Stress Signaling Pathways for the Pathogenicity of Cryptococcus

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Sensing, responding, and adapting to the surrounding environment are crucial for all living organisms to survive, proliferate, and differentiate in their biological niches. This ability is also essential for Cryptococcus neoformans and its sibling species Cryptococcus gattii, as these pathogens have saprobic and parasitic life cycles in natural and animal host environments. The ability of Cryptococcus to cause fatal meningoencephalitis is highly related to its capability to remodel and optimize its metabolic and physiological status according to external cues. These cues act through multiple stress signaling pathways through a panoply of signaling components, including receptors/sensors, small GTPases, secondary messengers, kinases, transcription factors, and other miscellaneous adaptors or regulators. In this minireview, we summarize and highlight the importance of several stress signaling pathways that influence the pathogenicity of Cryptococcus and discuss future challenges in these areas.

Cryptococcosis has been emerging as a major infectious disease globally, mainly due to the increasing number of aged and immunocompromised individuals (for reviews, see references 1, 2, 3, 4, and 5). There are two major pathogenic species of Cryptococcus, Cryptococcus neoformans (serotypes A and D) and Cryptococcus gattii (formerly known as serotype B/C C. neoformans var. gattii). C. gattii is particularly dangerous for its ability to attack healthy individuals (for reviews, see references 6, 7, 8, and 9). Cryptococcus has both saprobic and parasitic life cycles (10). Cryptococcus generates infectious spores in the natural environment, such as soils, avian habitats, or trees. Such infectious propagules (spores or dried yeasts) are transferred to the host through the respiratory system and eventually disseminated to the brain through the central nervous system by crossing the blood-brain barrier, causing fatal meningoencephalitis (4, 5).

The number of antifungal drugs is generally limited, compared to antibacterial agents, due to the conserved cellular structures between fungi and humans. However, the number of anticytotoxic drugs is even more limited in spite of their importance in clinical settings and to public health (for reviews, see references 11, 12). Hence, there have been extensive investigations to elucidate the virulence mechanisms of pathogenic Cryptococcus species with the hope of identifying novel anticytotoxic drug targets. Out of these efforts, several key virulence factors have been discovered. Of these virulence factors, a polysaccharide-based cell surface capsule and a polymerized polyphenol complex, melanin, have been recognized as two major virulence factors that help the pathogen resist the host immune system. Several excellent reviews are available on these virulence factors (13, 14, 15, 16, 17, 18, 19, 20).

Another virulence-related attribute of Cryptococcus is its ability to survive the harsh environmental stresses conferred in both natural and host settings. During the transition between separate natural and biological niches, Cryptococcus senses, responds, and adapts to environmental changes dynamically for its survival and proliferation. The unusual stress resistance of Cryptococcus is best represented by its ability to survive high radiation conditions (21); Cryptococcus species have even been isolated from the defunct Chernobyl nuclear reactors (22). A comprehensive understanding of complex stress signaling networks will pave new ground for development of novel and effective antifungal drugs and anticytotoxic agents. Here, we review known stress signaling pathways in Cryptococcus describing their conserved and unique features compared to those in other model yeasts and their impact on pathogenesis. We also discuss future challenges in better understanding the complex stress signaling pathways in Cryptococcus.

THE HIGH-OSMOLARITY GLYCEROL RESPONSE PATHWAY

The high-osmolarity glycerol response (HOG) pathway was first identified in the ascomycete budding model yeast Saccharomyces cerevisiae (for reviews, see references 23, 24, and 25). The core signaling components of the HOG pathway consist of a stress-activated mitogen-activated protein kinase (MAPK), Hog1, and its upstream kinases, the Pbs2 MAPK kinase (MAPKK), and the Skp2/22 MAPKK kinase (MAPKKK). MAPK is evolutionarily conserved from yeasts to mammals; the yeast Hog1 ortholog is orthologous to the mammalian p38 MAPK, which also plays a role in stress sensing and adaptation in humans (for reviews, see references 26, 27, 28, and 29). The divergent point between fungal Hog1 and mammalian p38 MAPK pathways is their upstream signaling module. Most yeasts and filamentous fungi have a His-Asp phosphorelay system, which is not observed in mammalian systems. The fungal phosphorelay system consists of a hybrid sensor histidine kinases (HHKs), a His-containing phosphotransfer protein (Hpt), and response regulators (RRs). Several excellent reviews are available on this topic (for reviews, see references 30, 31, 32, and 33).

Cryptococcus also has the evolutionarily conserved Hog1 MAPK, the Pbs2 MAPKK, and the Skp2 MAPKKK (34, 35). Notably, however, the regulatory mechanism of Cryptococcus Hog1 is distinct from that of Hog1 orthologs in S. cerevisiae and other fungi. In a number of clinical and environmental isolates, including the H99 strain (a serotype A platform strain), Hog1 is highly phosphorylated, even under unstressed conditions, and undergoes subsequent dephosphorylation in response to environmental stresses (34), which is in stark contrast to other fungal Hog1 orthologs that are normally unphosphorylated under unstressed conditions.
conditions and rapidly phosphorylated in response to certain stresses (23, 24, 25). Nevertheless, Hog1 phosphorylation completely depends on the Pbs2 MAPKK (34). Upstream of Pbs2 and Hog1, Cryptococcus possesses only a single MAPKKK, Ssk2, which is also in contrast to S. cerevisiae with its three MAPKKKs (Ssk2, Ssk22, and Ste11) for the regulation of the Pbs2-Hog1 kinase cascade. In fact, the Ssk2 MAPKKK was identified as a signaling component responsible for differential levels of basal Hog1 phosphorylation between the serotype D f1 sibling strains B-3501 (high basal Hog1 phosphorylation) and B-3502 (no basal Hog1 phosphorylation) through comparative analysis of meiotic maps (35). In this analysis, an SSK2 allele exchange between strains B-3501 and B-3502 changed Hog1 phosphorylation patterns and subsequent stress resistance patterns. Two nonsynonymous changes (Leu240 and Met738 in strain B-3501 to Phe240 and Val738 in strain B-3502) in the extended N-terminal region of Ssk2 appeared to allow the leaky activation of the Pbs2-Hog1 kinase in some strains of C. neoformans. Interestingly, such an increased basal Hog1 phosphorylation enhances resistance to diverse environmental stresses, such as osmotic shock, oxidative stress, and genotoxic stress in C. neoformans (35). Therefore, it is conceivable that C. neoformans strains having these types of mutations have been selected during evolution due to their increased fitness in their environmental niches or within the host.

A phosphorelay system has also been discovered upstream of the Hog1 module in Cryptococcus. Similar to the S. cerevisiae phosphorelay system, Cryptococcus contains two response regulators (Ssk1 and Skn7) and a single Hpt (Ypd1). Of the two RRss, Ssk1, not Skn7, positively regulates the Ssk2-Pbs2-Hog1 cascade because the ssk1Δ mutant is phenotypically similar, albeit not identical, to the ssk2Δ, pbs2Δ, and hog1Δ mutants (36). In contrast, Ypd1 negatively regulates Hog1 because deletion of YPD1 causes lethality in C. neoformans, similar to S. cerevisiae Ypd1, only in the presence of functional Hog1 (37). As Ypd1 is a fungus-specific protein and its deletion or inhibition generally causes lethality in many fungi, it is considered a good antifungal drug target (for a review, see reference 38).

However, unlike S. cerevisiae, which contains a single HHK (Snl1), Cryptococcus contains multiple HHKs, named Tco1 to Tco7 (two-component-like proteins) (36). In fact, the presence of multiple sensory HHKs is more common in fungi than a single HHK, reflecting the fact that the HOG pathway responds to a plethora of environmental stresses in most fungi (33). Among Tco HHKs, Tco1 and Tco2 have redundant and discrete roles in HOG signaling. Unlike the essential protein Snl1, deletion of TCO1 or TCO2 (or both) does not cause lethality in C. neoformans (36). Similar to Hog1, both Tco1 and Tco2 promote cellular susceptibility to fluoroquinol, a phenylpyrrole class of fungicide. In other phenotypic traits, however, Tco1 and Tco2 have distinct roles. Tco1 is negatively and positively involved in melanin synthesis and mating, respectively, whereas Tco2 is involved in stress responses against osmotic stress, oxidative damage, and toxic metabolites. Among other TCO genes, TCO6 has been suspected to be essential because its gene disruption has not been successful. Recently, we successfully deleted the TCO6 gene in the serotype A H99 strain background (Y.-S. Bahn, unpublished data) by employing a highly efficient split-marker gene disruption system (39). Therefore, none of the HHKs are essential in C. neoformans. However, it is possible that deletion of all seven HHKs may cause cell lethality in C. neoformans.

The Hog1 MAPK modulates numerous downstream effector proteins and governs a plethora of phenotypic traits in C. neoformans, including growth, stress response, differentiation, virulence factor production, and ergosterol biosynthesis. Transcriptome analysis through DNA microarrays has revealed that the basal expression levels of 545 genes are significantly changed more than twofold by the deletion of HOGL (40). Notably, expression of genes involved in ergosterol synthesis (e.g., ERG11 and ERG28) and ergosterol content are significantly increased in both ssk1Δ and hog1Δ mutants, strongly suggesting that the HOG pathway represses ergosterol biosynthesis under unstressed conditions (40). Supporting this finding, the HOGL mutants (hog1Δ, pbs2Δ, ssk2Δ, and ssk1Δ mutants) are highly susceptible to amphotericin B (AMB) but resistant to azole drugs, possibly due to increased AMB-binding sites (ergosterol) and ERG11 expression levels, respectively (40). As this role of the HOG pathway in ergosterol biosynthesis is observed in H99 and B-3501 strains (high basal Hog1 phosphorylation), but not in JEC21 strain (low basal Hog1 phosphorylation), basal Hog1 phosphorylation levels likely affect this ability.

On the basis of the transcriptome profiling data of the HOG pathway, Bahn and colleagues recently functionally characterized a subset of Hog1-downstream effectors. First, several genes involved in osmotic balance are modulated by the HOG pathway. Two osmotic transporters, ENA1 (a Na+/ATPase efflux pump) and NHA1 (Na+/H+ antiporter), are strongly and weakly induced, respectively, by osmotic stress (1 M NaCl, KCl, or sorbitol) in a Hog1-dependent manner (41). In C. neoformans, Ena1 serves as an efflux pump for both Na+ and K+ ions, whereas Nha1 is involved only in K+ pumping. Interestingly, the two cation pumps are also involved in proton and pH homeostasis, as Ena1 and Nha1 are required for adaptation to high and low pH, respectively (41). Reflecting their roles in pH homeostasis, the expression of ENA1 and NHA1 are also regulated by the pH-sensing Rim101 and Nrg1 signaling pathways (41). The absolute requirement of Ena1, but not Nha1, for the virulence of C. neoformans indicates that cation and pH homeostasis is critical for the pathogen to survive within the host (41, 42). Another potential candidate involved in the osmotic stress response, an aquaporin gene, AQP1, was also found to be regulated by the HOG pathway (40), although its physiological role is not known. Second, several genes involved in the oxidative stress response are controlled by the HOG pathway (40). Of these genes, a sulfiredoxin gene (SRX1) is most strongly induced by hydrogen peroxide (H2O2) in a Hog1-dependent manner (40, 43). Most recently, Srx1 was found to be critical not only for peroxide sensing and recycling of peroxide toxin (Tsa1) but also for promoting fluoroquinol susceptibility (44).

Thus far, only a few signaling regulators (e.g., kinases and transcription factors downstream of Hog1 have been reported. One is Hrk1 (Hog1-regulated kinase 1), which is involved in the synthesis of intracellular glycerols and osmotic balance, although it is dispensable for the virulence of C. neoformans (45). Other Hog1-regulated kinases include the Sch9 kinase; its expression also appears to be induced by oxidative stress in a Hog1-dependent manner, and Sch9 is indeed involved in the oxidative stress response (40). Known Hog1-regulated transcription factors include Atf1 and Mbs1. Atf1 is a bZIP transcription factor, orthologous to Schizosaccharomyces pombe Atf1 that is a well-known downstream transcription factor of Sty1 (a Hog1 ortholog) (46,
In *C. neoformans*, Atf1 has been identified by Lodge and colleagues to regulate peroxide sensing (48). Our subsequent study demonstrated that Atf1 is involved in sensing and responding to diverse environmental factors as well as virulence factor regulation in *C. neoformans* (49). Our recent unpublished results further demonstrated that Hog1 positively regulates *ATFI* expression. The other potential Hog1-regulated transcription factor is one of the APSES (named APSES for Asm1, Phd1, StuA, Efg1, and Sok2) proteins, Mbs1 (Mbp1- and Swi4-like protein 1) (50). MBS1 expression appears to be regulated by both Tco2 and Hog1. Mbs1 is one of the flucytosine-regulated genes that governs genotoxic stress response and is involved in ergosterol biosynthesis, membrane integrity, oxidative/osmotic stress response, virulence factor production, and the virulence of *C. neoformans*. Interestingly, Mbs1 is also involved in titan cell formation (50), which is a specific morphological form of *C. neoformans* in vivo with a 5- to 10-fold larger cell size (51, 52). Nevertheless, it remains unclear whether Hrk1, Sch9, Atf1, and Mbs1 are direct phosphorylation targets of Hog1.

There are many remaining questions regarding the *Cryptococcus* HOG pathway. It is particularly unclear how *Cryptococcus* negatively regulates the HOG pathway in the absence of external cues. This is an important issue because overactivation of the HOG pathway leads to lethality, as witnessed by the action of fludioxonil, which overactivates the HOG pathway and confers lethal effects (53). Therefore, any drugs targeting the negative regulators of the HOG pathway could be more efficient at killing this pathogen than those targeting their positive regulators. Ypd1 (a Hpt protein) could be one such target. Another important question is what other upstream signaling pathways *Cryptococcus* employs to activate the Ssk2-Pbs2-Hog1 signaling cascade. In our previous studies, we proposed that another upstream signaling pathway(s) that activates the HOG pathway must exist, as Hog1 can be phosphorylated in response to osmotic shock, even in the *ssk1Δ* mutant (36). The proposed regulatory mechanism of the *Cryptococcus* HOG pathway is summarized in Fig. 1.

**THE CYCLIC AMP/PROTEIN KINASE A AND Ras SIGNALING PATHWAYS**

Among cellular signaling pathways mediated by the second messenger, the cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway has gained the most attention from mycologists due to its critical role in the pathogenicity and morphological differentiation of diverse plant and animal fungal pathogens (for reviews, see references 10, 54, 55, 56, 57, and 58). In *Cryptococcus*, the cAMP/PKA signaling pathway is also essential for virulence and differentiation (for reviews, see references 18, 59, 60, and 61). The proposed regulatory mechanism of the Ras and cAMP/PKA signaling pathways in *C. neoformans* is summarized in Fig. 2. Interestingly, however, the cAMP/PKA pathway is dispensable for the vegetative growth of *C. neoformans*. The absence of intracellular cAMP (e.g., through deletion of an adenylyl cyclase gene) generally causes abnormal, defective growth in most fungi, but not in *C. neoformans*. Cells with deletion of *CAC1* (*Cryptococcus* adenylyl cyclase 1) grow as well as the wild type does (62). It is unclear whether *C. neoformans* utilizes other cAMP-like second messengers, such as cGMP, to support vegetative growth.

Although the role of the cAMP signaling pathway in melanin and capsule biosynthesis and mating has been extensively studied, its role in stress response in *C. neoformans* has only recently been reported (63). Besides its dispensable role in vegetative growth, the roles of the *Cryptococcus* cAMP/PKA signaling pathway in stress response are quite distinct from those in other fungi. In the ascomycete fungi, like *S. cerevisiae* and *Candida albicans*, the reduced intracellular cAMP perturbs normal vegetative growth but increases cellular resistance to a plethora of environmental stresses (for reviews, see references 56, 57, 58, 64, 65, and 66). For example, the hyperactivation of the cAMP pathway by the constitutive active *RAS* allele renders cells hypersensitive to heat shock in *S. cerevisiae*. In *C. neoformans*, however, the Ras and cAMP/PKA signaling pathways appear to be independently involved in stress response. For growth at high temperatures, which is critical for the survival of *Cryptococcus* within the human host, Ras is required, whereas the cAMP/PKA pathway is largely dispensable (63, 67). In the osmotic stress response, the ras1Δ mutant is highly susceptible to salt stresses under both nutrient-rich and -starved conditions, whereas the *aca1Δ, gap1Δ*, and *pka1Δ* mutants are as resistant as the wild type is (63).

Interestingly, however, two cAMP signaling components, Aca1 (adenylyl cyclase-gssociated protein 1) and Pka2 (PKA catalytic subunit 2), appear to play some role in the salt or osmosis stress response in *C. neoformans* (63). Deletion of *ACA1* increases sensitivity to salt stress independent of both Ras and cAMP signaling pathways (63). As described before, Pka2 is considered largely dispensable for the cAMP signaling pathway in the serotype A H99 strain background. Under glucose-starved conditions, however, the *pka2Δ* mutant is highly sensitive to osmotic stress, whereas the *pka1Δ* mutant is not (63). Interestingly, a deletion of *PKA1* (PKA catalytic subunit 1) suppresses the sensitivity of the *pka2Δ* mutant, suggesting that Pka1 and Pka2 play opposing roles in osmotic stress response under glucose-starved conditions in *C. neoformans*.

The roles of Ras- and *Aca1*-specific signaling pathways in osmotic and salt stress responses appear to stem from their roles in the maintenance of cell wall and membrane integrity, because the *ras1Δ* and *aca1Δ* mutants display synergistically enhanced sensitivity to cell wall/membrane-damaging agents, such as Congo red (CR), and sodium dodecyl sulfate (SDS) (63). The common features observed in both pathways are their roles in actin cytoskeleton regulation. The Alspaugh group reported a series of elegant studies showing that the Ras signaling pathway employs signaling components distinct from those of the cAMP signaling pathways for stress response and adaptation in *C. neoformans*. These signaling components are involved in regulation of actin cytoskeletal architecture. For example, Rac1, which belongs to the Rho family of G proteins that regulate the actin polymerization during the morphological development of mammals and other fungi, was reported to work downstream of Ras to control thermotolerance (68). However, deletion of *RAC1* alone does not cause high temperature sensitivity like the deletion of *ras1* does (68), indicating that other Rac1-like paralogs may exist and play redundant roles in the thermotolerance of *C. neoformans*. In fact, *C. neoformans* contains two more Rac1-like Rho proteins, Cdc42 and Dch2 (dual Cdc42 homolog). Overexpression of *CDC42* dramatically suppresses the thermosensitivity of the *ras1Δ* mutant (69).

Immediately downstream of Ras, another guanine nucleotide exchange (GEF) factor protein, Cdc24, was discovered to directly interact with Ras. Its deletion causes the *ras1Δ* mutant-like thermosensitivity, and its overexpression suppresses the *ras1Δ* mutant thermosensitivity (69). Downstream of Cdc24, Cdc42 (but not
Rac1 or Dch2) and Ste20 (but not Pak1) have a role in thermotolerance, because overexpression of CDC42 or STE20 rescues the thermosensitivity of the cdc24Δ mutant (69). Similar to the ras1Δ mutant, the cdc24Δ mutant exhibits swollen cell morphology and highly depolarized actin structures at high temperatures (69). In fact, all of the Ras-dependent stress phenotypes are similarly observed in the cdc24Δ mutant (63), suggesting that the Ras1/2-Cdc24-Cdc42-Ste20 pathway has major roles in the stress response of C. neoformans.

The role of Aca1 in the stress response also appears to be independent of the cAMP/PKA signaling pathway, because the stress-responsive phenotypes of the aca1Δ mutant are quite different from those of the cac1Δ, gpa1Δ, and pka1/2Δ mutants (63). Aca1 orthologs, cyclase-associated proteins (CAPs), are well-known in the maintenance of actin cytoskeletal structure in fungi (for a review, see reference 70). CAPs not only modulate adenyllyl cyclase activity, but they also bind to highly conserved monomeric actin in eukaryotes. C. neoformans Aca1 has all of the typical signature domains found in most CAPs: the RLEXA(T/V)XRLE motif for Ras/cAMP signaling and proper cellular localization, the central proline-rich domain, the SH3 domain for binding to Abp1 (actin-binding protein 1) and the highly conserved CAP signature sequence at the C terminus for actin-monomer binding activity (71). Abp1 regulates actin cytoskeleton structure in S. cerevisiae (72), and its ortholog appears to be conserved in the C. neoformans genome (CNAG_01375.2). Therefore, Aca1 could modulate the actin cytoskeleton by directing binding of the actin monomer and interacting with Abp1, which affects diverse stress responses in C. neoformans in parallel with the Ras signaling pathway. This possibility needs to be addressed in future studies.

The cAMP/PKA signaling pathway has some Ras-independent roles in the stress response of C. neoformans: resistance to the thiol-specific external oxidant diamide, toxic heavy metal (CdSO4), and toxic metabolites (e.g., methylglyoxal [MG]) (63).
In response to diamide and MG, the ras1Δ mutant and strains with mutations of genes encoding components of the cAMP pathway display opposing phenotypes. The cac1Δ, aca1Δ, gpa1Δ, and pka1Δ mutants, but not the pka2Δ mutant, show increased resistance to diamide and sensitivity to MG, whereas the ras1Δ mutant shows the opposite phenotype (63).

It also seems that the Ras signaling pathway has both positive and negative roles in the oxidative stress response depending on the type of reactive oxygen species (ROS) involved. The ras1Δ mutant is resistant to hydrogen peroxide (H₂O₂) and a superoxide generator (e.g., menadione) but is sensitive to alkyl peroxide (e.g., tert-butyl hydroperoxide [tBOOH]) and diamide. In response to toxic heavy metal (CdSO₄), both the cAMP and ras1Δ mutants show increased sensitivity to CdSO₄. Interestingly, however, the deletion of CAC1 partially rescues CdSO₄ resistance in the ras1Δ mutant (63). Similarly, the deletion of CAC1 also partly suppresses the osmosensitivity of the ras1Δ mutant (63). Therefore, it is possible that the Ras and cAMP signaling pathways negatively regulate each other under specific conditions such as osmotic and heavy metal stresses. Supporting this, a large portion of the transcriptome profiles of the ras1Δ mutant shows patterns opposite to those of strains with mutations of genes encoding proteins in the cAMP pathway (63). The regulatory mechanism and cross talk between the two pathways should be further studied in detail.

Downstream of PKA, a plethora of downstream target genes were discovered by several independent transcriptome analyses (63, 73, 74, 75). Supporting the aforementioned roles of the Ras and cAMP/PKA signaling pathways in the stress response, Maeng et al. reported that out of 1,959 environmental stress response (ESR) genes, including genes encoding small heat shock proteins, Hsp12 and Hsp122, a total of 225 are differentially regulated in the ras1Δ mutant and mutants in the cAMP pathway (63). Hu et al. also discovered a number of stress response genes, including the Hsp122 gene, by using serial analysis of gene expression (SAGE).
with the pka1Δ and pkr1Δ mutants (74). Cramer et al. discovered the Nrg1 transcription factor as a PKA target (75). Interestingly, Nrg1 is only distantly related to C. cerevisiae and C. albicans Nrg1 orthologs and uniquely contains a PKA consensus sequence for phosphorylation. Nrg1 is positively involved in a subset of cAMP phenotypes, such as capsule production and mating but not in melanin synthesis. In future studies, more downstream regulators of PKA, particularly PKA-dependent stress-responsive transcription factors, should be identified and characterized. Interestingly, a PKA-dependent Msn2/4-like general stress-responsive transcription factor found in C. cerevisiae and other fungi is missing in C. neoformans.

**OTHER Ras- and cAMP/PKA-RELATED PATHWAYS**

Other Ras- and cAMP/PKA-related signaling components involved in stress response have been reported in C. neoformans, including the Msl1-like (MSIL) protein and the Sch9 protein kinase. MSIL proteins, which contain six or seven WD40 repeat domains, are multifunctional, eukaryote-specific proteins (for a review, see reference 76). In C. cerevisiae, an MSIL protein, Msl1 (also known as Cac3), is one of the chromatin assembly factor 1 (CAF-1) components (others are Cac1 and Cac2). CAF-1, in turn, serves as a histone chaperone, mediating histone binding and chromatin assembly during DNA replication and in DNA damage repair (77, 78). Msl1 also negatively regulates the Ras/cAMP signaling pathway by sequestering the Npr1 protein kinase. Npr1 is activated by PKA and inhibits the ubiquitin-proteasome-dependent degradation of nutrient transporters, such as ammonium permease (79).

C. neoformans contains a single MSIL protein, Msl1, and some data have suggested a functional connection between Msl1 and the cAMP/PKA signaling pathway in C. neoformans (80). As some phenotypes of the msl1Δ mutant (e.g., increased melanin and mating efficiency) are opposite those of strains with mutations in genes encoding components of the cAMP pathway and as these phenotypes are suppressed by deletion of CAC1, Msl1 may negatively regulate cAMP signaling upstream of Cac1 (80). In contrast, most stress-related phenotypes observed for the msl1Δ mutant, including increased thermotolerance and defects in cell membrane stability, genotoxic resistance, and oxidative stress response, are mainly independent of the Ras and cAMP/PKA phenotypes (80). Supporting this, the msl1Δ ras1Δ double mutant exhibits a significant growth defect and is also synergistically defective under most of the environmental stresses described above (80). Therefore, it seems that Msl1 is a component of another novel stress signaling pathway in C. neoformans. In support of this hypothesis, several stress defense genes were identified as Ms11-regulated genes by DNA microarray-based transcriptome analysis of the msl1Δ mutant (80). For example, the expression of two small heat shock protein genes involved in polypeptide drug resistance, HSP12 and HSP122 (63), is downregulated in the msl1Δ mutant, whereas the mitochondrial matrix chaperone protein, Hsp78, is upregulated in the msl1Δ mutant (80).

An important issue is whether Cryptococcus contains an Npr1-like kinase, which is a major Ms11-interacting kinase in C. cerevisiae (79). The yeast npr1Δ mutant exhibits phenotypes opposite those of the MSII overexpression strain (79). Interestingly, C. neoformans appears to contain a single putative kinase that is orthologous not only to Npr1, but also to yeast Hrk1 (first hit), Rtk1, and Prf2 kinases in C. cerevisiae. The potential functional connection between Ms11 and the Npr1 ortholog is under investigation.

Yeast Sch9 is a member of the AGC (named after protein kinases A, G, and C) protein kinase family. It is orthologous to mammalian S6 kinase 1 (S6K1) and a direct target of TOR (target of rapamycin) complex 1 (TORC1) (81, 82). Phosphorylated by TORC1 and Pkh1, Sch9 regulates ribosomal biogenesis by directly phosphorylating RPS6 (ribosomal protein S6). The Sch9 kinase signaling pathway is also a nutrient-sensing signaling pathway (along with the Ras/cAMP/PKA and Tor pathways) that governs chronological life span by negatively regulating the cytoprotective role of autophagy in C. cerevisiae (83, 84, 85).

Related to its roles in nutrient sensing, aging, and autophagy, Sch9 is involved in stress response and adaptation in C. cerevisiae. The sch9Δ mutant exhibits increased cellular adaptation to oxidative and thermal stresses (86). This role of Sch9 in stress response correlates with its ability to cross talk with the cAMP/PKA signaling pathway. Sch9 inhibits PKA activity by destabilizing the catalytic subunit of PKA, particularly Tpk2 (87). Deletion of SCH9 increases protein stability of Tpk2 and delocalizes the cellular location of Tpk2 from the nucleus to the cytoplasm, which subsequently increases the phosphorylation of Cdc25. Hyperphosphorylation of Cdc25 lowers its GEF activity for Ras activation and eventually decreases glucose-induced CAMP production as part of negative-feedback regulation (87, 88).

The Cryptococcus Sch9 pathway shows both distinct and shared features with the C. cerevisiae Sch9 pathway. Unlike Sch9 in C. cerevisiae, the functional connection of Sch9 to the cAMP/PKA pathway is not clear in C. neoformans. Deletion of SCH9 increases capsule production, but not melanin production, and decreases mating efficiency. These phenotypic patterns are quite different from those of mutants deleted in either positive (e.g., CAC1 or PKA1) or negative components (e.g., PKR1) of the cAMP pathway. In addition, Sch9 does not affect cAMP levels (89). However, Sch9 is involved in diverse environmental stress responses (39, 40, 89). Deletion of SCH9 increases the thermotolerance of C. neoformans (39, 89), which is similar to the thermotolerance of the S. cerevisiae sch9Δ mutant. In contrast, however, Sch9 promotes the oxidative stress response in C. neoformans. The expression of SCH9 is induced upon oxidative stress (H2O2) in a Hog1-dependent manner, and interestingly, even basal expression levels of SCH9 appear to be controlled by Hog1 in C. neoformans (40). Supporting these results, the sch9Δ mutant is highly susceptible to external H2O2 (39, 40), indicating that Sch9 could be a downstream kinase regulated by the HOG pathway. In response to other environmental stresses, however, Sch9 mainly has pleiotropic roles in a Hog1-independent manner. The sch9Δ mutant exhibits increased susceptibility to toxic heavy metal (CdSO4) and cation (e.g., NaCl, but not KCl), a fungicide (fluodioxinol), and azole drugs (39). These stress-responsive patterns are quite distinct from those of other stress-activated pathways, indicating that the Sch9 protein kinase constitutes a unique stress-activated pathway in C. neoformans. Therefore, the functional and regulatory connection among Sch9, cAMP/PKA, Hog1, and TORC pathways needs to be addressed in future studies.

**THE Rim SIGNALING PATHWAY**

An extracellular pH change is a key environmental stress for fungi because it affects diverse cellular processes, including cell integrity and metabolic process. For instance, Candida albicans senses neu-
tral or slightly alkaline pH for its morphological transition from yeast to hyphae, which is critical for the virulence of the pathogen (90, 91). In fungi, the Rim (Pal in filamentous fungi) signaling pathway is a crucial circuit for the pH-responsive cellular process. The regulatory mechanism of the Rim pathway has been nicely covered by multiple reviews (reviewed in references 92, 93, and 94). In the Rim pathway, Rim101 (a PacC ortholog) is a key transcription factor with a Cys2His2 zinc finger domain. In response to alkaline or neutral pH, the C terminus of Rim101 is proteolytically cleaved and activated to induce target genes.

The ability to sense and adapt to pH changes is physiologically meaningful for C. neoformans, which encounters an acidic environment in the macrophage phagolysosome or a slightly alkaline environment in cerebrospinal fluid or serum (94, 95). Similar to S. cerevisiae, C. neoformans contains a single Rim101 ortholog, and its deletion increases cellular susceptibility to an alkaline pH (96). RIM101 was first discovered as a virulence-related gene through a large-scale deletion mutant analysis in C. neoformans (97). Unexpectedly, Rim101 has been independently identified as a putative PKA target transcription factor in C. neoformans (96), whereas in other fungi, such a PKA-mediated Rim101 regulation has not been reported. The rim101Δ mutant exhibits a slight melanin defect but increased fitness during infection (97). Reflecting the role of the CAMP/PKA pathway in capsule production, deletion of RIM101 reduces the size of capsules surrounding the cell (96). Supporting this finding, deletion of RIM20, which encodes a scaffolding protein required for Rim101 cleavage/activation (Fig. 3), reduces capsule size in a similar fashion. Interestingly, it was shown that the reduced capsule size observed in the rim101Δ mutant does not result from defective intracellular capsule synthesis per se, but from defective deposition of capsule secreted on the cell surface (96).

A Rim101 allele having a site-specific mutation in the PKA consensus site (S773A) not only fails to restore the capsule defect of rim101Δ but also causes mislocalization of Rim101, which normally localizes to the nucleus, suggesting that Rim101 is under the control of PKA (96). Interestingly, Rim101 undergoes additional cleavage under capsule-inducing conditions. The rim101Δ mutant is also hypersensitive to ion stress and...
iron deprivation. It has been shown that target genes, including UGD1 (a UDP-glucose dehydrogenase) and CMT1 (a mannosyl-transferase), ENA1, and iron transporter/permease (SIT1 and CFT1), are regulated through Rim101. In addition, Rim101 regulates the expression of a copper transporter (CTR4) (96). This is significant, as it has recently been reported that the copper detoxification machinery is critical for the virulence of C. neoformans (98, 99).

Unexpectedly, the deletion of RIM101 increases the fitness of C. neoformans (in specific murine host strains) regardless of defective capsule production and some stress-sensitive phenotypes (96). O’Meara and colleagues further discovered that the rim101Δ mutant causes a different cryptococcal disease by overactivating the host immune response (100). Rim101, under physiological conditions in the host, transcriptionally regulates a series of cell wall remodeling genes. Hence, the deletion of RIM101 alters cell surface antigens and decreases capsule deposition on the cell surface, triggering an excessive but ineffective host immune response that enhances host death (100).

Interestingly, Rim101 is also involved in regulation of the titan cell (100, 101). It still remains elusive, however, as to how Rim101 is regulated and activated in C. neoformans. In fact, the PKA-dependent regulation of Rim101 has not been observed in other fungi. Therefore, upstream activators or sensors of Rim101 need to be further discovered and functionally elucidated (Fig. 3).

Known components of the plasma membrane pH sensor complex in fungi include a seven-transmembrane Rim21-like receptor, a three-transmembrane Rim9-like protein, and an arrestin-like Rim8-like protein. Interestingly, orthologs for Rim21 and Rim9 appear to be missing in the Cryptococcus genome, suggesting the possibility that C. neoformans may employ a unique receptor to activate Rim101 (94). One possible upstream regulator of Rim101 is the Gpr5 G-protein-coupled receptor (GPCR), because both rim101Δ and gpr5Δ mutants exhibit severe defects in basal titan cell formation (101). However, it remains unknown whether Gpr5 is involved in sensing pH and whether it activates Rim101 through PKA. The current proposed model of the Cryptococcus Rim pathway is summarized in Fig. 3.

**THE Ca2+/CALCINEURIN SIGNALING PATHWAY**

The Ca2+/calcineurin pathway acts together and/or in parallel with the Rim signaling pathway to regulate pH response and the integrity of the cell wall. This pathway has been well characterized in mammals and fungi (for reviews, see references 59, 102, 103, 104, 105, 106, and 107). In response to certain environmental signals, intracellular Ca2+ increases by taking up extracellular Ca2+ and/or by releasing endoplasmic reticulum (ER)-stored internal Ca2+ into the cytoplasm and binds to calcmodulin. The calcmodulin proteins contain four EF-hand motifs, each of which can bind a single Ca2+. The Ca2+-bound calcmodulin either activates Ca2+/calcmodulin-dependent protein kinases (CaMK) or a Ser/Thr-specific phosphatase, calcineurin. The physiological outputs of the calcineurin pathway are as diverse as T cell activation in mammals and cell wall integrity and thermotolerance control in certain fungi.

In C. neoformans, the Ca2+/calcmodulin/calcineurin pathway is involved in diverse cellular functions, including thermotolerance, resistance to high CO2 and alkaline pH, cell wall integrity, and sexual differentiation (for reviews, see references 59, 104, 105, 106, and 107). Calcmodulin (Cam1) appears to have both Ca2+-dependent/independent and calcineurin-dependent/independent roles and does not require Ca2+ binding in the control of thermodurability and mating (108). Inhibition of calcineurin, consisting of the catalytic A (Cna1) and regulatory B (Cnb1) subunits, decreases the ability of C. neoformans to survive at 37°C, at a slightly alkaline pH, or in the presence of high CO2 (5%) (109, 110). A calcineurin-binding protein, Chop1, has been identified to be a target of both calcineurin and CaMK. The deletion of CBP1 causes only a modest defect in survival under ionomic stress, alkaline and high CO2 conditions, and in vivo virulence but causes no growth defect at 37°C (111). Two cyclophilin-like proteins, Cpa1 and Cpa2, play redundant roles in growth at high temperature (112).

It has long been thought that a bona fide Crz1 ortholog downstream of calcineurin is missing in C. neoformans. Recently, however, two independent studies have demonstrated conflicting results about the presence of a Crz1-like transcription factor in C. neoformans. In an effort to identify a Crz1 ortholog, Adler et al. found CNAG_00156 (named SP1 for specificity protein 1), a protein containing the zinc finger C2H2 type domain, which is phylogenetically similar to the yeast Crz1 (113). However, they concluded it is not a Crz1 ortholog on the basis of the following observations: (i) a lack of complementation on cna1Δ phenotypes (thermotolerance and SDS/Ca2+ sensitivity) by SP1 overexpression, (ii) a lack of calcineurin-dependent posttranslational modification, and (iii) a lack of calcium-dependent nuclear translocation of the Sp1-green fluorescent protein (GFP) (constitutive nuclear localization of Sp1). Instead, they suggested Sp1 is the downstream transcription factor of Pck1 (protein kinase C1), because the transcriptome profiles of the sp1Δ mutant are similar to those of the pck1Δ mutant under glucose starvation conditions and the overexpression of SPI partially restores cell wall integrity defects in the pck1Δ mutant (113). In contrast, Lev and colleagues provided several lines of evidence that Crz1/Sp1 is a transcription factor downstream of calcineurin (114). First, Crz1/Sp1 can physically interact with Cna1 in an inactive state based on the yeast two-hybrid assay. Second, the crz1Δ mutant exhibits an increased susceptibility to cell wall-damaging agents (Calcifluor white [CFW] and Congo red [CR]), but not to host physiological temperature and ion stress, and shows normal mating efficiency, suggesting that Crz1/Sp1 is involved in only a subset of calcineurin phenotypes. Supporting this result, the expression of CHS6, which encodes the chitin synthase gene and is induced at 37°C or by CFW treatment, is also regulated by Crz1. In stark contrast to the finding of Adler et al. (113), a functional Crz1-GFP fusion protein localizes to both the cytosol and nucleus under normal conditions but translocates into the nucleus in response to CFW, 37°C, or CaCl2 in a calcineurin-dependent manner, but not to NaCl or high temperature (42°C). Another independent study demonstrated that Crz1/Sp1 is required for growth under hypoxic conditions, calcium resistance at 37°C, biofilm formation, and azole resistance (115). Taken together, it is likely that Crz1 could be under dual regulation by the Ca2+/calcineurin and Pck1 pathways. The current proposed model of the Ca2+/calcineurin pathway is summarized in Fig. 3.

**THE Pck1 AND Mpk1 MAPK SIGNALING PATHWAYS**

Along with the Rim and calcineurin pathways, the Pck1 and Mpk1 MAPK pathways have a central role in controlling cell wall integrity. Pck1 plays pleiotropic roles in C. neoformans (59, 106). Pck1 was discovered as a key regulator of the lipid signaling pathway in
C. neoformans and is activated by diacylglycerol (DAG), which is generated from phosphatidylglycerol by inositol phosphoryl ceramide (IPC) synthase (Ipc1) (116). The DAG-activated Pkc1 promotes melamin and capsule biosynthesis in C. neoformans (116, 117). Such a DAG-mediated PKC activation in C. neoformans is similar to activation in mammals, but distinct from other yeasts, including S. cerevisiae and C. albicans. Ipc1 is considered a good anticyclococcal target, as the Ipc1-like enzyme is not present in mammals but is important for virulence of C. neoformans (for a review, see reference 118).

Pkc1 is also involved inazole resistance by controlling spherolipid content and is phosphorylated by an AGC family kinase, Pdk1 (also known as Pkh2-02) (119, 120). Pck1 also contains a consensus Pdk1 phosphorylation site, and both pck1Δ and pdk1Δ mutants exhibit increasedazole susceptibility. In addition, Pdk1/Phk2-02 promotes growth, thermoresistance, cell wall integrity, and oxidative and nitrosative stress responses via Pck1 and the Mpk1 MAPK module, thereby significantly affecting the virulence of C. neoformans (120). Furthermore, an inhibitor of human Pdk1 (hPDK1), which is highly orthologous to Pdk1, confers antifungal activity against Cryptococcus (121).

Pkc1 signaling appears to branch into multiple downstream signaling cascades (122). One of these cascades involves the Bck1-Mkk2-Mpk1 MAPK module. In response to oxidative and nitrosative stresses, Pck1 transmits its signal to the Bck1 MAPKKK, which subsequently activates the Mkk2 MAPKK (also known as Mkk1) and the Mpk1 MAPK by sequential phosphorylation. As expected, all of the mpk1Δ, mkk2Δ, and bck1Δ mutants exhibit increased susceptibility to oxidative and nitrosative stresses (35, 123). Furthermore, the Mpk1 MAPK mediates Pck1 in azole resistance because all mpk1Δ, mkk2Δ, and bck1Δ mutants exhibit an increase inazole susceptibility similar to the pck1Δ mutant (119). In response to thermal stress, however, PKC does not activate the Bck1-Mkk2-Mpk1 module (122). Nevertheless, Pck1 is still required for thermotolerance in C. neoformans because the pck1Δ mutant does not grow at 37°C or 39°C (123). Furthermore, the role of the Ipc1-DAG-PKC pathway in melanin and capsule biosynthesis does not appear to be mediated through the Mpk1 MAPK (123).

Thermal stress activates the Bck1-Mkk2-Mpk1 pathway through Rho-type GTPases, including Rho1, Rho10, and Rho11, without the involvement of Pck1 (122). Rho1 is an essential protein. The constitutive activation of Rho1 (the G-to-V change at position 15 encoded by rho1 [rho1G15V]) increases Mpk1 phosphorylation levels (via Bck1 and Mkk2) and perturbsthermol tolerance like the mpk1Δ mutant, indicating that the constitutive phosphorylation of Mpk1 also hampers normalthermol tolerance. In contrast, Rho10 and Rho11 are nonessential proteins. Rho11 appears to activate the Bck1-Mkk2-Mpk1 pathway and is repressed by upstream Rho10. Supporting this hypothesis, deletion of RHO10 increases Mpk1 phosphorylation at both basal and induced levels, but double deletion of RHO11 in the rho10Δ mutant restores the wild-type level of Mpk1 phosphorylation (122). However, it seems that Rho10 also controls thermol tolerance and cell wall integrity independent of the Mpk1 pathway because the rho10Δa rho1ΔΔ double mutant also shows increasedthermol tolerance and cell wall integrity defect like the rho10Δa mutants (122). Other than thermol tolerance, the Bck1-Mkk2-Mpk1 pathway is also involved in resistance to the fungicide fludioxonil and a toxic metabolite methylglyoxal (MG) with unknown mechanism (35). In summary, the Pkc1 and Mpk1 MAPK signaling pathways play distinct and shared roles in regulating a variety of environmental stress responses in C. neoformans (Fig. 3).

THE UNFOLDED PROTEIN RESPONSE (UPR) SIGNALING PATHWAY

In eukaryotic cells, the endoplasmic reticulum (ER) is the hub where most secreted and transmembrane proteins undergo post-translational modifications, including glycosylation and disulfide bond formation, to undergo proper folding and maturation. When unfolded or misfolded proteins accumulate in the ER (called ER stress), the cells activate an evolutionarily conserved unfolded protein response (UPR) to alleviate ER stress (124). The regulatory mechanism of the UPR pathway has been well characterized in S. cerevisiae and humans. In the budding yeast, the UPR pathway consists of an Ire1 sensor kinase and its downstream transcription factor, Hac1 (125). Ire1, which is a Ser/Thr kinase with an endonuclease (RNase) domain, senses ER stress, and its activation leads to the removal of an unconventional intron in the mRNA of HAC1 (an active basis domain/leucine zipper [bZIP] transcription factor) without involvement of spliceosomes (126). The Rlg1 RNA ligase splices the two exons of the HAC1 mRNA to induce UPR target genes, such as molecular chaperones, and relieve ER stress (127).

Cryptococcus has an evolutionarily conserved Ire1 kinase but a unique bZIP transcription factor, Hxl1 (HAC1 and XBPI-like gene 1) (128). Strikingly, Hxl1 is phylogenetically distinct from Hac1 and Xbp1 and could serve as a prototypic UPR transcription factor for the basidiomycete and plants. Supporting this, HXL1 cannot complement the yeast hac1Δ mutant (128). Cheon et al. demonstrate that Ire1 removes the unconventional intron (56 nucleotides [nt]), which is much shorter than the intron of the HAC1 mRNA (252 nt), from the HXL1 mRNA and generates the active Hxl1 transcription factor upon ER stress (128). The splicing mechanism between two exons of the HXL1 mRNA remains elusive, although Cryptococcus has a single Rlg1-like ortholog, which is essential for the growth of S. cerevisiae. However, the conserved nucleotides CAG at the border of the unconventional intron in HAC1 and XBPI is also observed in the HXL1 intron and is specifically recognized by Ire1 (128), suggesting that the Cryptococcus UPR pathway is evolutionarily conserved and has unique features. Besides its role in ER stress response, the UPR pathway not only is involved in other environmental stress responses, including thermotolerance and maintenance of cell wall integrity, but it also modulates virulence factor production (e.g., capsule synthesis) and in vivo virulence of C. neoformans (128). Notably, however, Ire1 and Hxl1 have both redundant and distinct roles in certain cellular functions (128), indicating that Ire1 and Hxl1 are not strictly in the linear signaling cascade (Fig. 4). Recently, Kar2/ binding immunoglobulin protein (BiP), which is an ER-resident molecular chaperone and a major downstream UPR target, was functionally characterized (129). Kar2/BiP is an essential protein and governs a subset of UPR-dependent phenotypes, including ER stress response, high temperature growth, genotoxic stress response, and cell wall integrity.

Besides Ire1 and Hxl1, other signaling components and pathways are also involved in UPR and ER stress response. The deletion of CNA1, a catalytic subunit of calcineurin, affects HXL1 splicing event and induction of KAR2 during adaptation to the physiological temperature of the host (37°C) (128). In Candida
**glabrata**, KAR2 expression is controlled by the calcineurin pathway, but not by the UPR pathway (130), indicating context-dependent roles of calcineurin and UPR pathways in fungal ER stress response.

Recently, Bloom et al. (131) and Havel et al. (132) reported that mRNA degradation machinery controls stability of ER stress-responsive transcripts during host temperature adaptation in *C. neoformans*. Decay of ER stress-responsive transcripts, including KAR2, OST2 (a subunit of the ER oligosaccharyltransferase complex), and ALG7 (lipid-linked N-oligosaccharyltransferase), is delayed in cells lacking Ccr4 (which encodes mRNA deadenylase and is involved in mRNA stability) during ER stress response and host temperature adaptation (132). Furthermore, Rpb4, which encodes a RNA polymerase II subunit, controls the destabilization of KAR2 during temperature upshift (131). Taken together, the mRNA degradation machinery is likely also involved in the UPR and ER stress responses.

Another notable function of the UPR pathway is its role in azole resistance (128, 129). Inhibition of the UPR pathway dramatically increases azole susceptibility, suggesting that a UPR inhibitor could be a good antifungal agent, if fungus specific, as a mono- or combinatorial therapy with azoles. This phenomenon is also observed in *Aspergillus fumigatus* but in a distinct manner (133). The UPR pathway in *A. fumigatus* affects expression of ERG3 and ERG11, whereas the *Cryptococcus* UPR pathway does not regulate these genes (129). Instead, *Cryptococcus* appears to sense azole treatment as an ER stress, as azole treatment activates the Hxl1 splicing event and thereby induces KAR2 expression (129). Supporting this, the constitutive expression of KAR2 partially restores azole resistance in ire1Δ and hxl1Δ mutants.

The current proposed model of ER stress response and UPR pathways is summarized in Fig. 4. The most urgent question to answer is how Hxl1, which is structurally distinct from yeast Hac1, regulates ER stress genes, considering its unique structure. Furthermore, it remains to be answered which upstream regulator, other than Ire1, regulates Hxl1. In addition, the identity of other transcription factors regulated by Ire1 in an Hxl1-independent manner remains to be determined. Finally, genome-wide transcriptome analysis should be performed to identify Ire1/Hxl1-, Ire1-, or Hxl1-dependent genes in *C. neoformans*.

**CONCLUSIONS**

Besides unanswered topics described above, there are further issues that future studies of stress signaling pathways in *Cryptococcus* should address. The first and foremost important issue is pathway cross talk. Despite a growing amount of evidence suggesting...
that there is extensive cross talk between multiple stress signaling cascades, it remains unclear how pathway cross talk is coordinated in response to incoming stresses. For example, although the HOG pathway cross talks with the cAMP/PKA, Rim101, and Mpk1 pathways, their cross talk points are not clear. Second, regardless of the large amount of information for the roles and regulation of core signaling components in many stress-sensing pathways, how cells sense diverse stress signals and which transcription factors are critical in controlling downstream gene expression remain largely unclear. Therefore, additional upstream sensor/receptor-like proteins and transcription factors in these pathways must be discovered and characterized in future studies to understand the complete signaling cascade of each pathway. Third, other evolutionarily conserved and unique stress signaling pathways should be discovered and investigated. The well-known TOR signaling pathway is one example, as its role in the pathogenicity of Cryptococcus has not been intensively studied. Cryptococcus appears to have a number of unique transcription factors and other signaling components, suggesting it may employ unique signaling cascades to be a successful ubiquitous human fungal pathogen. Finally, a comparative analysis of C. gattii and C. neoformans stress signaling networks should be performed to understand their mechanisms of pathogenesis, particularly considering the recent outbreak of C. gattii. This data will be needed to understand the complex stress signaling networks, enabling the development of novel anticytotoxic and antifungal therapeutic methods.

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