with the addition of exogenous cAMP (3). In contrast, addition of exogenous cAMP to a *cir1*Δ mutant strain is not sufficient to restore capsule (108). Despite this separation, there is evidence of cross talk between the Cir1 transcription factor and elements of the cAMP pathway. Cir1 transcriptionally regulates Gpr4, which can associate with Gpa1 to activate cAMP signaling (108). Downstream of Pka1, there are

further connections to iron homeostasis. The Rim101 transcription factor is directly activated by Pka1 phosphorylation and transcriptionally regulated by both Cir1 and HapX. Cir1 induces Rim101 transcripts under both low- and high-iron conditions, and HapX induces both Cir1 and Rim101 in LIM (106). Finally, Pka1, Cir1, and HapX are all involved in the transcriptional regulation of many iron transporters and siderophore uptake genes (Cft1, Cfo1, and Sit1), and this is likely mediated through Rim101 activation (92, 106–108, 156, 195). However, *rim101*Δ cells do not have a defect in capsule production under low-iron conditions. Currently, the direct connections between these transcription factors and the elements that regulate surface capsule induction are still unknown.

To further examine this relationship, it is possible to compare the downstream targets of these pathways. Similar to the downstream responses regulated by Cir1 and HapX, Pka1 does not appear to induce the transcription of known capsule biosynthesis genes under low-iron conditions. In a *pka1*Δ strain incubated in low-iron medium, *CAS35* and *CAP10* transcripts were decreased (92). The level of *UGD1* transcripts was not significantly differentially regulated between the wild-type and mutant strains. Interestingly, *UXS1* transcripts were increased in both *pka1*Δ and *pkr1*Δ strains (92). Overall, the majority of the other capsule synthesis genes were not differentially expressed in the *pka1*Δ mutant. Instead, multiple genes potentially involved in secretion and cell wall remodeling demonstrated significantly different expression (92). The Ags1 α-glucan synthase, Fks1 β-glucan synthase, and other glucan-modifying enzymes were differentially regulated in the *pka1*Δ mutant under low-iron conditions (92). RNA sequencing experiments performed on the *pka1*Δ mutant after incubation in DMEM confirmed the differential regulation of the Fks1, Ags1, Agn1, Kre6, Kre61, and Skn1 glucan synthesis-related genes. The genes involved in cell wall remodeling processes are presented in Table 2. These results suggest that Pka1 is involved in regulating primarily the secretion and attachment of capsule under low-iron conditions instead of the expression of capsule biosynthesis genes.

Host CO_2 Levels

Increased carbon dioxide is a strong host-specific signal that is used by many fungal pathogens to trigger phenotypes that allow for invasion and disease. In *Candida albicans*, 5% CO_2 triggers invasive hypha formation and disease (112, 184). *C. neoformans* also responds strongly to host levels of carbon dioxide—in this pathogen, the important phenotype is the induction of the polysaccharide capsule (77, 112, 199, 225). Both *C. albicans* and *C. neoformans* use the cAMP pathway to respond to CO_2, and the response bypasses the membrane-bound G proteins (11, 112). Instead, the dissolved bicarbonate can directly stimulate adenylyl cyclase to induce cAMP synthesis (112, 230). See Fig. 2 for the detailed structure of the cAMP-PKA pathway.

Two parallel studies on the Can2 carbonic anhydrase protein demonstrated that *C. neoformans* uses Can2 to convert CO_2 to HCO_3 (11, 143). Can2 is required for growth in the environment but is dispensable for growth in the host, ostensibly due to the high levels of carbon dioxide in the host environment (11). The natural conversion of CO_2 to HCO_3 at physiological pH with 5% environmental CO_2 provides sufficient bicarbonate to stimulate cAMP production (112). This direct activation of cAMP production most likely acts in concert with the other activating signals of the cAMP-PKA pathway to induce a robust downstream response leading to the production of encapsulated yeast in the host.

Interestingly, a study by Zaragoza et al. demonstrated that a *pka1*Δ mutant incubated in DMEM with 10% CO_2 was able to produce capsule (225). Although this concentration is higher than human physiological concentrations of CO_2, it is possible that there are other signals that respond either to cAMP levels or directly to bicarbonate that are involved in inducing capsule. By examining the differential transcriptional response of cells incubated in DMEM with or without additional bicarbonate, it will be possible to define the CO_2-responsive regulon. It is likely that this will overlap significantly with the genes that are regulated by the cAMP-PKA pathway. Further examination of the specific transcriptional response to 10% versus 5% external CO_2 is necessary to determine how the cell bypasses Pka1 phosphorylation. These experiments will give clues to the additional pathways involved in the CO_2 response.

Ambient pH

There is a strong physiological connection between iron availability, CO_2, and pH. At human physiological pH (pH 7), iron is often found in insoluble compounds, as ferric iron, thus creating a low-iron signal inside the host in addition to the pH signal (199). The CO_2-HCO_3 equilibrium is also vital for maintaining the pH of the host, which then influences both the available CO_2 level and the environmental pH sensed by the fungus (175).

Most fungi use a conserved pH-sensing pathway to respond to environmental pH, and this response is required for virulence in the host (22). In other fungi, this Pal/Rim signal cascade is initiated through activation of a seven-transmembrane-domain protein (Rim21) that senses pH (6). The Rim9 three-transmembrane-domain protein may assist Rim21 localization to the cell membrane (23, 27, 47). Under neutral to alkaline pH, Rim21 is activated, and this activation can trigger the Rim8 arrestin-like protein to be phosphorylated and ubiquitinated, although other fungi, such as *S. cerevisiae*, have constitutively monoubiquitinated Rim8 proteins that may be regulated by localization (84, 86). The entire Rim21-Rim8 complex can then be transported via the ESCRT system to the endosomes (46, 85, 87). Rim20 then associates with this endosomal structure and forms a scaffold on the Rim101 transcription factor by direct interaction with the C-terminal region of the Rim101 protein (202, 217). This Rim20 scaffold mediates cleavage of Rim101 by the Rim13 calpain-like protease by bringing the protease into association with Rim101 (128, 129, 217). Dissociation of the ESCRT complex is required for removal of the proteolytic Rim20/Rim13 scaffold (81). Proteolytic cleavage is necessary for Rim101 activation (20, 142, 157, 165). In some species, the transcription factor undergoes a second cleavage event, which may be mediated by the proteasome or by other proteases, depending on the species (8, 54, 202). The Rim101 transcription factor is then able to induce the responses necessary for adaptation to host pH, which allows for fungi to cause disease (8, 20, 52, 121).

In contrast to many model fungi, *C. neoformans* has integrated the conserved pH-sensing pathway with the cAMP pathway. The *C. neoformans* Rim101 (CnRim101) transcription factor requires phosphorylation by Pka1 in addition to Rim20-mediated cleavage for localization and activation (156). Vps25 is part of the ESCRTII complex, and *vps25*Δ mutants have a similar capsule size to that of a *rim101*Δ mutant, consistent with activation of Rim101 through

the ESCRT pathway (42). Although other members of the pathway, such as Rim13 and Rim8, have putative homologues encoded in the genome, the homologue of the Rim21 pH-sensing receptor has not been identified. Additionally, the regulation of the Rim13 and Rim8 proteins, including the role of ubiquitination and localization, has not been explored fully. Figure 2 displays the putative elements of the pH-responsive pathway in *C. neoformans.* This model provides a platform to further explore the link between pH and cAMP levels. Because physiological pH is regulated in part by CO_2 levels and because the cell can sense CO_2 levels using Cac1, this may also serve as a signal for neutral pH (5, 11, 112, 143). Therefore, the Rim101 transcription factor may act by synergizing the pH-related inputs from both the conserved pH-sensing pathway and the cAMP pathway.

To determine the Rim101-dependent processes, O'Meara et al. performed a comparative transcriptional analysis comparing the wild-type and *rim101*Δ mutant strains after incubation under tissue culture conditions. Downstream of Rim101, there are a number of cell wall integrity proteins but not many capsule biosynthesis proteins. Of the 12 chitin-related processes, 8 are differentially regulated in the *rim101*Δ strain. Correspondingly, the *rim101*Δ mutant has a defect in maintaining capsule at the cell wall, but the strain can secrete capsule similarly to the wild-type strain (156). The *rim101*Δ strain also has a growth defect in alkaline pH, confirming its role in neutral/alkaline pH responses. Rim101 transcriptionally regulates the Ena1 sodium transporter, and this protein is required for growth in alkaline pH and the CSF (95, 122, 156).

To look specifically at capsule biosynthesis genes induced by physiological pH, Zaragoza et al. examined the expression of *CAP10*, *CAP59*, *CAP60*, and *CAP64* after incubation in 10% Sabouraud's medium buffered to pH 7.3 with morpholinepropanesulfonic acid (MOPS). Similar to what was observed in the *rim101*Δ mutant strain, there was no significant change in expression for any of these genes (227). This suggests that other processes, such as cell wall remodeling or capsule structure alterations, must be responsible for the dramatic encapsulation of cells under these inducing conditions.

Despite the potential activation of both the low-iron and cAMP pathways, rich media buffered to physiological pH are insufficient as a signal to trigger capsule induction (77). The cells must also receive input from another signaling pathway, such as the response to nutrient limitation. Multiple studies have demonstrated that incubation of cells under nutrient-poor conditions at physiological pH results in a strong induction of capsule around the cell (60, 223). The role of limited nutrients is discussed below.

Low Glucose and Low Nitrogen

The upstream elements of the *C. neoformans* cAMP-PKA pathway also respond to low glucose and low nitrogen. G proteins and G-protein-coupled receptors sense the environmental signals of low glucose and low nitrogen and activate the pathway by inducing the production of cAMP (3, 158, 180, 218, 219). Interestingly, signals of nutrient poorness do not consistently result in the induction of capsule around the cell. Granger et al. determined that incubation of cells in DMEM with glucose concentrations between 5 and 50 mM had no effect on capsule (77). However, incubation in Sabouraud's medium with approximately 50 mM glucose at pH 7 resulted in induced capsule (60). It is likely that multiple inputs are necessary for capsule induction, even within a single signaling cascade.

The requirement for multiple inputs is clearly demonstrated by the role of Gpr4 in capsule induction. Under low-nitrogen conditions, Gpr4 interacts with Gpa1 to activate the signaling cascade (218). However, a *gpr4*Δ mutant has no melanin defect, despite the clearly defined role for Gpa1 in melanin production (218). Additionally, low nitrogen transcriptionally induces both Gpr4 and Gpa1, and methionine triggers Gpr4 internalization, but low glucose induces only Gpa1 expression. By adding both the nitrogen and glucose signals, it is possible to induce higher cAMP levels than those induced by adding each signal alone (218).

Another example of specificity in downstream responses to the cAMP-PKA cascade is the Nrg1 transcription factor. *nrg1*Δ mutants have a defect in capsule induction similar to that of *pka1*Δ mutants, and the Pka1 phosphorylation consensus sequence is important for Nrg1 activation. However, comparison of the Nrg1-dependent targets under low-glucose and tissue culture conditions revealed very little overlap, despite both conditions acting as activators of the cAMP-PKA pathway (49; our unpublished data). Under tissue culture conditions, the Nrg1 transcription factor did not appear to be regulated by Pka1, as determined by examining the correlation in downstream targets (our unpublished data).

Additionally, *C. neoformans* appears to repress capsule formation under low-glucose conditions, unless other inducing signals are also present. This is exemplified by the repression of capsule by Ssa1, which is a member of the Hsp70 heat shock family of transcriptional coactivators. An *ssa1*Δ mutant has an increased capsule diameter after incubation in malt agar without glucose (a starvation condition). However, this condition does not induce encapsulation in the wild-type strain (228). The exact mechanism by which Ssa1 represses capsule in response to specific glucose starvation signals has not been explored fully.

For both low nitrogen and low glucose, the respective signal is not sufficient to induce capsule without an additional host environmental cue. This can be demonstrated by examining the *pkr1*Δ mutant. This mutant strain causes constitutive activation of Pka1 and results in the production of a capsule even in rich media. However, the capsule of the *pkr1*Δ strain is even larger when the strain is incubated under tissue culture conditions with CO_2 (59) (Fig. 2). These results demonstrate that a single signal transduction cascade can respond to multiple inputs and regulate multiple downstream outputs, presumably by coordination with parallel signaling cascades.

Stress

Certain stresses on the cell also play a role in repressing capsule induction, potentially by altering cell wall integrity. For example, high osmolarity can repress capsule formation, even when the cells are incubated under the otherwise inducing conditions of low glucose at pH 7 (60). To understand the role of cell stress in capsule formation, it is important to examine two conserved osmotic stress response pathways—the Hog1 pathway and the protein kinase C (PKC) pathway.

The Hog1 signaling pathway responds to a number of cell stresses, and mutations in this pathway lead to alterations in the ability of *C. neoformans* to regulate capsule under normal conditions. The Bahn lab has determined many of the elements of the Hog1 pathway in *C. neoformans* and their roles in responding to

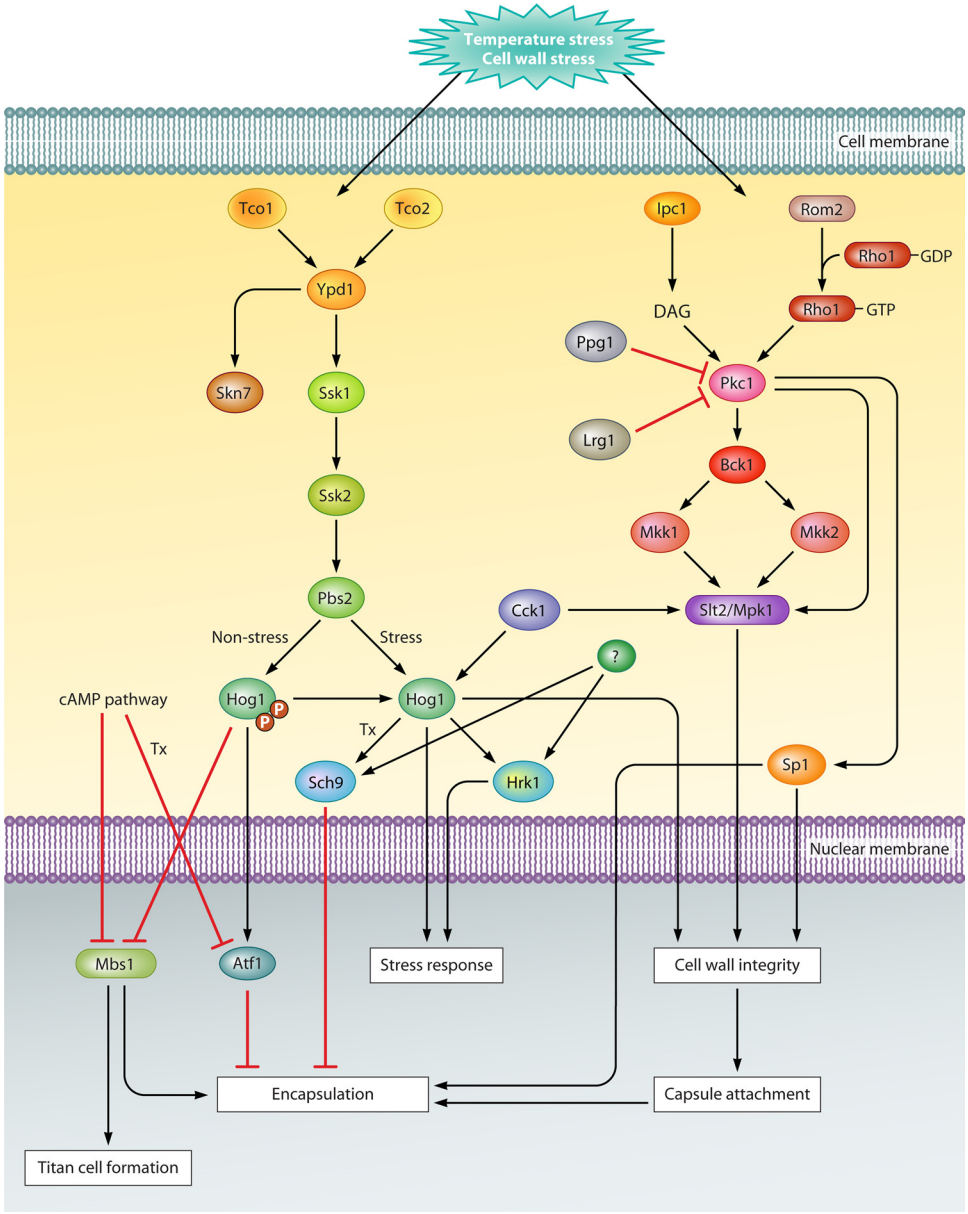

FIG 3 Elements of MAPK cascades in *C. neoformans* and their roles in capsule regulation.

environmental conditions. The known elements of these signal transduction cascades are presented in Fig. 3.

The role of Hog1 in capsule regulation was first documented by the observation of a hypercapsular phenotype of a *hog1*Δ mutant strain (10). The two-component sensor kinases Tco1 and Tco2 respond to environmental conditions and signal through the Ypd1 histidine kinase relay protein to phosphorylate the Ssk1 response regulator (13, 41, 109, 125). Ssk1 then phosphorylates Pbs2, which phosphorylates Hog1 (10, 13). Under stress conditions, Hog1 is rapidly dephosphorylated. However, phosphorylated Hog1, which is present under normal conditions, acts to repress capsule and melanin (10, 12, 13). The phosphorylation status and localization of Hog1 under various capsule-inducing conditions, such as DMEM with 5% CO_2 or low iron, have not been established, but it is likely that Hog1 is dephosphorylated under these conditions to allow for induction of capsule. Examination of a constitutively dephosphorylated Hog1 strain placed under capsule-inducing conditions would shed light on the processes necessary to repress capsule.

Downstream of Hog1 are a number of kinases and transcription factors, and the interaction and regulation of these proteins are still being explored. The Sch9 kinase is likely regulated by Hog1; however, it is also likely controlled by additional inputs because Sch9 regulates only a subset of the Hog1 phenotypes. Additionally, Sch9 is transcriptionally induced only under oxidative stress (117). Similar to the *hog1*Δ mutant, an *sch9*Δ mutant has increased capsule, suggesting that Sch9 also normally represses capsule (117, 208). In contrast, the Hrk1 protein kinase does not regulate capsule and plays only a minor role in melanin, despite being downstream of Hog1 (110).

There are currently two known transcription factors that are regulated by Hog1. The Atf1 transcription factor was first connected to Hog1 because an *atf1*Δ mutant has increased capsule and melanin production and increased sensitivity to osmotic stresses. The connection was confirmed by microarrays demonstrating that Hog1 regulates the expression of Atf1 (109, 117). However, the *atf1*Δ mutant has some drug sensitivities that are not shared with the upstream Hog pathway mutants, which suggests that Atf1 is also regulated by other elements. Because Atf1 is also transcriptionally regulated by Can2, Pka1, and Rim101, it is likely that the cAMP pathway is involved in the regulation of this transcription factor (109).

The Mbs1 transcription factor is repressed by Hog1, and *mbs1*Δ mutants have a minor defect in encapsulation (188). These data imply that Hog1 represses capsule under normal conditions by repressing the Mbs1 activator of encapsulation. To determine the differentially regulated processes responsible for repressing capsule under normal conditions, Ko et al. examined the transcriptional profile of the *hog1*Δ mutant strain (117). The capsule-associated genes *CAP59*, *CAP60*, and *CAP64* demonstrated 1.5- to 1.9-fold increased expression in the *hog1*Δ mutant strain, and the *CAP10* gene was induced 1.8- to 2.2-fold in this strain background. This modest increase in expression of four capsule genes suggests that increased capsule biosynthesis may not be the major biological process responsible for increased encapsulation in the *hog1*Δ mutant (117). In contrast, Hog1 may have its main effect on capsule by regulating various cell wall components. Microarray studies indicate that several cell wall modifiers display Hog1-dependent transcription, including five chitin and chitosan proteins, the Agn1 glucosidase, and two Kre glucan synthases. Confirmation of the Mbs1 downstream targets by transcriptional profiling and examination of Mbs1 binding sites will provide further insight into how Hog1 is able to specifically repress capsule production.

Cross talk between the Hog1 pathway and other capsule-inducing pathways was examined by comparative transcriptional profiling. Interestingly, many of the iron transporter genes (*SIT1*, *CFO1*, *CFO2*, and *CFT1*) were highly induced in the *hog1*Δ mutant strain (117). These microarray studies demonstrated parallel downstream regulation of ergosterol biosynthesis by the cAMP pathway and the Hog1 pathway. Additionally, the arrays revealed transcriptional regulation of Tco2 by cAMP pathway components. However, these experiments were performed under rich medium conditions where the cAMP pathway is not necessarily activated (135). The relationship between the cAMP and Hog1 pathways needs to be explored further, especially with the potentially coordinated regulation of the Mbs1 and Atf1 transcription factors.

The PKC pathway is the other major cell stress-responsive pathway, and it is responsible for maintaining cell wall integrity and chitin distribution in the cell. The structure of the Pkc1 signaling cascade is illustrated in Fig. 3. Environmental stresses such as osmotic or cell wall stresses are sensed by an unknown cellular component. Inositol-phosphorylceramide synthase (Ipc1) regulates the levels of phytoceramide and diacylglycerol (DAG), which act as intracellular signaling molecules that are able to activate the Pkc1 protein kinase (89). Pkc1 can also be activated by the Rho1 GTPase, which is itself activated by the Rom2 protein. Activated Pkc1 then initiates the MAPK cascade, which activates the Bck1 MAPK kinase kinase (MAPKKK), the Mkk2 MAPKK, and finally the Mpk1 MAPKK (74). Separate from the MAPK cascade, Pkc1 can also regulate the Sp1 transcription factor (2).

Mutations in Pkc1 cause overproduction of capsular polysaccharides that are not maintained at the cell surface. Deletion of the entire coding region of the *PKC1* gene results in dramatically increased capsule production, as measured by packed cell volume and the mucoid appearance of the cells on plates. However, examination of the cells using India ink did not reveal capsule around the cell, demonstrating that this strain has a defect in capsule attachment. In a strain missing just the C1 domain of the Pkc1 protein, preventing activation by DAG, capsule diameter around the cell was decreased 42% compared to that of the wild type, and the density of the remaining fibrils was also decreased (88). Additionally, this strain has significant growth defects (73). In *pkc1*Δ mutants, chitin and chitosan levels are similar to wild-type levels, but the distribution of these components in the cell is altered. Additional regulation of the PKC pathway comes from the Lrg1 and Ppg1 proteins, which were discovered by comparing the *C. neoformans* pathway to its *S. cerevisiae* counterpart. In both *ppg1*Δ and *lrg1*Δ mutant strains, capsule production was decreased (74).

Of the downstream targets of Pkc1, the Sp1 transcription factor may be the main negative regulator of capsule in this pathway (2). The *sp1*Δ mutant strain exhibits large amounts of surface capsule even under noninducing conditions, such as growth in yeast extract-peptone-dextrose (YPD) with sorbitol. The increased amount of surface capsule is greater than that in the *pkc1*Δ mutant, potentially due to basal levels of Sp1 in the *pkc1*Δ mutant (2). Analysis of the downstream targets of the Sp1 transcription factor implicated carbohydrate metabolism and cell wall integrity defects. Additionally, the Fks1 β-glucan synthase is reduced in an *sp1*Δ *pkc1*Δ double mutant strain and an *mpk1*Δ mutant strain; this decrease may result in fewer attachment sites for the secreted polysaccharide.

Recent work has shown that the Hog1 and PKC pathways are intimately connected. In the *hog1*Δ mutant, the PKC/MAPK pathway is constitutively activated (10). Dephosphorylation of Hog1 under stress conditions is regulated by the Pkc1-dependent Cck1 casein kinase I protein (209). Although *cck1*Δ mutants have no defect in capsule, Cck1 also regulates the phosphorylation of Mpk1, further connecting these two cell wall integrity pathways.

The third MAPK cascade in *C. neoformans*, the pheromone response pathway, is also involved in regulating capsule production, although it does not appear to be one of the primary mediators of the stress response. The Ste12α transcription factor induces the expression of capsule biosynthesis genes in glucose media, and both *ste12*αΔ and *ste12a*Δ mutants have decreased capsule *in vivo* (34, 35). Ste12 can activate the Cpk1 MAPK cascade, which regulates mating in *C. neoformans* (51). Downstream of the Cpk1 MAPK cascade is the Cwc1-Cwc2 complex, and this complex negatively regulates Ssn801-Ssn8 (CNAG_00440). Ssn8 is a homologue of the cyclin subunit of the Mediator complex, and an *ssn8*Δ mutant has increased capsule (132, 207). Additionally, *ssn8*Δ mutants show a dramatic alteration in morphology that can be attributed to a defect in cell wall construction and integrity. Chitin and chitosan localization is disrupted in this strain, and there is increased β-1,3 glucan on *ssn8*Δ cells (207). Wang et al. identified this cell wall defect as being similar to the phenotypes seen in a *rom2*Δ mutant, potentially connecting Ssn8 to the Pkc1 pathway as well (207).

Hypoxic Stress

In the microenvironment of the human lung, *C. neoformans* cells are frequently exposed to hypoxic stress. Under these conditions, the fungus uses the conserved SREBP pathway to regulate adaptations to this stress. In *C. neoformans*, the Scp1 protein (CNAG_01580) processes the Sre1 transcription factor (CNAG_04804) (29, 41). Processed Sre1 is then imported into the nucleus by the Kap123 protein (CNAG_05884) and phosphorylated by the Gsk3 kinase (CNAG_06730) (30). The Dam1 protein regulates the turnover of Sre1, thus regulating the transcriptional response to low oxygen. Although there are more elements in the mammalian hypoxic response pathway that regulate the processing, trafficking, and degradation of the SREBP complex, these proteins are still being identified and examined in *C. neoformans* for their role in the response to low oxygen and in capsule induction.

In the H99 background, *sre1*Δ mutants have slight capsule defects in 10% Sabouraud's medium at pH 7.3, although this is not a low-oxygen environment (41). In the B3501 background, *sre1*Δ mutants have capsule defects *in vivo* (29). Although Tco1 is also involved in the hypoxic response, there is evidence that the Hog1 and Sre1 pathways act in parallel. For example, a *tco1*Δ *sre1*Δ double mutant is more sensitive to low oxygen than either single mutant (41). Additionally, *sre1*Δ mutants have decreased melanin instead of the increased melanin of Hog1 pathway mutants.

To understand the biological processes that are regulated by the Sre1 transcription factor, Chun et al. performed comparative transcriptional profiling of the *sre1*Δ mutant strain. These experiments revealed that neither capsule nor cell wall biogenesis proteins were differentially regulated in the *sre1*Δ mutant strain in response to hypoxia (41). However, Sre1 did play an important role in the regulation of ergosterol synthesis. The connection between ergosterol (a membrane component) and capsule has not been explored fully.

Downstream of Sre1 is the Gat1 GATA-type transcription factor (29, 124). In both RPMI medium and DMEM, a *gat1*Δ mutant has a decreased capsule (124), similar to the phenotype of the *sre1*Δ mutant. Interestingly, this protein negatively regulates the secretion of exopolysaccharide under noninducing conditions (116). In minimal medium, Gat1 represses the expression of genes involved in capsule biosynthesis, including *UGD1* and *UXS1* (116). However, the size of the capsule surrounding the *gat1*Δ mutant cell under these conditions is similar to that in the wild type, supporting the hypothesis that secretion of exopolysaccharide is separate from encapsulation. It is possible that the Sre1 transcription factor also regulates secreted as opposed to attached capsule, but this has not yet been established.

UNCONNECTED GENES AND CONDITIONS

Some genes that are required for proper capsule formation are not obviously connected to one of the known capsule-inducing pathways. In the literature, there are also genes and environmental conditions identified as regulating capsule that have not been investigated further (see Table S1 in the supplemental material). This section highlights some of these genes and environmental conditions, proposing connections that should be addressed in future experiments.

Tup1

A *C. neoformans tup1*Δ mutant has an increased amount of capsule compared to that in isogenic wild-type strains, and this capsule difference is maximized by incubation in RPMI medium (123). In *S. cerevisiae*, Tup1 is a transcriptional repressor that acts by establishing repressive chromatin in response to Hog1 regulation of the Sko1 protein (167). However, *C. neoformans* does not have an obvious homologue of the Sko1 protein, and the Tup1 protein does not appear to be downstream of Hog1 (117, 123). Currently, the upstream regulator of Tup1 is unknown, although the *tup1*Δ strain is sensitive to cell wall stressors. Unlike the case for many other capsule regulators, the expression of specific, known capsule genes (*CAP10*, *CAP64*, and *CAS35*) is at least 3-fold higher in the *tup1*Δ mutant than in the wild type (123).

Although certain genes involved in iron transport (*SIT2*, *CTR4*, *FRT1*, and *CIG1*) have decreased expression in the *tup1*Δ strain, Tup1 does not transcriptionally regulate the *CIR1* transcription factor or the *CFT1* and *CFT2* iron uptake genes (123). Additionally, the induction of capsule in LIM appears to be separate from Tup1, because the capsule of the *tup1*Δ strain can be induced further in LIM (123). Tup1 is also distinct from the cAMP pathway, as addition of exogenous cAMP does not alter capsule production in the *tup1*Δ strain and Tup1 does not transcriptionally regulate elements of the cAMP pathway (123). In *S. cerevisiae*, Tup1, Sko1, and Hog1 are involved in the recruitment of the SAGA chromatin-remodeling complex, and this remodeling is important for transcriptional regulation (134, 138, 167, 168, 222). However, due to the distinction between Hog1- and Tup1-dependent phenotypes as well as the lack of a Sko1 protein gene in the *C. neoformans* genome, the role of CnTup1 in the regulation of chromatin remodeling is unclear.

GCN5, *ADA2*, and Chromatin Remodeling

The Gcn5 protein is a conserved acetyltransferase in the SAGA complex, which controls chromatin structure and the associated expression of many genes. A *gcn5*Δ mutant displays markedly decreased capsule attachment. However, distinct from strains with mutations in other stress-responsive elements, such as Hog1, the *gcn5*Δ mutant displays no change in susceptibility to osmotic stresses (155). Interestingly, the expression of Gcn5 and Ada2, another component of the SAGA complex, is altered in the *hog1*Δ mutant strain, demonstrating repression of these two factors under normal growth conditions (82, 117). The *ada2*Δ mutant strain has a similar capsule defect to that of the *gcn5*Δ mutant strain, but Ada2 plays a role in mating, while Gcn5 does not (82).

To determine the connection between Gcn5, Ada2, and Hog1, we examined the downstream targets that are shared by these transcriptional regulators. This analysis revealed 79 genes that are coordinately regulated by Gcn5 and Hog1; however, the strains were incubated under different conditions before microarray profiling, limiting this type of analysis. Therefore, there may still be further connections between the pathways (117, 155). One of the genes that is transcriptionally induced by Gcn5 is Tco2, but whether that is sufficient to activate the Hog1 kinase is unclear (155). However, in the *hog1*Δ mutant, the transcription of the *ADA2* gene increases, although the level of *GCN5* is not altered. Currently, the SAGA complex seems to be regulated by the Hog1 MAPK cascade, but the connections between dephosphorylated Hog1 and SAGA activity are still being examined.

Further analysis of the downstream targets of Gcn5 did not reveal significant differences in expression of the capsule biosynthesis genes, complementing the experiment demonstrating wild-type levels and electrophoretic mobility of secreted capsule in the

*gcn5*Δ mutant strain (155). The *ada2*Δ mutant, however, showed differential regulation of the *CAS3*, *CAS32*, *CAS1*, and *MAN1* genes. Therefore, the SAGA complex may still be involved in the differential regulation of some polysaccharide biosynthetic processes. Overall, the mechanism by which the SAGA chromatin-modifying complex regulates capsule has not been elucidated completely, especially in the separation of Gcn5 and Ada2 targets.

Other members of a histone deacetylase complex are also involved in capsule regulation. Mutation of Set301 and Hos2 results in an increased amount of capsule (132). However, in *S. cerevisiae*, Hos2 is associated with highly expressed genes, acting as an activator instead of a repressor (206). Currently, these proteins and their function in a histone deacetylase complex have not been confirmed in *C. neoformans.*

Zds3

The Zds3 protein was identified through insertional mutagenesis, and this protein negatively regulates the production of capsular polysaccharides (130). Zds proteins in *S. cerevisiae* regulate protein phosphatase activity, and Zds1 and Zds2 are involved in cell polarity and the cell cycle. Interestingly, the overproduction of capsule in a *C. neoformans zds3*Δ mutant is tightly linked with pH, with the most capsule produced at pH 4. However, limiting glucose, which presumably limits the pool of available carbohydrate precursors, can prevent the overproduction of capsule. Similar to the case in the *pkc1*Δ mutant strain, increased production of capsule does not correlate with an increased diameter of encapsulation around the cell. Additionally, the *zds3*Δ mutant is also sensitive to cell wall stresses (73, 74, 130). However, the phenotypes of the *zds3*Δ mutant cannot be rescued by sorbitol, making this mutation more severe than *pkc1*Δ pathway mutations and implying basal activity of the unphosphorylated Zds3 protein. Currently, the elements downstream of the Zds3 protein are unknown.

Copper

In addition to the response to low iron, the low-copper regulon has also been implicated in capsule regulation. In the *C. neoformans* genome, there are genes for two copper transporters, i.e., Ctr2 (CNAG_01872) and Ctr1 (CNAG_07701) (42, 55). Chun and Madhani determined that a *ctr1*Δ mutant strain has a defect in encapsulation and in growth in low-copper medium. However, the capsule-deficient phenotype of the *ctr1*Δ mutant was not replicated in further experiments by Ding et al. (55). The Ccc2 protein is a copper transporter that is involved in negatively regulating capsule (103, 205). However, Ccc2 may be required for the assembly of the Fet3/Cft1 iron transporter, and altered iron homeostasis may be the primary capsule-inducing signal. Additionally, the Hxt1 protein negatively regulates capsule production (38). An *hxt1*Δ mutant has increased capsule compared to the wild type after incubation in malt agar. Although Hxt1 is a copper chaperone in other species, *C. neoformans* Hxt1 is not involved in copper resistance (38).

Ire1

The Ire1 kinase is involved in the cellular response to unfolded proteins (37). Activated Ire1 removes an unconventional intron from a downstream transcription factor, either Hxl1 in *C. neoformans* or Hac1 in ascomycetous fungi. The spliced transcription factor can then induce genes necessary for responding to cell stress. Cheon et al. determined that both *ire1*Δ and *hxl1*Δ mutants are sensitive to cell wall stressors, but only Ire1 plays a role in inducing encapsulation (37). By examining the levels of Hxl1 splicing in *cac1*Δ, *cna1*Δ, *cpk1*Δ, *hog1*Δ, and *mpk1*Δ mutant strains, Cheon et al. were able to determine that Hxl1 regulation is independent of these signaling pathways (37). However, because Ire1 regulation of capsule is separate from Hxl1 splicing, there may still be some cross talk between known capsule-regulating pathways and the Ire1-mediated response.

In *C. albicans*, the SAGA complex demonstrates direct binding to the Ire1 promoter to increase expression of the Ire1 protein. However, analysis of the downstream targets of the SAGA complex revealed no change in Ire1 expression in either the *gcn5*Δ or *ada2*Δ mutant strain (82, 155). Interestingly, expression of Ire1 was decreased 2-fold in a *rim101*Δ mutant, potentially linking Ire1 with Rim101. Further experiments are necessary to determine the activator of Ire1 and whether it is connected with known signaling cascades.

Cpl1

Cpl1 is a putative secreted protein that was discovered to result in a capsule defect in a systematic deletion screen (132). It is transcriptionally induced in response to oxidative stress (117), but its regulation and function are currently unknown.

Cin1

Cin1 is a putative intersectin homologue that has a demonstrated role in vesicle transport, resulting in alterations in chitin deposition, cell morphology, and surface capsule in a mutant strain (181). Although the Cin1 protein interacts *in vitro* with Cdc420, the interaction appears to be dispensable for Cin1 action, which is consistent with the wild-type capsule of a *cdc420*Δ mutant strain (17). Cin1 also interacts with the Wsp1 WASP protein, and a *wsp1*Δ mutant strain also has a defect in capsule and chitin deposition (182). Additionally, the localization of the Sav1/Sec4 vesicle transporters is disrupted in this mutant strain, suggesting a potential role in the regulation of capsule secretion (182). The regulation of the Cin1-Wsp1 complex is still being examined.

ClcA

In addition to the Rim and cAMP pathways, the ClcA chloride channel protein is required for growth at alkaline pH. A *clcA*Δ mutant also has a significant defect in encapsulation compared to the wild-type strain after incubation on malt extract agar (229). However, this inducing medium is acidic, so the effect of pH on capsule production in the *clcA*Δ mutant has not been examined fully. Due to the similarity in phenotypes between the *clcA*Δ and *vph1*Δ mutant strains, it is possible that the *clcA*Δ mutant also has a defect in capsule secretion (62). Further analysis of the capsule defect in the *clcA*Δ mutant strain will provide more insight into the role of this chloride channel in capsule regulation.

Serum

In addition to genes that have not been connected to known signaling cascades, there are some environmental cues that alter *C. neoformans* capsule production whose responsible genes are unknown. For example, the signaling pathways that respond specifically to mammalian serum are unclear. Although serum contains iron-binding components, it is unlikely that the cell uses low-iron-responsive signaling pathways to induce capsule in response to serum, because supplementation with iron does not repress

capsule formation under this condition (225). Difficulties in understanding the signaling response to serum derive in part from the complexity of serum. Recently, Chrisman et al. determined that serum lipids might be responsible for the induction of capsule, and the induction may depend on interactions with cryptococcal phospholipase proteins (39).

Carbohydrate Source

Another cue that regulates capsule is supplementation with mannitol. Mannitol is able to cause a different presentation of capsular antigens, and growth in mannitol causes a larger capsule than that for growth in glucose (48, 80). Furthermore, initial reports indicated that high glucose is able to induce the production of exopolysaccharide, if not encapsulation (43, 80). However, other studies indicate that a high glucose level represses both encapsulation and exopolysaccharide production, potentially by preventing activation of the cAMP cascade (39).

Nitrogen Source

In addition to the methionine-regulated activation of Gpr4, the nitrogen source can influence capsule regulation. Early work on the induction of capsule ascertained that ammonium sulfate [$(NH_4)_2SO_4$] can suppress capsule (77), but the mechanism is unknown. However, recent work has shown that other nitrogen sources can induce large amounts of surface capsule, as long as ammonium sources are not present (124). The repression of capsule in the presence of ammonium is likely due to Gat1-mediated nitrogen catabolite repression (124).

Amino acid transport is also regulated by the nitrogen source, as demonstrated by the auxotrophies of an *ilv2*Δ mutant strain (111). The *ilv2*Δ strain has a significantly smaller capsule diameter than the wild-type strain after growth in DMEM. However, the *ilv2*Δ mutant also has a growth defect under these conditions, even when supplemented with isoleucine and valine, making the mechanism for the decreased capsule unclear (111). The capsule defect of a *met6*Δ mutant is also associated with poor growth under capsule-inducing conditions (162). The growth defect may be due to the toxic accumulation of homocysteine, which cannot be converted to methionine in the *met6*Δ mutant (162). However, it is also possible that the altered amino acid transport in these mutant strains interferes with the normal nitrogen regulation of capsule.

TITAN CELLS—A SPECIAL CASE

The titan cell is a newly described *C. neoformans* morphological form observed primarily in the lungs of the infected host. In addition to their very large size (12 to 50 μm), titan cells have a very different capsule structure from that of normal yeast cells. The titan cell capsule appears to be especially effective at dampening the host immune response. Although wild-type capsule can suppress the host inflammatory response *in vitro*, titan cell capsules appear to be more efficient at this process, as evidenced by the negative correlation between the degree of inflammation and the number of titan cells in the fungal population (201, 226).

One of the differences in titan cell capsule is the increased size and number of chitin-like structures in the capsule (70). These structures may be related to the increased resistance of titan cell capsule to radiation, organic solvents, and dimethyl sulfoxide (DMSO) (226). It is possible that these structures increase the cross-linking and density of the capsule. Additionally, titan cells have extremely thickened cell walls, reaching between 2 and 3 μm instead of the normal size of 50 to 100 nm for cells grown *in vitro* (226). Another important feature of titan cells is that they are polyploid (152, 226). In titan cells, the nuclear content, cell wall, and capsule cross-linking must be regulated specifically.

The inducing factors for this unique morphology have been the subject of recent investigations. Host conditions appear to be extremely important in the formation of titan cells. Previously, titan cells could be induced only during infection of mammalian lungs (152, 226). Recently, Chrisman et al. demonstrated that increased cell and capsule diameters can be induced by amoeba membrane lipid components, especially phosphatidylcholine (PC), in a dose-dependent manner (39). Under these conditions, the cells also induce twice as much surface capsule and six times more exopolysaccharide, consistent with separate pathways regulating the production of these two types of capsule. The response appears to be mediated through the action of the Plb1 phospholipase. Phospholipase B mutants (*plb1*Δ) produce capsule in response to serum but not in response to amoeba extracts, showing that Plb1 is required for responding to amoebae (39). Additionally, infection of *Galleria mellonella* wax moth larvae with *C. neoformans* can also induce titan cell formation (70). However, incubation of cells with whole *Galleria* extract is sufficient to trigger enlargement, presumably due to the lipid components and not the process of colonization (70).

The cryptococcal signaling pathways responsive to these environmental cues are also being examined. The initial reports on titan cells demonstrated that the mating and pheromone pathway is important in the induction of titan cells, with coinfections of mating type **a** and α cells producing the most titan cells. However, only **a** cells undergo increased titan cell formation, suggesting that there is a specific response to the α pheromone (152). Additionally, deletion of the Ste3a pheromone receptor did not abolish basal levels of titan cell formation in **a** cells, implying that multiple signals are required for titan cell induction (152).

Screening through the seven GPCR mutants of *C. neoformans* revealed that only Gpr5 is required for titan cell formation (153). In the *gpr5*Δ mutant strain, there is a decreased rate of titan cell formation, and the cells that do undergo an increase in size are not as large as wild-type titan cells. Gpr5 likely signals through Gpa1 and Ste3a, with Ste3a interacting directly with Gpa1. The Ste3α protein, which plays no role in titan cell formation, interacts with Gpa2 and Gpa3 to regulate mating (153). The other members of the Gpa1 complex (Gib2, Gpg1, and Gpg2) have not been investigated for their role in titan cell formation. Downstream of Gpa1, the Rim101 transcription factor also plays a major role in titan cell formation (153). The *rim101*Δ mutant strain has a more severe defect in titan cells than an *ste3a*Δ mutant, suggesting that uninduced Rim101 activity is sufficient for basal levels of titan cell formation (153).

Other factors have also been implicated in the regulation of titan cells and their concomitant increase in capsule formation. Mbs1 is required for titan cell formation, but the impact of the upstream regulators of Mbs1 on titan cell formation has not been investigated (188). *cdc420*Δ and *gap1*Δ mutants have defects in titan cell formation, and the Cnc1560, Pcl103, and Rho104 proteins act to repress titan cell formation (153, 226). However, the upstream signals for these elements and the downstream biological process that they regulate to influence titan cell formation are unknown. Currently, there are many unanswered questions about this process of host-induced morphological change, but the

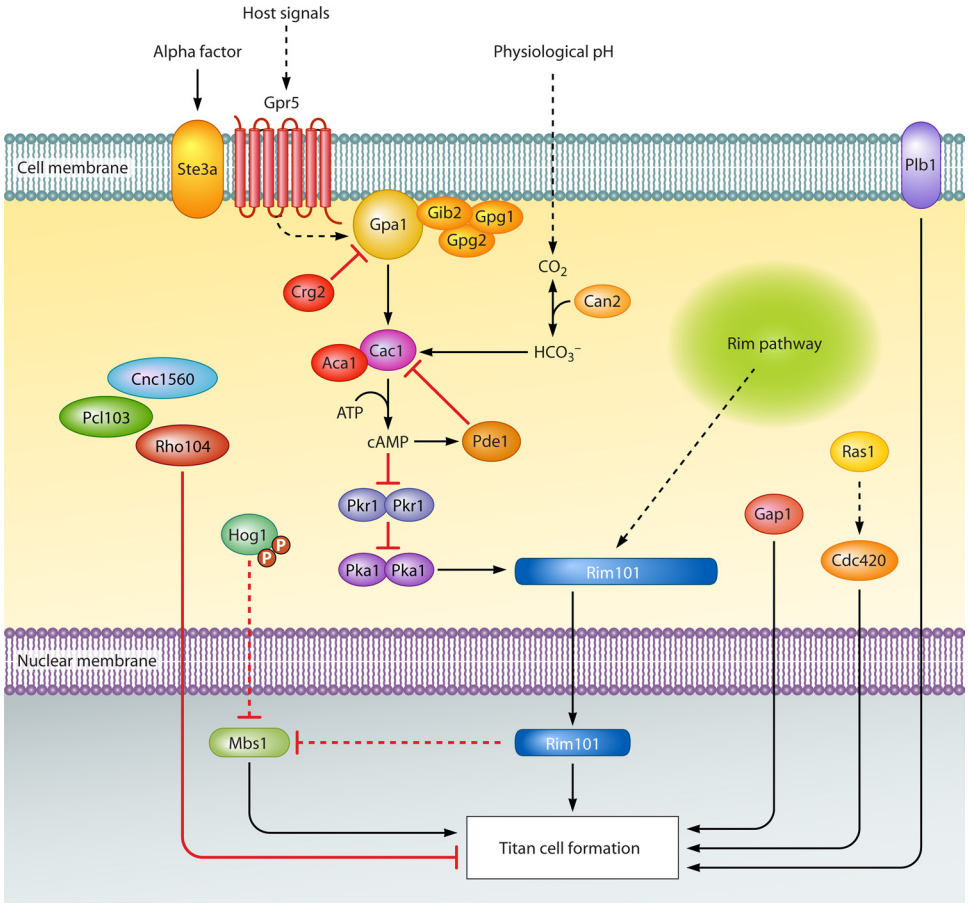

FIG 4 Signal transduction cascades that regulate titan cell formation. Although some elements of the Pka1 cascade have not been tested explicitly for titan cell formation, the involvement of multiple proteins from this pathway in titan cell formation demonstrates the importance of cAMP/PKA signaling in regulating this phenotype.

known elements that control the titan cell transition are presented in Fig. 4. Future experiments to better understand titan cell biology, including transcriptional profiling of titan cells, are now more feasible due to the ability to induce the morphotype *in vitro*.

CLINICAL CONSIDERATIONS OF *C. NEOFORMANS* CAPSULE AND ITS REGULATION

The biology of the polysaccharide capsule has been linked intimately to our clinical understanding of *C. neoformans* disease. As mentioned previously, the surface capsule is a distinctive cellular phenotype that clinicians and microbiologists intimately associate with this fungal pathogen. The recognition of encapsulated yeasts in CSF or other clinical samples allows clinicians to rapidly diagnose the presence of *C. neoformans* at the site of disease.

The capsule has also served as a clinical epidemiological tool. Exposure to *C. neoformans* results in the production of specific antibodies to various capsule epitopes. Most individuals in a region of endemicity develop anticryptococcal serum antibodies by the age of 5 years (76). Because related strains tend to react *in vitro* to similar antibodies in human serum, investigators were able to group *C. neoformans* strains into "serotypes" long before detailed analysis of the fungal cell surface was possible. In this way, *C. neoformans* strains were historically divided into four major serotypes: A, B, C, and D (21, 176). The serotype distinctions were based upon conserved structural features in the capsule that are shared among related strains (58, 61).

Subsequent molecular epidemiology based on DNA sequencing has supported many of the strain classifications derived initially by these capsule-based serological studies (215, 216). However, the recent combination of DNA and phenotypic analyses has resulted in a rigorous reclassification of "*Cryptococcus neoformans*" strains into two different species (*C. neoformans* and *Cryptococcus gattii*), as well as two varieties within the species *C. neoformans* (*C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii*) (120). This renaming of *Cryptococcus* strains has important clinical relevance. For example, most cryptococcal disease occurs in highly immunocompromised patients. However, *C. gattii* strains are able to cause disease in apparently immunocompetent individuals. Moreover, *C. gattii* infections often result in small, focal brain abscesses (cryptococcomas) as opposed to diffuse meningoencephalitis (189). These distinctive clinical features were first noted in subtropical regions of the world, such as Australia, where *C. gattii* is routinely isolated. However, new outbreaks of *C. gattii* infections have recently occurred in more temperate regions, such as British Columbia and the U.S. Pacific Northwest (26, 193). Therefore, it is important for clinicians to be aware of the potentially varying presentation of infections caused by the different cryptococcal species.

The polysaccharide capsule is also the basis for the very sensitive and specific cryptococcal antigen test used widely in clinical practice. This assay is more sensitive than routine culture and documents the presence of *C. neoformans* in clinical specimens, especially CSF and serum. For highly immunocompromised patients, such as patients with late-stage AIDS, this test has >95% sensitivity for systemic *C. neoformans* infections (63).

Prospective testing for *C. neoformans* capsular antigen is also being studied in certain HIV-infected populations to prevent excessive immune reconstitution inflammatory syndrome (IRIS). Patients with AIDS who have clinically asymptomatic cryptococcal infections can develop very serious symptoms mimicking progressive *C. neoformans* infections after being initiated on antiretroviral therapy (ART) (185). Investigators therefore hope to identify patients with early, asymptomatic cryptococcal antigenemia or CNS infections prior to beginning antiviral therapy. In this way, clinicians may be able to coadminister antifungal therapy and ART, hopefully preventing or minimizing cryptococcus-based IRIS during the period of rapid improvement in immune function. While IRIS can be managed medically, this syndrome may also be life-threatening, especially in patients with CNS symptoms and limited medical access. The availability of an inexpensive cryptococcal antigen test suitable for urine or serum samples would allow the application of this type of preemptive screening for clinically inapparent cryptococcal infections in resource-limited settings, where the confluence of AIDS and cryptococcosis is most striking (99).

CONCLUSIONS

Induction of the *C. neoformans* capsule is a complex biological process. As such, there are many ways that the cell can fail to produce capsule and many redundant pathways that allow for robust encapsulation around the cell. Despite these complications, significant progress has been made in the illumination of the signal transduction networks that regulate capsule. In future research on this important virulence factor, it will be necessary to define the specific aspect of capsule that has failed in a particular mutant strain. Does the cell produce GXM? Are these GXM molecules structurally sound? Are alternative moieties incorporated into the capsular material? Is the capsule secreted across the cell wall? Is it maintained appropriately at the cell surface? What aspect of the mutant cell surface prevents capsular attachment? By defining the capsular defects of mutant strains more precisely, we will be better able to dissect the mechanism by which the cell is able to regulate the induction of capsule.

In conjunction, the importance of transcriptional profiling cannot be debated. By examining the elements downstream of a transcription factor, it is possible to determine the biological processes that are the primary targets of a signaling cascade. Using this approach to dissect the capsule phenotype, we can determine that many signaling pathways that control the appearance of capsule on the cell surface do not transcriptionally regulate many of the classical capsule biosynthesis genes. Instead, many of the differentially regulated genes in the current array of capsule-defective mutants are involved in cell wall structure and integrity. The direct connections between cell wall phenotypes and capsule attachment are still being examined.

Finally, there is mounting evidence that the specific inducing condition plays a significant role in regulating the amount and structure of the resulting capsule. This is exemplified by the variability in antibody binding to the capsules of cells collected from different organs in a single infected host. This plasticity of capsule structure in response to various host conditions underscores the complex relationship between host and parasite. Not only do infectious microorganisms need to evade host defenses, but they also often shape their microenvironment for better survival. By precisely regulating the composition of its cell surface and its secreted polysaccharides, the pathogenic fungus *C. neoformans* has developed intricate ways to establish chronic infection and dormancy in the human host. Recent events, including new outbreaks of cryptococcal infections and the staggering prevalence of *C. neoformans* disease in untreated AIDS patients, underscore the success of this encapsulated pathogen. Understanding capsule biosynthesis, assembly, and regulation will allow us to develop new strategies to better diagnose and treat these serious infections.

ACKNOWLEDGMENTS

This work was supported by NIH grants R56-AI074677 and R01-AI074677 to J.A.A. T.R.O. was supported by a predoctoral fellowship from the American Heart Association.

All gene annotation was facilitated by the FungiDB website (http://FungiDB.org), created by Jason Stajich.

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