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Functional Characterization of cAMP-Regulated Gene, *CAR1*, in *Cryptococcus neoformans*

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The cyclic AMP (cAMP) pathway plays a major role in growth, sexual differentiation, and virulence factor synthesis of pathogenic fungi. In *Cryptococcus neoformans*, perturbation of the cAMP pathway, such as a deletion in the gene encoding adenylyl cyclase (*CAC1*), causes defects in the production of virulence factors, including capsule and melanin production, as well as mating. Previously, we performed a comparative transcriptome analysis of the Ras- and cAMP- pathway mutants, which revealed 163 potential cAMP-regulated genes (38 genes at a 2-fold cutoff). The present study characterized the role of one of the cAMP pathway-dependent genes (serotype A identification number CNAG_ 06576.2). The expression patterns were confirmed by Northern blot analysis and the gene was designated cAMP-regulated gene 1 (*CAR1*). Interestingly, deletion of *CAR1* did not affect biosynthesis of any virulence factors and the mating process, unlike the cAMP-signaling deficient *cac1*Δ mutant. Furthermore, the *car1*Δ mutant exhibited wild-type levels of the stress-response phenotype against diverse environmental cues, indicating that Car1, albeit regulated by the cAMP-pathway, is not essential to confer a cAMP-dependent phenotype in *C. neoformans*.

KEYWORDS : *CAR1*, *Cryptococcus neoformans*, Cyclic AMP, Transcriptome analysis

The ability to sense and respond to diverse environmental stresses is indispensable for all living organisms, and is particularly critical for pathogenic fungi that have to survive in harsh host conditions. This adaptation process is mediated by multiple signaling pathways. Among these, the cyclic AMP (cAMP) pathway plays an important role in maintenance of cellular homeostasis, sexual differentiation, and virulence factor production in pathogenic fungi [1, 2]. The general signal transduction mechanism of the cAMP-signaling pathway that has been studied in fungi can be summarized as follows. Plasma membrane-associated adenylyl cyclase converts ATP to cAMP, which is a key second messenger, in response to extracellular signal via small or heterotrimeric GTP-binding proteins coupled with G-protein coupled receptor (GPCR). The resulting high concentrations of cAMP lead to activation of protein kinase A (PKA), which subsequently triggers diverse cellular responses. To maintain the cAMP levels under a lethal threshold, the cAMP-signaling pathway is negatively controlled by phosphodiesterases, which degrade cAMP to AMP.

In *Saccharomyces cerevisiae*, activated adenylyl cyclase complex composed of Cyr1 and the cyclase-associated protein increases the intracellular levels of cAMP, which activate PKA by dissociating the PKA catalytic subunit (Tpk1/Tpk2/Tpk3) from PKA regulatory subunits (Bcy1) that repress the PKA catalytic subunit under normal conditions [3, 4]. Upon removal or adaptation to external signals, cAMP is degraded to AMP by low affinity phosphodiesterase (PDE) 1 and high affinity PDE2 [5, 6]. The cAMP-signaling pathway regulates diverse cellular functions including cellular growth, stress responses, and morphological differentiation in *S. cerevisiae* [7].

With the advent of acquired immunodeficiency syndrome, treatment of the opportunistic and life-threatening fungal infections and diseases caused by pathogens such as *Cryptococcus neoformans* has become urgent. The basidiomycetous *C. neoformans* infects the central nervous system in immunocompromised individuals through the respiratory system, which ultimately results in fungal meningitis [8]. Recently, *C. gattii* atypically appeared in British Columbia in Canada, causing disease in a number of immunocompetent individuals [9]. Two well-known virulence factors in *C. neoformans* are antiphagocytic polysaccharide capsule and antioxidant melanin [10, 11]. The capsule enhances the intracellular survival by preventing *C. neoformans* from being phagocytosed by host immune cells and from being dehydrated [12]. Melanin allows *C. neoformans* to strongly resist oxidative damages, ultraviolet irradiation, and high temperature exposure [13, 14]. Mutants defective in capsule or melanin production are avirulent [15, 16]. These two virulence factors are controlled by the cAMP-pathway in *C. neoformans* [17, 18]. Furthermore, the mating process is also governed by the cAMP-pathway in *C. neoformans* [17, 18]. The cAMP-pathway in *C. neoformans* comprises Cac1 (adenylyl cyclase), Aca1 (adenylyl cyclase-associated protein), Gpa1 (Gα subunit), GPCR Gpr4, catalytic subunits (Pka1 and Pka2) and regu-

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latory subunit (Pkr1) of PKA, and phosphodiesterases (PDE1 and PDE2) [19, 20].

Recently, we identified a number of cAMP-regulated genes in *C. neoformans* through a comparative transcriptome analysis of cAMP pathway-dependent mutants [21]. DNA microarray analysis was utilized to compare the genome-wide transcriptome patterns of the *C. neoformans* mutants deleted of a major Ras/cAMP-signaling component, including Cac1, Aca1, Gpa1, Pka1/2 (catalytic subunits of protein kinase A), and Ras1 with those of the serotype A H99 wild-type strain [21]. Through this analysis, the cAMP-pathway was also found to control expression of diverse stress response genes and was linked to certain environmental stress responses [21]. Notably, it has been reported that the cAMP-pathway is required for promoting resistance to polyene drugs including amphotericin B [21], suggesting that a potential inhibitor of the cAMP-pathway will confer a synergistic antifungal activity along with amphotericin B. Furthermore, we discovered 163 unique genes (38 genes at a 2-fold cutoff), whose expressions are regulated by the cAMP pathway.

The present study further characterized one of the cAMP-dependent genes, whose serotype A ID is CNAG_06576.2, since it appeared dramatically downregulated in all of the cAMP mutants but its function had not been hitherto characterized. Presently, Northern blot analysis was used to confirm that the gene was indeed positively regulated by the cAMP-pathway. Appropriately, it was designated cAMP-regulated gene 1 (*CAR1*). The results indicate that Car1 does not play any significant roles in growth, differentiation, and virulence factor production of *C. neoformans*.

Materials and Methods

Strains and media. Strains used in this study are listed in Table 1. Yeast extract-peptone-dextrose (YPD) medium was used for culturing *C. neoformans* strains. L-DOPA or Niger seed medium for melanin synthesis and agar-based Dulbecco modified Eagle (DME) medium for capsule production were prepared as previously described [18, 25].

Table 1. *Cryptococcus neoformans* strains used in this study

Strain	Genotype	Parent	Reference
Serotype A			
H99	*MAT*α		[22]
KN99	*MAT***a**		[23]
YSB6	*MAT*α *aca1Δ::NAT*-STM#43	H99	[18]
YSB42	*MAT*α *cac1Δ::NAT*-STM#159	H99	[18]
YSB64	*MAT*α *hog1Δ::NAT*-STM#177	H99	[24]
YSB639	*MAT*α *car1Δ::NAT*-STM#288	H99	This study
YSB640	*MAT*α *car1Δ::NAT*-STM#288	H99	This study
YSB641	*MAT*α *car1Δ::NAT*-STM#288	H99	This study
YSB83	*MAT*α *gpa1Δ::NAT*-STM#5	H99	[18]
YSB200	*MAT*α *pka1Δ::NAT*-STM#191 *pka2Δ::NEO*	YSB188	[18]

Each *NAT-STM*# indicates the Nat^r marker with a unique signature tag.
MAT, mating type.

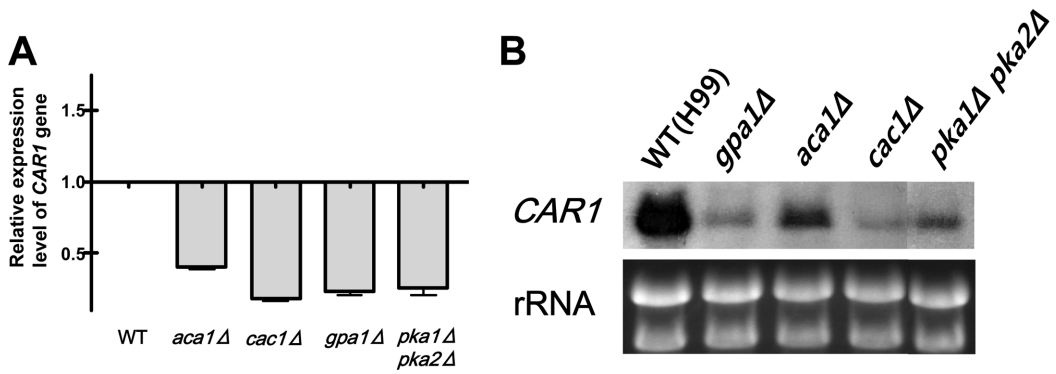

Fig. 1. Transcript levels of the cAMP-regulated gene 1 (*CAR1*) gene in cAMP-signaling mutants. A, Each bar indicates reduction of the *CAR1* gene in microarray data in *aca1Δ* (YSB6), *cac1Δ* (YSB42), *gpa1Δ* (YSB83), and *pka1Δ pka2Δ* (YSB200) mutants compared to the wild-type strain (H99) of our previous report [16]. B, Transcript levels of the *CAR1* gene in the wild-type strain (H99) and *gpa1Δ*, *aca1Δ*, *cac1Δ*, and *pka1Δ pka2Δ* mutants were verified by Northern blot analysis.

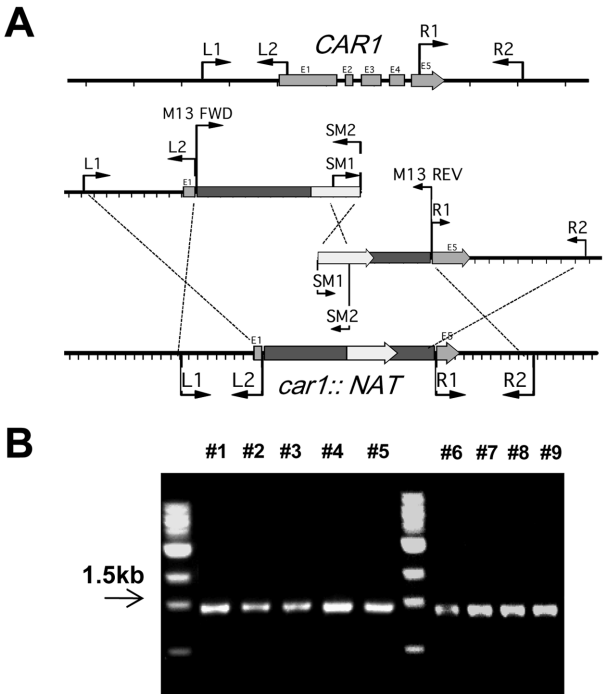

Fig. 2. Construction of the *car1Δ* mutant. A, Diagram for disruption of the cAMP-regulated gene 1 (*CAR1*) gene. Primers for the first-round PCR and double joint (DJ)-PCR are indicated as bent arrows. Through triple recombination between 5'- and 3' flanking regions of *CAR1* and split *NAT* selectable marker, the *CAR1* gene was specifically replaced with the intact *NAT* selectable marker. B, Nourseothricin-resistant transformants were selected and screened by diagnostic PCR with the *CAR1*-specific screening primer as listed in Table 2.

Gene disruption of the *CAR1* gene. For *CAR1* gene disruption, information about the *CAR1* genomic structure was obtained from the *C. neoformans* genomic database. (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html). The *CAR1* gene-disruption cassette was constructed by double joint polymerase chain reaction (DJ-PCR) with *NAT*-split markers as previously described [26]. The *car1Δ* mutants were generated by introduction of the *CAR1*-deletion cassette into the *C. neoformans* serotype A H99 strain by biolistic transformation [26]. Primers for amplification of the 5' and 3' flanking regions of the *CAR1* gene (Fig. 2A) are described in Table 2. The primers were used to amplify dominant selectable *NAT* (nourseothricin acetyltransferase) marker. Stable transformants selected on YPD medium containing nourseothricin were screened by diagnostic PCR and their correct genotypes were verified by Southern blot analysis.

Northern blot analysis. Total RNAs that have been prepared for the previous DNA microarray analysis [21] were used for Northern blot analysis. Electrophoresis, washing, membrane transfer, and hybridization were carried out by following standard protocols [27]. To construct the specific probe for the *CAR1* gene, specific primers summarized in Table 2 were used.

Melanin and capsule assay. Each mutant and wild type strain were grown in YPD liquid medium overnight at 30°C. Three microliters of cells were spotted on agar-based DME medium for capsule production and on agar-based L-DOPA or Niger seed medium for melanin production, which all contained the indicated concentrations of glucose. The plates were incubated at 30°C or 37°C, daily monitored for up to 4 days, and photographed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with camera (Diagnostic Instrument Inc., Sterling Heights, MI, USA). For visual inspection of capsule production, cells scraped from DME medium were resuspended in distilled water and stained with India ink. Relative capsule size was quantitatively measured as previously described [18, 25].

Mating assay. Mating assay was performed as previously described [18, 25]. Briefly, all *C. neoformans* strains for mating were grown overnight at 30°C in liquid YPD medium. Next, the same concentration of mating type (*MAT*)α and *MAT***a** cells (10^7 cells/mL) were mixed, spotted (5 μL of the

Table 2. Primers used in this study

Primer name	Sequence	Comment
B79	TGTGGATGCTGGCGGAGGATA	Screening primer on ACT promoter
B1026	GTAAAACGACGGCCAGTGAGC	M13 forward (extended)
B1027	CAGGAAACAGCTATGACCATG	M13 reverse (extended)
B1694	GGAAGACTTGGTAGGCAAAAC	CAR1 - 5' screening primer
B1690	GAGTTTAGAGTGGCGGAAG	CAR1 - left flanking primer 1
B1691	GCTCACTGGCCGTCGTTTTACCGTAGTAGTGAACCCAGTGTG	CAR1 - left flanking primer 2
B1692	CATGGTCATAGCTGTTTCCTGCAAGACCCAGGCTCTCTAAG	CAR1 - right flanking primer 1
B1693	TTTTTTGGCAAGGGGAGC	CAR1 - right flanking primer 2
B1694	GGAAGACTTGGTAGGCAAAAC	CAR1 - probe primer 1
B1695	CAAAGGGTAAGGCGAAAG	CAR2 - probe primer 2
B1454	AAGGTGTTCCCCGACGACGAATCG	NSL-2 5'-region of NAT (SM1)
B1455	AACTCCGTCGCGAGCCCCATCAAC	NSR-2 3'-region of NAT (SM2)

mixture) onto the V8 medium adjusted to pH 5, and then incubated in the dark at room temperature for 2 weeks. All colonial and cellular images were monitored and photographed using Olympus BX51 microscope (Olympus).

Stress sensitivity test. Each mutant and wild type strain were cultured overnight at 30°C in YPD liquid medium. These strains were washed, serially diluted (1~10^4), and spotted (4 mL) onto solid YP or YPD medium containing diamide (diazenedicarboxylic acid bis (*N*,*N*-dimethylamide)), hydrogen peroxide (H_2O_2), or menadione for oxidative stress test, $CdSO_4$ for heavy metal stress test, Congo-red for cell wall integrity test, and NaCl or KCl for osmosensitivity. To test antifungal drug sensitivity, cells were spotted onto YPD medium containing indicated concentrations of fluconazole, itraconazole, ketoconazole, amphotericin B, or fludioxonil. The plates were incubated at 30°C for 2~5 days and photographed.

Results

Identification of the *CAR1* gene in *C. neoformans*. Among the cAMP-dependent genes previously identified through transcriptome analysis [21], the expression patterns of CNAG_06576.2 was confirmed by Northern blot analysis. Expression levels of the CNAG_06576.2 gene significantly decreased in the *cac1Δ*, *gpa1Δ*, and *pka1Δ pka2Δ* mutants compared to the wild type strain, echoing DNA microarray data previously reported [21]. Appropriately, the gene was designated *CAR1* (Fig. 1).

To further characterize the role of Car1, mutant analysis was carried out by constructing the *car1Δ* mutant in the background of the serotype A H99 strain. For amplifying each 5'- and 3' flanking region of the *CAR1* gene and truncated *NAT* selectable marker, the specific primers summarized in Table 2 were used. Through triple homologous recombination between 5'- and 3' flanking regions of the *CAR1* gene and *NAT*-split markers that were produced by double joint PCR as previously described [26], the *CAR1* gene was specifically disrupted (Fig. 2A). Nine transformants were selected by diagnostic PCR with the *CAR1*-specific screening primer (Fig. 2B). Among these, six transformants were verified by Southern blot analysis (data not shown).

Role of Car1 in capsule and melanin production and mating. The cAMP pathway plays a major role in production of virulence factors such as capsule and melanin

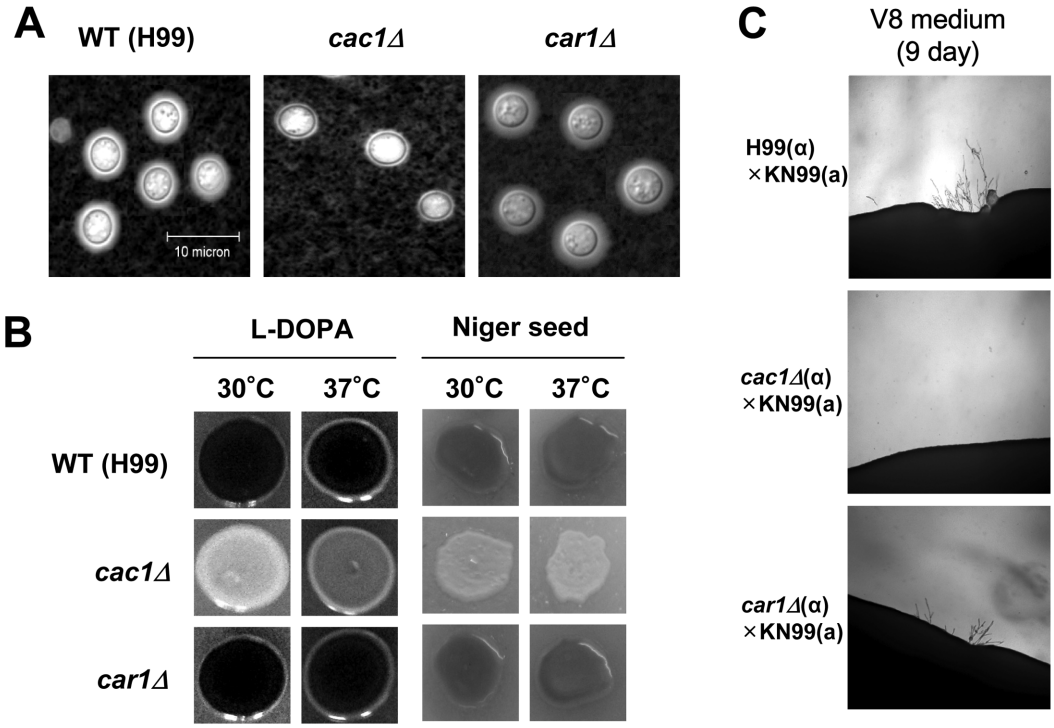

Fig. 3. Car1 is not involved in capsule and melanin production and mating response. A, Capsule production in the wild-type strain (H99) and *cac1Δ* and *car1Δ* mutants was visualized by staining with india ink and cellular images were monitored and recorded. B, For melanin assay, the wild-type strain (H99) and *cac1Δ*, and *car1Δ* mutants were spotted onto agar-based L-DOPA and Niger seed media and grown at either 30°C or 37°C for 5 days. Colony images of melanin production in the wild-type strain and *cac1Δ*, and *car1Δ* mutants were acquired. C, The following mating type (*MAT*)α and *MAT***a** strains were co-cultured on V8 medium at dark room temperature up to 2 weeks: H99 × KN99 ($\alpha \times$ **a**), YSB639 × KN99 (*car1Δ* × **a**), and YSB42 × KN99 (*cac1Δ* × **a**) and photographed after 9 days.

in *C. neoformans*. The *cac1Δ* mutant is defective in capsule and melanin production as well as in mating [17]. Similarly, deletion of the *ACA1* gene results in reduction of capsule and melanin production, and causes decreased levels of sexual differentiation [18]. To test the hypothesis that Car1 may play a role in regulation of virulence factors, a capsule production assay was carried out. Interestingly, deletion of the *CAR1* gene did not lead to any capsule defects, unlike that of *CAC1* gene (Fig. 3A). The possible involvement of Car1 in production of antioxidant melanin was assessed using L-DOPA and Niger seed media (Fig. 3B). Unlike the *cac1Δ* mutant, melanin production levels in the *car1Δ* mutant were similar to those in the wild-type strain (Fig. 3B). Finally to address the role of Car1 in mating response, a mating assay was performed. Contrary to the *CAC1* gene, the *CAR1* gene was not required for sexual differentiation (Fig. 3C). The observations were consistent with the suggestion that Car1 is not directly involved in capsule and melanin production, as well as in mating.

Role of Car1 in stress response of *C. neoformans*. We recently reported that *cac1Δ*, *aca1Δ*, *gpa1Δ*, and *pka1Δ* cAMP-dependent mutants display hypersensitivity to a polyene drug, amphotericin B, and an azole drug, itraconazole [21]. Furthermore, these cAMP mutants were more susceptible to methylglyoxal (MG), a toxic metabolite, and $CdSO_4$, a heavy metal stress inducer, than the wild-type strain. However, these mutants exhibited more resistance to diamide than the wild-type strain. Taken together, the results are consistent with the suggestion that cAMP-pathway contributes to diverse stress responses in *C. neoformans*. To address the role of Car1 in stress response in *C. neoformans*, we performed diverse stress response and antifungal drug sensitivity test. The *car1Δ* mutants exhibited similar resistance to osmotic shock as the wild-type strain (Fig. 4). Furthermore, the *car1Δ* mutants exhibited wild-type levels of resistance to antifungal drugs, unlike the other cAMP dependent mutants (Fig. 4) [18]. In response to oxidative stress-inducing agents including menadione, diamide, and hydrogen peroxide, the *car1Δ* mutants showed wild type levels of sensitivity (Fig. 4). Furthermore, Car1 was not involved in maintenance of cell wall integrity (Congo-red), genotoxic stress (methyl methanesulfonate, MMS), toxic metabolite stress (MG), and heavy metal stress ($CdSO_4$). Taken together, these data support

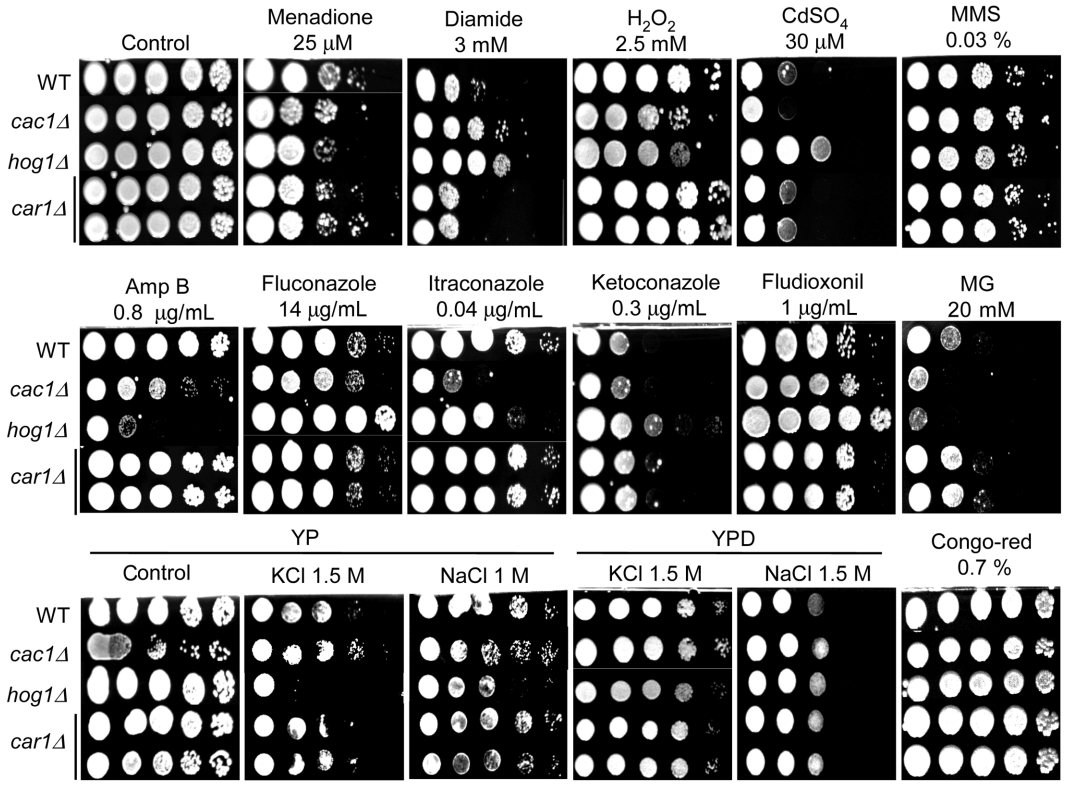

Fig. 4. Car1 is not involved in a variety of stress response against oxidative, osmotic, and genotoxic agents and treatment of cell wall destabilizer, antifungal drug, and toxic metabolites. For stress-sensitivity test, each *C. neoformans* strain (wild-type strain [H99] and *cac1Δ* [YSB42], *hog1Δ* [YSB64], *car1Δ* [YSB639 and YSB640] mutants) was cultured overnight at 30°C in liquid yeast extract-peptone-dextrose (YPD) medium and 4 μL of cells were spotted with 10-fold serial dilution onto YPD agar containing indicated concentrations of NaCl, KCl, H_2O_2, diamide, menadione, $CdSO_4$, methyl methanesulfonate, Congo-red, methylglyoxal, and antifungal drugs (amphotericin B, fluconazole, itraconazole, ketoconazole, and fludioxonil).

the suggestion that Car1 is not involved in diverse stress responses of *C. neoformans*.

Discussion

The present study aimed to functionally characterize the role of one of cAMP-regulated genes, CNAG_06576.2, that we previously discovered in the human fungal pathogen *C. neoformans* using comparative transcriptome analysis [21]. Here, the expression patterns were confirmed by Northern blot analysis and the gene was named *CAR1*. Unlike other cAMP dependent mutants, however, the deletion of the *CAR1* gene did not affect the production levels of two major *Cryptococcus* virulence factors, including capsule and melanin. Furthermore, the *car1Δ* mutant was as normal in mating as the wild-type strain. In addition, the *car1Δ* mutant exhibited wild type-like stress-response phenotypes against diverse environmental stresses, such as osmotic and oxidative shock, treatment of antifungal drugs, cell wall integrity destabilizers, and toxic metabolite. Taken together, the data support the view that Car1 is regulated by the cAMP-pathway, but appears not to play any significant role in *C. neoformans*.

However, it is still possible that Car1 may have some functional roles in the cAMP-pathway. In *S. cerevisiae*, the transcriptional levels of three genes named *GRE1* (Genes de Respuesta a Estres [stress responsive genes]), *GRE2*, and *GRE3* increase in response to hyperosmotic, oxidative, ionic, and heat shock stress [28]. These three genes are controlled by the HOG pathway, and *GRE1* and *GRE3* are regulated negatively by the cAMP-PKA pathway [28]. However, the functional roles of *GRE* genes have not been characterized during diverse stress responses. Furthermore, a similar case is found in other genes of *C. neoformans*. Expression of the *SMG1* gene is repressed in the *gpa1Δ* mutant and *SMG1* overexpression can restore melanin deficiency of the *gpa1D* mutant. However, the *smg1Δ* mutant exhibits wild type-levels of melanin production, unlike the *gpa1Δ* mutant [29, 30]. Therefore, it is possible that the function of *CAR1* gene may overlap with that of other genes regulated by the cAMP pathway, which explains why disruption of the *CAR1* gene does not result in any phenotypes in *C. neoformans*. Our previous transcriptome analysis identified many other candidate cAMP-regulated genes, which do not have any known function or orthologs in other fungi. This indicates that some of the cAMP-dependent phenotypes could be controlled by multiple target genes. Therefore, to further address the function of Car1, it may be necessary to overexpress the *CAR1* gene in some of the cAMP mutants and observe whether any of the cAMP-related phenotypes could be restored (at least partly) by *CAR1* overexpression.

Another possibility is that Car1 may play a role in some of cAMP-signaling related phenotypes that have not yet been elucidated. It is unlikely that all of the cAMP-signaling dependent phenotypes have been discovered in *C. neoformans*. Therefore, it is too early to conclude that Car1 does not play any role in the cAMP-signaling pathway of *C. neoformans*, since only a limited number of phenotypic analyses has been performed. Particularly, Car1 may be required for conferring full virulence during host infection and disease progress. To address this question, animal studies should be done.

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References

1. Bahn YS, Xue C, Idnurm A, Rutherford JC, Heitman J, Cardenas ME. Sensing the environment: lessons from fungi. Nat Rev Microbiol 2007;5:57-69.
2. Pukkila-Worley R, Alspaugh JA. Cyclic AMP signaling in *Cryptococcus neoformans*. FEMS Yeast Res 2004;4:361-7.
3. Pan X, Heitman J. Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Mol Cell Biol 1999;19:4874-87.
4. Robertson LS, Fink GR. The three yeast A kinases have specific signaling functions in pseudohyphal growth. Proc Natl Acad Sci U S A 1998;95:13783-7.
5. Sass P, Field J, Nikawa J, Toda T, Wigler M. Cloning and characterization of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 1986;83:9303-7.
6. Wilson RB, Tatchell K. *SRA5* encodes the low-Km cyclic AMP phosphodiesterase of *Saccharomyces cerevisiae*. Mol Cell Biol 1988;8:505-10.
7. Buck J, Sinclair ML, Schapal L, Cann MJ, Levin LR. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc Natl Acad Sci U S A 1999;96:79-84.
8. Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS: 100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 1995;8:515-48.
9. Hoang LM, Maguire JA, Doyle P, Fyfe M, Roscoe DL. *Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre (1997-2002): epidemiology, microbiology and histopathology. J Med Microbiol 2004;53:935-40.
10. Chang YC, Penoyer LA, Kwon-Chung KJ. The second capsule gene of *Cryptococcus neoformans*, *CAP64*, is essential for virulence. Infect Immun 1996;64:1977-83.
11. Nosanchuk JD, Rudolph J, Rosas AL, Casadevall A. Evidence that *Cryptococcus neoformans* is melanized in pigeon excreta: implications for pathogenesis. Infect Immun 1999; 67:5477-9.
12. Aksenov SI, Babyeva IP, Golubev VI. On the mechanism of adaptation of micro-organisms to conditions of extreme low humidity. Life Sci Space Res 1973;11:55-61.
13. Rosas AL, Casadevall A. Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. FEMS Micro-

biol Lett 1997;153:265-72.
14. Wang Y, Aisen P, Casadevall A. *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect Immun 1995;63:3131-6.
15. Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol Cell Biol 1994;14:4912-9.
16. Kwon-Chung KJ, Polacheck I, Popkin TJ. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J Bacteriol 1982;150:1414-21.
17. Alspaugh JA, Pukkila-Worley R, Harashima T, Cavallo LM, Funnell D, Cox GM, et al. Adenylyl cyclase functions downstream of the Galpha protein Gpa1 and controls mating and pathogenicity of *Cryptococcus neoformans*. Eukaryot Cell 2002;1:75-84.
18. Bahn YS, Hicks JK, Giles SS, Cox GM, Heitman J. Adenylyl cyclase-associated protein Aca1 regulates virulence and differentiation of *Cryptococcus neoformans* via the cyclic AMP-protein kinase A cascade. Eukaryot Cell 2004;3:1476-91.
19. Hicks JK, Bahn YS, Heitman J. Pde1 phosphodiesterase modulates cyclic AMP levels through a protein kinase A-mediated negative feedback loop in *Cryptococcus neoformans*. Eukaryot Cell 2005;4:1971-81.
20. Xue C, Hsueh YP, Chen L, Heitman J. The RGS protein Crg2 regulates both pheromone and cAMP signalling in *Cryptococcus neoformans*. Mol Microbiol 2008;70:379-95.
21. Maeng S, Ko YJ, Kim GB, Jung KW, Floyd A, Heitman J, et al. Comparative transcriptome analysis reveals novel roles of the Ras- and cAMP-signaling pathways in environmental stress response and antifungal drug sensitivity in *Cryptococcus neoformans*. Eukaryot Cell 2010. DOI:10.1128/EC.00309-09.
22. Perfect JR, Ketabchi N, Cox GM, Ingram CW, Beiser CL. Karyotyping of *Cryptococcus neoformans* as an epidemiological tool. J Clin Microbiol 1993;31:3305-9.
23. Nielsen K, Cox GM, Wang P, Toffaletti DL, Perfect JR, Heitman J. Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic **a** and α isolates. Infect Immun 2003;71:4831-41.
24. Bahn YS, Kojima K, Cox GM, Heitman J. Specialization of the HOG pathway and its impact on differentiation and virulence of *Cryptococcus neoformans*. Mol Biol Cell 2005;16:2285-300.
25. Hicks JK, D'Souza CA, Cox GM, Heitman J. Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans*. Eukaryot Cell 2004;3:14-26.
26. Kim MS, Kim SY, Yoon JK, Lee YW, Bahn YS. An efficient gene-disruption method in *Cryptococcus neoformans* by double-joint PCR with NAT-split markers. Biochem Biophys Res Commun 2009;390:983-8.
27. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. New York: Greene Publishing Associates and John Wiley & Sons; 1994.
28. Garay-Arroyo A, Covarrubias AA. Three genes whose expression is induced by stress in *Saccharomyces cerevisiae*. Yeast 1999;15:879-92.
29. Palmer DA, Thompson JK, Li L, Prat A, Wang P. Gib2, a novel Gβ-like/RACK1 homolog, functions as a Gbeta subunit in cAMP signaling and is essential in *Cryptococcus neoformans*. J Biol Chem 2006;281:32596-605.
30. Pukkila-Worley R, Gerrald QD, Kraus PR, Boily MJ, Davis MJ, Giles SS, et al. Transcriptional network of multiple capsule and melanin genes governed by the *Cryptococcus neoformans* cyclic AMP cascade. Eukaryot Cell 2005;4:190-201.