RESEARCH ARTICLE

Cross Talk between the Cell Wall Integrity and Cyclic AMP/Protein Kinase A Pathways in *Cryptococcus neoformans*

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ABSTRACT ***Cryptococcus neoformans* is a fungal pathogen of immunocompromised people that causes fatal meningitis. The fungal cell wall is essential to viability and pathogenesis of *C. neoformans*, and biosynthesis and repair of the wall is primarily controlled by the cell wall integrity (CWI) signaling pathway. Previous work has shown that deletion of genes encoding the four major kinases in the CWI signaling pathway, namely, *PKC1*, *BCK1*, *MKK2*, and *MPK1* results in severe cell wall phenotypes, sensitivity to a variety of cell wall stressors, and for Mpk1, reduced virulence in a mouse model. Here, we examined the global transcriptional responses to gene deletions of *BCK1*, *MKK2*, and *MPK1* compared to wild-type cells. We found that over 1,000 genes were differentially expressed in one or more of the deletion strains, with 115 genes differentially expressed in all three strains, many of which have been identified as genes regulated by the cyclic AMP (cAMP)/protein kinase A (PKA) pathway. Biochemical measurements of cAMP levels in the kinase deletion strains revealed significantly less cAMP in all of the deletion strains compared to the wild-type strain. The deletion strains also produced significantly smaller capsules than the wild-type KN99 strain did under capsule-inducing conditions, although the levels of capsule they shed were similar to those shed by the wild type. Finally, addition of exogenous cAMP led to reduced sensitivity to cell wall stress and restored surface capsule to levels near those of wild type. Thus, we have direct evidence of cross talk between the CWI and cAMP/PKA pathways that may have important implications for regulation of cell wall and capsule homeostasis.**

IMPORTANCE ***Cryptococcus neoformans* is a fungal pathogen of immunocompromised people that causes fatal meningitis. The fungal cell wall is essential to viability and pathogenesis of *C. neoformans*, and biosynthesis and repair of the wall are primarily controlled by the cell wall integrity (CWI) signaling pathway. In this study, we demonstrate that deletion of any of three core kinases in the CWI pathway impacts not only the cell wall but also the amount of surface capsule. Deletion of any of the kinases results in significantly reduced cellular cyclic AMP (cAMP) levels, and addition of exogenous cAMP rescues the capsule defect and some cell wall defects, supporting a direct role for the CWI pathway in regulation of capsule in conjunction with the cAMP/protein kinase A pathway.**

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Cryptococcus neoformans is a fungal pathogen of immunocompromised people that causes an estimated 1 million infections each year among HIV-positive patients globally, resulting in 600,000 deaths annually (1). Much of the disease burden occurs in sub-Saharan Africa, where deaths from cryptococcal infections may exceed those from tuberculosis in some areas (1). Cryptococcal infections can be successfully treated with antifungal agents, but the mortality rate remains 15 to 30%, even in the context of antiviral treatments for HIV (2–4). The closely related species *Cryptococcus gattii* can infect immunocompetent people and animals. Although *C. gattii* is more common in Australia, it received global attention in 1999 when it emerged on Vancouver Island in Canada and has since spread into other areas of the Pacific Northwest (5–7).

Infection by *C. neoformans* occurs upon inhalation of spores or desiccated yeast cells, which can then disseminate to the brain and cause fatal meningitis (reviewed in references 8, 9, and 10). *C. neoformans* rapidly establishes an infection in lung alveoli, where low iron availability and increased carbon dioxide levels induce the formation of a polysaccharide capsule on the surface of the cryptococcal cell. The *C. neoformans* capsule has been shown to be protective against phagocytosis and oxidative stress as well as reducing the T-cell immune response (11, 12). The capsule is a key virulence factor, and strains with disruptions or deletions of capsule-related genes are usually avirulent or demonstrate attenuated virulence in murine models of infection (reviewed in reference 13).

C. neoformans is a soilborne pathogen found ubiquitously in

TABLE 1 Number of differentially expressed genes

Strain(s)	No. of genes			No. of unique genes		
	Total	Up[a]	Down[a]	Total	Up	Down
*bck1*Δ strain	627	340	287	244	171	73
*mkk2*Δ strain	511	121	390	203	27	176
*mpk1*Δ strain	453	278	175	206	127	79
*bck1*Δ and *mkk2*Δ strains	164	39	123			
*bck1*Δ and *mpk1*Δ strains	103	85	13			
*mkk2*Δ and *mpk1*Δ strains	28	12	11			
*bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains	116	44	71			

[a] For genes found in more than one strain, they were listed as upregulated (Up) or downregulated (Down) only if the direction of the expression difference was concordance.

the environment of temperate zones that can also infect and persist in mammalian hosts. Thus, *C. neoformans* must be able to grow under a wide range of pHs and temperatures and be able to cope with a variety of exogenous stresses secreted by other soil organisms or mammalian host cells. The ability to survive in such diverse environments requires that the cell be able to sense and process multiple signals from the environment that result in activation of various signaling cascades to allow adaption to and growth under different conditions (reviewed in reference 14).

The cryptococcal cell wall is vital for maintaining cell morphology and forms the scaffold for capsule attachment. Cell wall homeostasis is maintained by the cell wall integrity (CWI) pathway, which has 4 core kinases, protein kinase C (Pkc1), bypass of protein kinase C (Bck1), mitogen-activated kinase kinase (Mkk2), and mitogen-activated protein kinase (Mpk1), all of which are necessary for a fully functional cell wall. The primary kinase for this pathway is Pkc1, a diacylglycerol-activated protein kinase C essential for responding to a variety of cell wall stresses and whose deletion results in severe cell wall defects, reduced melanin, and disrupted capsule (15, 16). Strains with a deletion of the *BCK1* or *MKK2* gene show increased sensitivity to cell wall stressors and high temperature as well as delayed melanin formation (17). Deletion of the gene encoding the putative terminal kinase in the cell wall integrity pathway, *MPK1*, results in attenuated virulence (18) and has been shown to reduce virulence when deleted in *C. gattii* (19). Previous studies of the CWI pathway have used phosphorylation of the terminal kinase, Mpk1, as a marker for activation of the pathway (18). We have demonstrated that in response to nitrosative or oxidative stress, *PKC1* is required for phosphorylation of the terminal kinase, Mpk1 (16). However, in a *pkc1*Δ strain, Mpk1 is still phosphorylated in response to heat shock, but not when the *BCK1* or *MKK2* gene is deleted (20), suggesting that inputs into this signaling cascade can bypass some of the core kinases and still result in activation of the pathway. We hypothesize that this pathway is likely to interact with other signaling cascades, such as the HOG1 and cyclic AMP (cAMP)/protein kinase A (PKA) pathways that respond to various exogenous stresses. To address this hypothesis, we used RNA sequencing to examine the genome-wide expression differences between the wild type and deletions in three of the core CWI kinases, *BCK1*, *MKK2*, and *MPK1*. We find strong evidence of cross talk between the cAMP/PKA and CWI pathways based on the genes differentially expressed in the kinase deletion strains. We demonstrate that deletion of any of the three kinases results in significantly decreased cAMP levels and reduced surface capsule and that exogenous cAMP can partially rescue the capsule- and cell wall-related defects of the *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains.

RESULTS

Impact of CWI kinases on the transcriptome. The expression profiles of the three kinase deletion strains (*bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains) were compared to wild-type *C. neoformans* KN99 MAT**a** (KN99a) when grown at 30°C in yeast extract-peptone-dextrose (YPD) medium plus 1 M sorbitol to an optical density (OD) of 1.0. We initially included a deletion of the *PKC1* gene in the experiment, which necessitated the use of sorbitol to act as a stabilizer (16). The RNA expression data revealed that the *pkc1*Δ strain was aneuploid, and thus, it was excluded from further analyses. Overall, 1,064 genes showed statistically significant differential expression with a corrected *P* value cutoff of <0.05 (Table 1; see Table S2 in the supplemental material) (21). More genes were differentially expressed in the *bck1*Δ strain (627 genes) than in the *mkk2*Δ (511 genes) and *mpk1*Δ (453 genes) strains. Of the 1,064 differentially expressed genes, 653 genes were found in only one of the three strains with a fairly even distribution: 244 genes were unique to the *bck1*Δ strain, 203 were unique to the *mkk2*Δ strain, and 206 were unique to the *mpk1*Δ strain (Fig. 1).

There were 116 transcripts differentially expressed in all three of the deletion strains, including genes involved in carbohydrate metabolism, cell wall biogenesis, transport, and signaling (see Table S3 in the supplemental material). Of the 116 genes, 115 were in concordance among the three mutant strains, with 71 upregulated genes and 44 downregulated genes compared to the wild type. The one gene that was not in concordance was a gene encoding a hypothetical protein of unknown function. We identified 10 of the

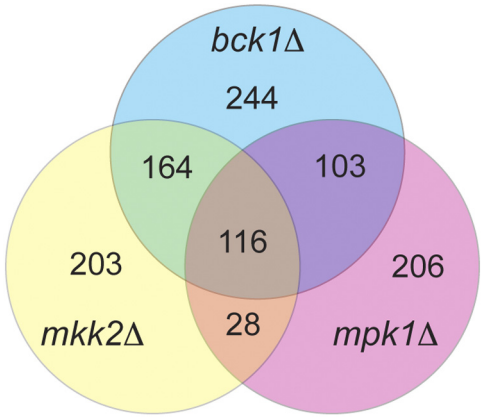

FIG 1 Comparison of the distribution and overlap of the differentially expressed genes in the CWI kinase deletion strains, the *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains.

TABLE 2 Cell wall- or capsule-related genes

Gene type and Broad ID[a]	Annotation	Log$_2$ fold change[b]			Reference(s)
		*bck1*Δ/WT strains	*mkk2*Δ/WT strains	*mpk1*Δ/WT strains	
Cell wall-related genes					
CNAG_00261	MPN10 putative mannoprotein			0.43	
CNAG_00546	CHS4 chitin synthase activity	−1.22	−1.33	−1.01	25
CNAG_00914	KRE6 β-glucan synthase	0.42			23
CNAG_01230	CDA2 chitin deacetylase			−0.61	22
CNAG_01234	SPO71 similar to *S. cerevisiae* spore wall assembly protein	−1.07	−1.81		
CNAG_01341	MPI1 putative mannose-6-phosphate isomerase		−1.17		
CNAG_02071	BNI4 scaffold protein	0.47			50
CNAG_02217	CHS7 chitin synthase	−0.87		−1.15	25
CNAG_02225	EXG1 cellulase	1.11		0.61	51
CNAG_03099	CHS1 chitin synthase	1.03	1.15	0.59	25
CNAG_03223	MPN6 putative mannoprotein	−1.30	−1.25	−1.35	
CNAG_03345	SSD1 translational repressor	−0.63			17
CNAG_03465	LAC1 laccase melanin synthesis	−0.75	−1.18	−0.66	52
CNAG_03782	MPN11 putative mannose protein		−3.34		
CNAG_05663	SCW1 similar to *S. cerevisiae* cell wall integrity protein	0.37	0.55		
CNAG_05818	CHS5 chitin synthase	−1.46	−1.31	−1.34	25
CNAG_06291	FPD1 polysaccharide deacetylase	−0.68	−1.19	−0.66	22, 51
CNAG_06487	CHS6 chitin synthase	0.51	0.50		25
CNAG_06835	KRE61 β-glucan synthase	−1.33	−2.79	−1.03	23
CNAG_07499	CHS8 chitin synthase			−0.56	25
Capsule-related genes					
CNAG_00600	CAP60 capsule-associated protein	0.81			53
CNAG_00721	CAP59 capsule-associated protein	0.75	0.67	0.31	54
CNAG_01172	PBX1 parallel β-helix repeat protein			0.35	55
CNAG_01371	CRG2 regulator of G-protein signaling	0.46			56, 57
CNAG_01654	CAS34 capsule structure protein	0.61			58
CNAG_02581	CAS33 capsule-associated protein	0.39			59
CNAG_03735	CAP4 glycosyltransferase	0.67	0.56		58
CNAG_05081	PDE1 phosphodiesterase		−0.89		60
CNAG_07554	CAP10 glycosyltransferase	0.59			61

[a] Broad ID, Broad identification or locus tag.
[b] The log$_2$ fold changes in the level of expression for the *bck1*Δ, *mkk2*Δ, and *mpk1*Δ deletion strains compared to that of the wild-type (WT) strain are shown.

115 genes as related to cell wall or capsule homeostasis, reflecting the importance of these kinases working in concert to maintain cell wall integrity (CWI).

Differential expression of cell wall- and capsule-related genes. Among all of the differentially expressed transcripts, we identified 29 genes that are known or predicted to be involved in cell wall biogenesis, cell wall regulation, or capsule synthesis (Table 2). Six of the eight chitin synthase genes, *CHS1*, *CHS4*, *CHS5*, *CHS6*, *CHS7*, and *CHS8*, show altered expression in two or more of the kinase deletion strains (Table 2). The *CHS1*, *CHS4*, and *CHS5* genes show altered expression in all three deletion strains, with *CHS1* upregulated and *CHS4* and *CHS5* showing decreased expression. A deacetylase gene, *FPD1*, for which no function has yet been identified (22) was downregulated in all three deletion strains, suggesting a possible adaptive response to the cell wall stress induced by the deletions. The *KRE6* gene, which is involved in β-1,6-glucan synthesis (23), was modestly upregulated in the *bck1*Δ strain, but *KRE61*, a homolog of *KRE6* with no known function, was highly repressed in all three deletions, again suggesting a possible adaptive response or regulation of this gene by the CWI pathway. Of the nine capsule-related genes that are differentially expressed, seven are changed in the *bck1*Δ strain, but only one (*CAP59*) is found in all three deletion strains, precluding a significant role for the CWI pathway in transcriptional regulation of capsule biosynthesis. Although *CAP59* has been associated mostly with capsule defects, it may have a role in general secretion, rather than a capsule-specific role (24).

Reduced chitin levels in the CWI kinase deletion strains. All of the deletion strains had significant cell wall phenotypes, which likely affect cellular chitin and chitosan levels. We determined the chitin and chitosan content of the cell walls, and all three had significantly more chitin and significantly less chitosan than did the wild-type KN99a strain (Fig. 2). The chitin synthase gene, *CHS3*, responsible for the majority of chitosan synthesis during vegetative growth was unchanged in these deletion strains, as were two of the three chitin deacetylase genes. Three chitin synthase genes, *CHS1*, *CHS4*, and *CHS5*, had altered expression in all three of these deletion strains (Table 2). Previous work on the *CHS* genes revealed no phenotypes when *CHS1* was deleted, although *CHS1* transcript could be detected in vegetatively growing cells (25). Strains with deletion of either *CHS4* or *CHS5* have reduced chitin and increased chitosan content, but otherwise had no other *in vitro* cell wall phenotypes (25). These data suggest a possible role for *CHS1*, *CHS4*, and/or *CHS5* in maintaining cell wall morphology but one that is dependent on the CWI kinases. Previous work has demonstrated an important role for chitin oligomers in

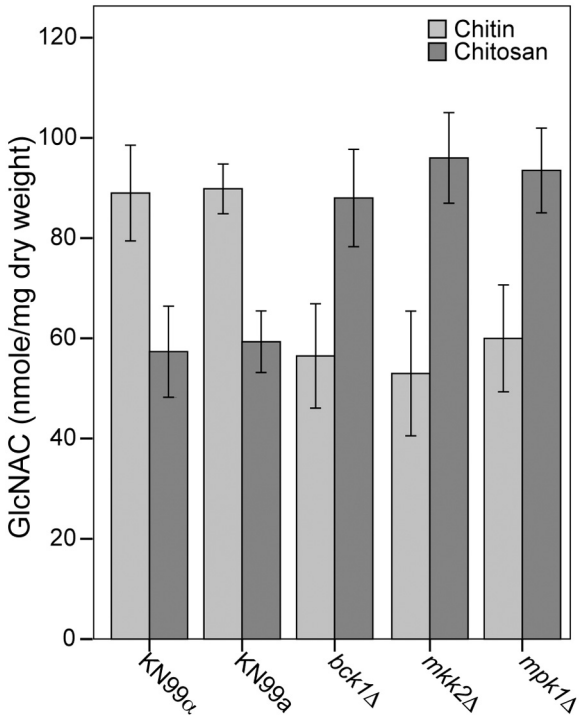

FIG 2 *C. neoformans BCK1*, *MKK2*, and *MPK1* are required for full chitin production. Chitin and chitosan levels of *C. neoformans* KN99a, KN99α, and *bck1*Δ, *mkk2*Δ, and *mpk1*Δ deletion strains. Measurements of strains were taken after 70 h of growth in liquid YPD at 25°C and reported as average nanomoles per milligram (dry weight) of chitin and chitosan as indicated. Each measurement represents the average from three independent cultures for each strain, and error bars indicate standard deviations. The levels of chitin and chitosan for the *bck1*Δ, *mkk2*Δ, and *mpk1*Δstrains were compared to either KN99a or KN99αusing a one-way ANOVA and Dunnet's posthoc test with the KN99a strain as the control. The chitin and chitosan values for all three deletion strains were highly significantly different ($P < 0.001$) from the value for the KN99a strain.

capsule attachment (26, 27). However, the source of the chitin for the oligomers has not been determined. It is plausible that *CHS1*, *CHS4*, and/or *CHS5* may play a role in producing the chitin necessary for capsule attachment.

Overlap of differentially expressed genes with other stress-responsive signaling pathways. Because of the cell wall and stress phenotypes associated with the CWI kinase deletion strains, we compared our differentially expressed gene lists with those generated from deletions in genes that regulate the cAMP/PKA pathway (28), the HOG1 stress pathway (29), and capsule synthesis (30–32). There is a substantial overlap in genes identified as differentially expressed in all of these studies, which is expected given that these gene lists include several hundred to thousands of genes. We narrowed the search to examine the overlap of genes differentially expressed in all three of our deletion strains with these other data sets (see Table S3 in the supplemental material). We found the greatest overlap (54 and 56 genes, respectively) with genes dependent on *HOG1*, a mitogen-activated protein (MAP)-type kinase involved in osmoregulation (33) and *SSK1*, a response receiver protein that modulates Hog1 activity (34). We found substantial overlap (38 genes) with *RIM101*-dependent genes (31), with genes dependent on *NRG1* (31 genes) (32) and with genes dependent on *SKN7* (27 genes), another response receiver protein that regulates sensitivity to Na^+ ions (34). Out of the 115 genes differentially expressed in all three CWI kinase deletion strains were 10 genes that have a known or predicted role in cell wall or capsule biosynthesis or regulation. Of the 10 genes, 6 genes overlap with the genes of a *skn7*Δ strain, 5 overlap with those of a *hog1*Δ strain (29), and 5 overlap with those of a *nrg1*Δ strain (32) (Table S3). Although these high-throughput experiments were conducted under different conditions, the genes differentially expressed in various deletion strains suggest a central role for these genes in response to perturbation of the different signaling pathways and potential mechanisms of cross talk between the pathways.

Reduced intracellular cAMP in CWI kinase deletion strains. Recent work has demonstrated the importance of the *RIM101* transcription factor in the cAMP/PKA-dependent regulation of capsule production, defining a novel interaction between the Rim and PKA signaling pathways in *C. neoformans* (30). PKA1 activates the transcription factor NRG1, leading to the transcriptional activation of genes directly involved in capsule assembly (35). The Hog1 kinase is known to be a negative regulator of capsule synthesis in serotype A (33), a process that requires cAMP. In addition, the gene encoding Smg1, identified as a suppressor of Gpa1, the G alpha subunit that functions in cAMP signaling (36), was significantly repressed in all three CWI kinase deletion strains. The overlap between genes differentially expressed in the kinase deletion strains and those differentially expressed in gene deletion strains related to the PKA/cAMP pathway led us to ask if loss of the CWI kinases might negatively impact cellular cAMP levels. We measured cAMP levels in the three deletion strains and wild-type KN99a during log-phase growth in YPD medium. These experiments were done on two separate days, with three replicates for each strain. Since we observed the differential gene expression under log growth in nutrient-rich conditions and not in response to stress, we wanted to measure the cAMP levels under similar conditions. The kinase deletion strains, *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains, all showed significantly less cAMP compared to the KN99a wild-type strain (Fig. 3A). The experiments do not address the effect of cAMP in response to stress; however, the significant decrease in cAMP levels among all three deletion strains provides strong evidence for an impact on basal cAMP activity.

We tested whether the addition of exogenous cAMP could mitigate the sensitivity of the deletions to SDS, calcofluor white, and Congo red. Exogenous cAMP almost completely rescues the sensitivity to SDS for *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains after 5 days (Fig. 3B), but it has a lower effect on sensitivity to calcofluor white and does not rescue sensitivity to Congo red (data not shown). It is possible that the different stresses activate the CWI and cAMP/PKA pathways by different mechanisms, with various degrees of overlap between the activating mechanisms. An alternative explanation may be that cell membranes are more permeable in the presence of SDS. cAMP does not readily cross cell membranes, and the fact that we saw an effect with SDS but not with Congo red may reflect increased permeability in the presence of SDS, which does not happen with Congo red treatment.

Capsule defect in the CWI kinase deletion strains. To further investigate the cross talk between the cAMP/PKA and CWI pathways, we examined phenotypes associated with disrupted cAMP levels, specifically, capsule formation when grown in capsule-inducing low-iron medium (LIM) (37). When the deletion strains were grown in LIM, all three had visibly reduced capsule com-

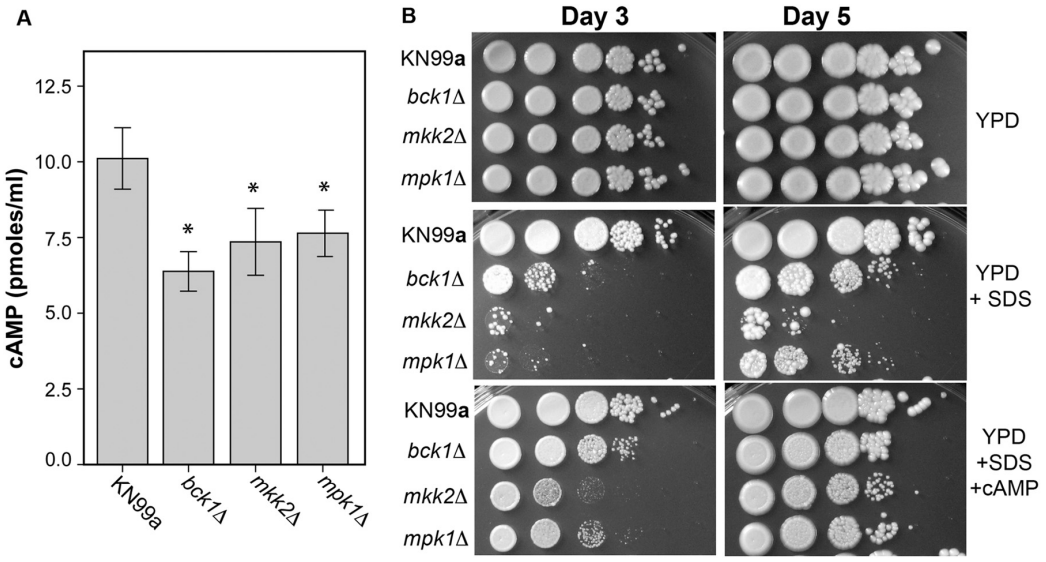

FIG 3 The downstream kinase deletion strains produce less cAMP than KN99a cells. (A) The cAMP levels were measured in the different deletion strains. Values that are statistically significantly different from the value for KN99a strain as determined by a one-way ANOVA with Dunnet's posthoc test are indicated by an asterisk. Error bars represent the standard deviations. (B) Phenotypes of *bck1*Δ, *mkk2*Δ, and *mpk1*Δ deletion strains grown on YPD, YPD plus 0.01% SDS, or YPD plus 0.01% SDS plus 10 mM cAMP at 3 and 5 days.

pared to wild-type KN99a (Fig. 4A and C), and measurement of the capsule diameters showed significantly reduced capsule diameters for *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains compared to the wild-type KN99a strain (Fig. 4B and C and Table 3). The addition of 10 mM cAMP to the medium increased the size of the capsule in all three deletion strains to wild-type levels while having essentially no effect on the KN99a strain (Fig. 4B and C and Table 3). These data point to an important, and previously unknown, role for the CWI kinases in regulation of cAMP levels. It suggests that production of cAMP is dependent on a functioning CWI pathway, as disruption of the pathway leads to decreased cAMP levels and causes impairment of capsule induction in LIM.

Analysis of shed capsule in the CWI kinase deletion strains. The defect in the increase of capsular polysaccharide in the mutant strains under capsule-inducing conditions suggests that the disruption of the CWI pathway affects the biosynthesis of the capsule, secretion of the capsule material, or attachment of the secreted capsule to the fungal cell wall. To differentiate between these possible explanations, we collected the shed capsular material from the wild-type and mutant strains. Secreted capsular material corresponding to an equal number of yeast cells was resolved on an agarose gel, transferred to a nylon membrane, and probed to determine the amount and relative size of the capsular material with an antiglucuronoxylomannan (anti-GXM) antibody (monoclonal antibody 3C2). We found that the amounts of capsule shed by the *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains were nearly equal the amount shed by wild-type cells; however, they exhibited slight differences in the migration pattern on an agarose gel, suggesting potential differences in the physical-chemical properties of the shed capsule material (Fig. 5). Overall, these data suggest that the observed capsule defects in these strains are more likely due to a defect in attachment, not in synthesis or secretion.

DISCUSSION

Eukaryotic cellular signaling cascades receive inputs from several proteins simultaneously and then balance the signals to generate an appropriate response. One of the kinases in the CWI pathway, *PKC1*, plays a central role in the response to various cell wall-perturbing agents and production of virulence factors, capsule, and melanin (15, 16). However, the CWI pathway can be induced by some stresses, such as heat shock, in a *PKC1*-independent manner (20). To explore possible alternative pathway connections or cross talk, we determined the global transcription response to the loss of three other CWI kinases, *BCK1*, *MKK2*, and *MPK1*. We found that over 1,000 genes were differentially expressed in one or more of the deletion strains (see Table S2 in the supplemental material), including 29 cell wall- or capsule-related genes (Table 2) and 115 genes with concordant differential expression in all three strains (Table S3). These data are consistent with a significant adaptive response of the cell to the loss of the CWI kinases.

We hypothesized that disruption of three core kinases in the cell wall integrity pathway affect signaling in other pathways that impact cell wall and capsule homeostasis and looked for overlap between the 115 genes showing differential expression in all three kinase deletion strains (see Table S3 in the supplemental material) with genes dependent on other stress response-related genes as well as those that regulate capsule and cell wall homeostasis. We found a substantial overlap with genes dependent on stress-responsive *HOG1* (29) and *SSK1* (34). We also found >30 genes that overlapped with genes dependent on *RIM101* (31) and *NRG1* (32), which suggested a connection to the cAMP/PKA pathway. We found a significant reduction in the cellular cAMP levels in all three of the CWI kinase deletion strains compared to wild-type cells (Fig. 3A). The addition of exogenous cAMP reduced the sensitivity to some, but not all, cell wall-perturbing agents (Fig. 3B), suggesting multiple inputs for maintaining cell wall homeostasis that are dependent on both the cAMP/PKA and CWI pathways.

We observed a loss of surface capsule with deletion of the CWI kinases and restoration of the surface capsule in those strains with addition of exogenous cAMP (Fig. 4). This is the first experimental evidence of a direct connection between the CWI pathway and

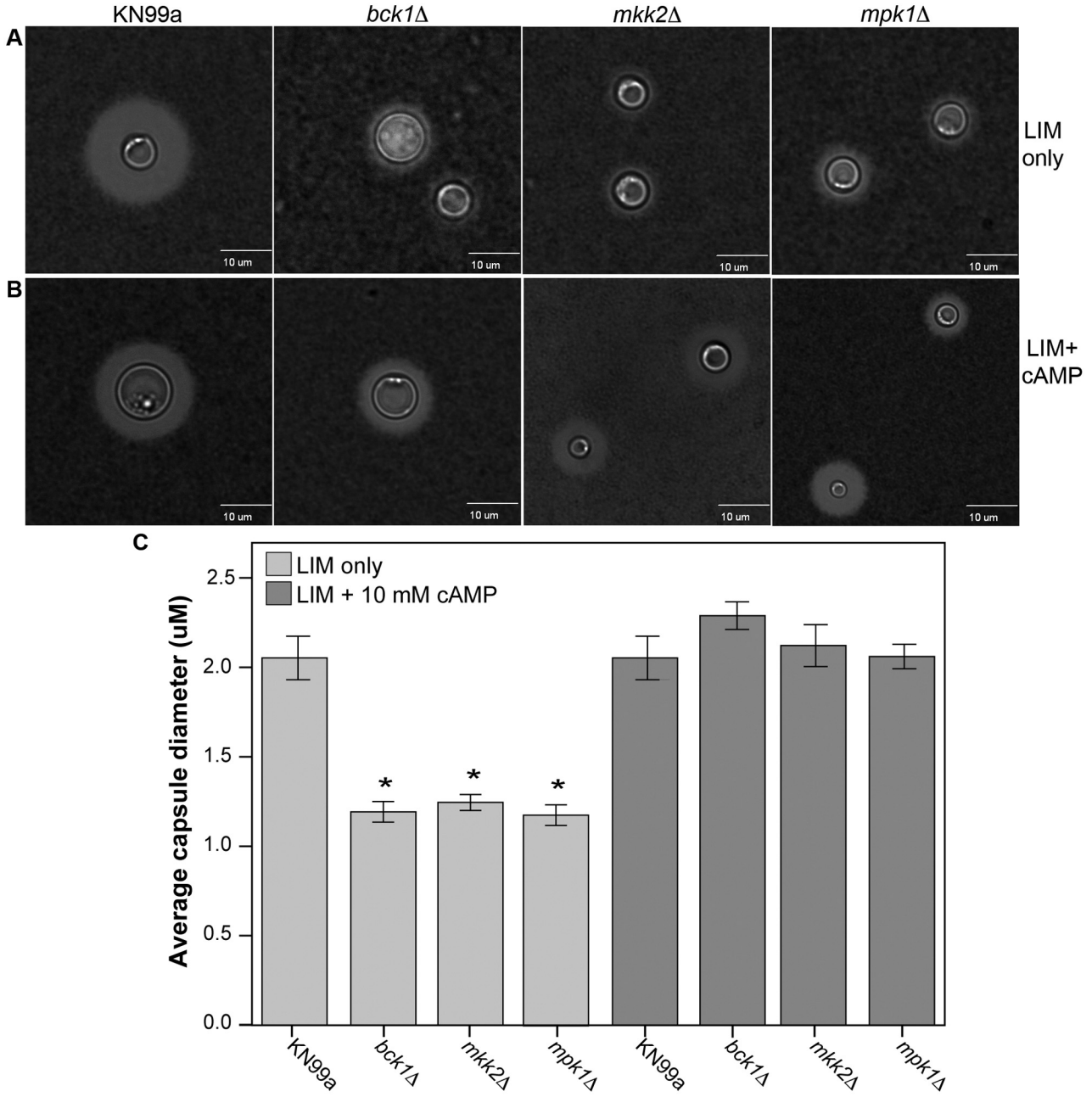

FIG 4 *C. neoformans BCK1*, *MKK2*, and *MPK1* are required for capsule production. (A and B) Cells were incubated under capsule-inducing conditions for 4 days without cAMP (A) or with 10 mM cAMP (B). Capsule size was assessed by staining with India ink and visualizing the zone of exclusion at a magnification of ×60. The capsule diameters of the *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains were reduced compared to that of the KN99a strain, and capsule size was restored when grown with exogenous cAMP. (C) Comparison of capsule diameters between strain KN99a and *bck1*Δ, *mkk2*Δ and *mpk1*Δ strains grown in low-iron medium (LIM) with and without the addition of 10 mM cAMP. Measurements were taken for a minimum of 100 cells for each strain. Asterisks represent statistically significant differences as determined by a one-way ANOVA with Dunnet's posthoc test. Error bars represent the 95% confidence intervals.

capsule regulation in *C. neoformans*. The reduced capsule does not appear to be due to a defect in synthesis or secretion but is more likely due to a defect in attachment to the cell wall, as we did not observe a significant difference in the amount of shed capsule between the wild-type and kinase deletion strains (Fig. 5). The nature of this defect is not clear, but it is known that α-glucan and chitin oligomers or chitin-like structures are required for capsule attachment (26, 27, 38). The CWI kinase deletion strains have reduced chitin/chitosan ratio compared to wild-type cells (Fig. 2), and a similarly reduced chitin/chitosan ratio was also observed in *chs4*Δ and *chs5*Δ strains (25). Expression of both *CHS4* and *CHS5* was downregulated in all three kinase deletion strains (Table 2); however, neither the *chs4*Δ nor *chs5*Δ strain had a measurable capsule defect when grown in LIM (data not shown). A third chitin synthase gene, *CHS1*, was upregulated in all three deletion strains (Table 2), but deletion of *CHS1* has no discernible cell

TABLE 3 Average induced capsule and cell diameters

Strain or genotype	Capsule diam LIM[a]	*P* value[b]	Capsule diam LIM + cAMP[a]	*P* value[c]	Cell diam LIM[a]	Cell diam LIM + cAMP[a]	*P* value[d]
KN99a	2.05 ± 0.61		2.83 ± 0.79	1.00	6.01 ± 1.10	6.16 ± 1.77	0.45
*bck1*Δ	1.19 ± 0.31	<0.001	2.28 ± 0.39	1.00	4.92 ± 0.61	5.58 ± 0.87	<0.001
*mkk2*Δ	1.24 ± 0.24	<0.001	1.83 ± 0.51	0.78	4.56 ± 0.72	5.22 ± 0.74	<0.001
*mpk1*Δ	1.18 ± 0.31	<0.001	1.48 ± 0.53	1.00	4.36 ± 0.69	5.39 ± 1.2	<0.001

[a] Capsule and cell diameters of cells grown in LIM alone or in LIM plus cAMP are reported as averages ± standard deviations of at least 100 measurements.
[b] *P* value comparing the capsule diameters of deletion strains to that of the WT strain (KN99a) in low-iron medium (LIM) only.
[c] *P* value comparing the capsule diameters between deletion strains and WT in LIM plus cAMP.
[d] *P* value comparing the cell diameters for each strain with and without cAMP in the medium.

wall-related phenotype (25) or capsule-related phenotype in LIM (data not shown).

The *RIM101* transcription factor has been shown to play a significant role in the regulation of cell wall morphogenesis (31). Despite the overlap of genes dependent on all three CWI kinases with those dependent on *RIM101*, the *rim101*Δ strain has significantly different phenotypic characteristics compared to the CWI kinase deletion strains. The *rim101*Δ strain is sensitive to LiCl and NaCl but insensitive to SDS, calcofluor white, or Congo red. More interestingly, the *rim101*Δ strain has an increased chitin/chitosan ratio, and two chitin synthase genes, *CHS4* and *CHS5*, are upregulated in the *rim101*Δ strain but downregulated in the CWI kinase deletion strains. This raises the possibility that *RIM101* and the CWI kinases may play antagonistic roles in the regulation of cell wall homeostasis.

Only three genes with a likely role in transcription regulation were differentially expressed in all three CWI deletion strains; the three genes were CNAG_01103, CNAG_05290, and CNAG_07775 (see Table S3 in the supplemental material). Of the three genes, only one, CNAG_05290 which encodes SPt3, a subunit of the SAGA transcriptional regulatory complex seems likely to have a role in cell wall or capsule regulation (32, 39). The CNAG_05290 transcript for the putative transcription factor, SPt3, is upregulated in an *ada2*Δ strain at 37°C with CO_2 compared to the wild type (32), but no further characterization of this putative transcription factor has been published.

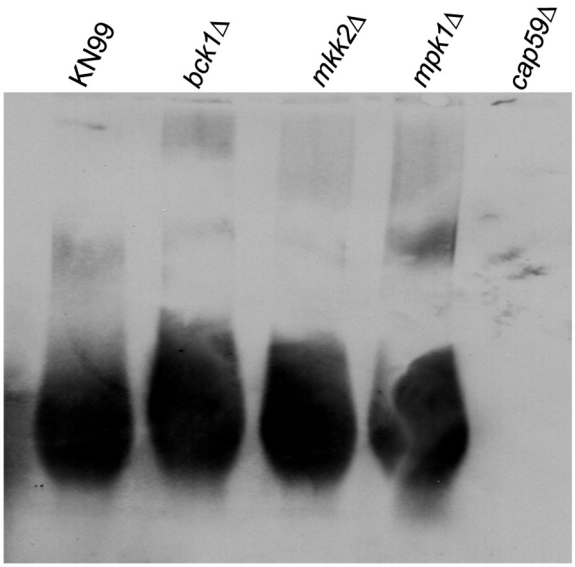

FIG 5 The levels of capsule shed by the *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains were similar to the level shed by the wild-type KN99a strain. The wild-type, *bck1*Δ, *mkk2*Δ, *mpk1*Δ,and *cap59*Δ strains were grown in YPD medium for 5 days, and the number of cells was determined by absorption at 650 nm. The supernatants were collected, and the cell counts were normalized before loading. The gel was transferred to a blot and probed with the 3C2 anti-GXM monoclonal antibody.

The regulation of capsule production in *C. neoformans* is very complex. Capsule synthesis is responsive to iron and CO_2 levels via the cAMP/PKA signaling pathway (reviewed in reference 13), and the downstream changes in capsule biosynthetic and trafficking gene expression are controlled by at least five different transcription factors, which are also responsive to a number of other proteins (30, 32, 35, 40–42). Therefore, the hypothesis that the CWI pathway might have a role in the regulation of capsule production is plausible. One possible mechanism of capsule regulation is that the CWI kinases and some subset of chitin synthases interact to regulate the synthesis of the chitin oligomers that are necessary for capsule attachment (26, 27). However, experimentally discerning the mechanism of that regulation is beyond the scope of this study.

In previous studies, deletions of *C. neoformans* genes with homology to regulators of the *Saccharomyces cerevisiae* CWI pathway, *ROM2*, *LRG1*, and *SIT4* (reviewed in reference 43) resulted in moderate to severe cell wall-related phenotypes and increased sensitivity to cell wall stressors (17). However, we saw no differential expression of these genes in any of the CWI kinase deletion strains, nor did we observe differential expression of the regulators of the HOG pathway, *SKN7*, *SSK1*, and *TCO1* or *TCO2* (33, 34) in the kinase deletion strains. However, we did observe considerable overlap between genes differentially expressed in the CWI kinase deletion strains with genes that are differentially expressed in response to the deletion of the stress-responsive genes *HOG1*, *SSK1*, and *SKN7* (29, 33, 34). The lack of differences in expression of genes previously identified as critical for stress response may reflect the fact that regulation of their activity occurs primarily via protein modifications, not by transcriptional changes. We also conducted our expression analysis of the CWI kinase deletion strains under nutrient-rich conditions in the absence of exogenous stress. It is possible that if the CWI kinase deletion strains were exposed to an external stress, we would observe transcriptional changes in some of these other pathway regulators.

In summary, we have demonstrated a direct connection between the CWI and cAMP/PKA pathways in the regulation of intracellular cAMP levels. These data support a role for the CWI pathway in the regulation of surface capsule. The mechanisms and transcription factors, which mediate the cAMP and capsule response to the CWI pathway, remain to be elucidated.

MATERIALS AND METHODS

Strains and media. Wild-type (WT) *C. neoformans* var. *grubii* strain KN99 (serotype A, MAT**a**) was used in this study (see Table S1 in the supplemental material). Strains containing deletions in *BCK1* or *MKK2* in the KN99 MAT**a** (KN99a) background were generated by overlap PCR (44) using the primers listed in Table S1. A strain deleted for *MPK1* in the KN99 MAT**a** background was generated by amplification of the *mpk1*Δ cassette (pNAT-STM150) from strain KK3, containing *mpk1*Δ in the H99 *MAT*α background (generously provided by Joseph Heitman, Duke University) (45). Each fragment was biolistically transformed into strain KN99a (46), and isolates were selected and verified by PCR screening as described previously (17) utilizing the primers listed in Table S1. Southern blotting was performed, using the selectable marker as a probe to rule out ectopic integrations.

RNA preparation and sequencing. The KN99a, *bck1*Δ, *mkk2*Δ, and *mpk1*Δ cells were grown for 3 days on plates containing yeast extract-peptone-dextrose (YPD) medium supplemented with 1 M D-sorbitol. A 50-ml flask of YPD medium supplemented with 1 M sorbitol was inoculated and grown overnight at 30°C with shaking (300 rpm). Cells were diluted to an optical density at 650 nm (OD_{650}) of 0.004 and grown overnight to an OD_{650} of 0.75 to 1.0. A strain with a deletion in the *PKC1* gene, which required sorbitol to grow, was originally part of this study, but it was later determined to have an aneuploid chromosome. It was excluded from further analyses (data not shown). RNA was prepared from three biological replicates using the Qiagen RNeasy plant minikit (Qiagen, Valencia, CA) with minor modifications (details of methods available upon request). RNA quality was assessed on a formaldehyde agarose gel, and the ratio of 25S/18S rRNA was quantified using ImageQuant software (GE Healthcare Biosciences, Pittsburgh, PA). Total RNA (10 μg) from each sample was used as starting material to prepare sequencing libraries with the Illumina mRNA sequencing sample preparation kit (Illumina, San Diego, CA) following the manufacturer's instructions. Samples were barcoded and sequenced on a single lane of an Illumina HiSeq sequencing system to generate nondirectional, single-ended 50-bp reads. The library preparation and RNA sequencing were performed by the Genome Access Technology Center at Washington University (http://gtac.wustl.edu). The bar code sequences were trimmed to 42 bp, and quality-filtered reads were mapped to the H99 reference genome sequence prepared by the Broad Institute (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html) using Bow tie 2.1.0 as implemented in the TopHat-Cufflinks suite (21, 47).

Differential gene expression. The *C. neoformans* H99 transcript file prepared by the Broad Institute was used as the reference. Transcript files for each biological replicate were generated from each alignment file using Cufflinks, merged into a single transcript file using Cuffmerge, and differentially expressed transcripts were identified using Cuffdiff (21). Genes were considered differentially expressed if P values were <0.05 after Benjamini-Hochberg correction for multiple testing.

Capsule measurements. Strains were grown in low-iron medium [LIM plus ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA)] (37) with and without exogenous 10 mM cAMP for 4 days at 30°C. All mutant strains were compared to the wild-type KN99a strain. Capsule-induced strains were suspended in a 1:4 India ink-H_2O solution and photographed on an Olympus BX61 microscope at a magnification of ×60. Cell diameters (one including the capsule and one excluding it) were measured for a minimum of 100 cells per strain using SlideBook, version 5.0. Capsule length was calculated as the difference between the radii obtained from the diameters. All values were averaged, and comparisons were considered significant if the P value was <0.05 using a one-way analysis of variance (ANOVA) with a Dunnet's posthoc test (one-tailed comparison; < control).

Chitin and chitosan assays. The cellular chitin and chitosan levels were determined as previously described (22). Samples were divided into two aliquots. One aliquot was treated with acetic anhydride to measure chitin plus chitosan. The other aliquot was left untreated to measure total chitin. Chitosan was estimated from the difference between the two measurements.

Measurement of cAMP. Overnight cultures of the WT and the deletion strains were diluted in 50 ml YPD medium to an OD_{650} of 0.05 and grown for 20 h at 30°C with shaking (300 rpm). Cells were harvested, washed, and resuspended in MES-EDTA buffer (10 mM morpholineethanesulfonic acid [MES], 0.1 mM EDTA, pH 6.0). Cells were counted using a hemocytometer, centrifuged, and resuspended at a concentration of 5×10^8 cells/ml. Cells (0.5 ml) were lysed by bead beating in 2.5% trichloroacetic acid at 4°C with 0.5-mm glass beads for 1 min, followed by a 2-min rest, for a total of 4 cycles. Crude lysates were centrifuged at 20,800 × *g* for 15 min. Lysates were extracted four times with water-saturated diethyl ether and centrifuged for 20,800 × *g* at 4°C for 2 min between extractions, frozen in liquid nitrogen, and lyophilized overnight. The lyophilized samples were suspended in 0.5 ml of 0.1 M HCl, and cAMP assays were performed according to Sigma cAMP enzyme immunoassay kit (catalog no. CA200; Sigma, St. Louis, MO). Assays were performed twice with three technical replicates. cAMP concentrations were calculated for individual samples, and statistical significance was determined using a one-way ANOVA with Dunnet's posthoc test (two-tailed test) using IBM SPSS Statistics (version 20).

SDS viability assays. Overnight cultures of wild-type strain KN99a and *bck1*Δ, *mkk2*Δ, and *mpk1*Δ strains in YPD medium were diluted to an OD_{650} of 1.0 with phosphate-buffered saline (PBS). Tenfold serial dilutions were plated on YPD medium plus 0.01% SDS and YPD medium plus 0.01% SDS and 10 mM cAMP. The plates were incubated at 30°C for up to 5 days and photographed. The SDS concentration used was based on levels determined to be deleterious, but not lethal, to the mutant strains.

Immunoblotting of the secreted capsule. Immunoblotting of the shed capsular material was performed as described previously (48). Briefly, conditioned medium for each strain corresponding to an equal number of yeast cells grown in YPD medium was passed through a 0.22-μm filter, mixed with DNA loading dye, and separated on a 0.6% agarose gel using Tris-acetic acid-EDTA buffer (pH 8.0). The gel was transferred onto a positively charged nylon membrane by capillary transfer and immunoblotted with 1 μg/ml of antiglucuronoxylomannan (anti-GXM) 3C2 antibody (generously provided by Thomas Kozel, University of Nevada) (49).

Microarray data accession number. RNA sequences/microarray data were deposited in the NCBI GEO database and assigned accession number GSE57217.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01573-14/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.
Table S2, PDF file, 0.3 MB.
Table S3, PDF file, 0.1 MB.

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