Divergence of Protein Kinase A Catalytic Subunits in *Cryptococcus neoformans* and *Cryptococcus gattii* Illustrates Evolutionary Reconfiguration of a Signaling Cascade

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Gene duplication and divergence via both the loss and gain of gene activities are powerful evolutionary forces underlying the origin of new biological functions. Here a comparative genetics approach was applied to examine the roles of protein kinase A (PKA) catalytic subunits in three closely related varieties or sibling species of the pathogenic fungus genus *Cryptococcus*. Previous studies revealed that two PKA catalytic subunits, Pka1 and Pka2, control virulence factor production and mating. However, only one of the two plays the predominant physiological role, and this function has been exchanged between Pka1 and Pka2 in strains of the *Cryptococcus neoformans* var. *grubii* serotype A lineage compared to divergent *C. neoformans* var. *neoformans* serotype D isolates. To understand the basis for this functional plasticity, here the activities of Pka1 and Pka2 were defined in the two varieties and the related sibling species *Cryptococcus gattii* by gene disruption and characterization, heterologous complementation, and analysis of serotype AD hybrid mutant strains. The findings provide evidence for a shared ancestral role of PKA in governing mating and virulence factor production and indicate that the exchange of catalytic subunit roles is attributable to loss of function. Our studies illustrate the plasticity of signaling networks enabling rapid rewiring during speciation of a clade of common human fungal pathogens.

Gene duplication and divergence are significant forces in the evolution of new genes (21). Gene duplication events can occur both on a macrogenomic scale, such as that involving the ancestral whole genome duplication that gave rise to *Saccharomyces cerevisiae* and related sensu stricto strains (31), and on the microgenomic scale of individual genes. Several models regarding the mechanisms by which new functions arise following gene duplication have been postulated. The model originally postulated by Ohno (21), now termed “neofunctionalization,” suggests that following duplication, one gene of the pair retains the original function while functional constraints on the other gene are relaxed, allowing it to undergo accelerated evolution and eventually develop novel functions.

A well-documented example of neofunctionalization is that of the *ORC1/SIR3* gene pair that arose in *S. cerevisiae* following the whole-genome duplication that occurred approximately 100 to 300 million years ago (mya) (15, 28). In this instance, *S. cerevisiae* Orc1 shares 48% identity with a protein found in the related species, *Saccharomyces kluveri*, while Sir3 shares only 24% identity with the protein, suggesting that Orc1 has retained the ancestral function while Sir3 has been subject to accelerated evolution. Consistent with this is the observation that the *S. kluveri ORC1/SIR3* gene can complement the *S. cerevisiae orc1Δ* mutant but not the *S. cerevisiae sir3Δ* mutant.

Another example of gene duplication in *S. cerevisiae* involves the genes encoding the three protein kinase A (PKA) catalytic subunits, Tpk1, Tpk2, and Tpk3. All three catalytic subunits share a redundant function yet also have novel functions as well. While all three catalytic subunits share redundant roles in viability, as demonstrated by the fact that a *tpk1Δ tpk2Δ tpk3Δ* triple mutant is inviable, they play opposing roles in pseudohyphal growth, with Tpk2 activating and Tpk1 and Tpk3 repressing the filamentous dimorphic transition (22, 24). The abundance of well-studied gene duplications in *S. cerevisiae* and the presence of a well-characterized lineage (including the closely related sensu stricto strains that can be used as outgroups) make *S. cerevisiae* an ideal model system for examining gene duplication events in ascomycete fungi.

Similarly, *Cryptococcus neoformans* has several attributes that render it a facile system in which to study gene duplication events in basidiomycete fungi. First, there are multiple examples of gene duplications in this fungus, including the laccase genes *LAC1* and *LAC2*, the Ras genes *RAS1* and *RAS2*, the carbonic anhydrase genes *CAN1* and *CAN2*, and the cyclophilin A genes *CPAI* and *CPA2* (2, 4, 23, 29, 30). Another example of gene duplication in *Cryptococcus* involves the protein kinase A catalytic subunits, Pka1 and Pka2, which share 35% identity at the protein level. Our previous work showed that both the serotype A and D lineages have both subunits but that the functions of these subunits differ between the two lineages. Deletion of the *PKA1* gene in serotype A results in a loss of mating and melanin and capsule production, whereas it is the deletion of the *PKA2* gene in serotype D that results in the loss of these functions (3, 11). The second attribute that renders *Cryptococcus neoformans* an amenable model system for studying gene duplication and evolution in basidiomycete fungi is...
the presence of full genome sequences for the closely related varieties *C. neoformans* var. *neoformans* (serotype D) and *C. neoformans* var. *grubii* (serotype A) and sister species *Cryptococcus gattii* (18). Much like the sensu stricto strains of *C. neoformans* the presence of full genome sequences for the closely related *C. neoformans* (serotype D) and *C. neoformans* (serotype A), *Pka1* has retained its roles in melanin and capsule production and mating, whereas *Pka2* has lost all three functional roles. Finally, in *C. neoformans* var. *neoformans* (serotype D), *Pka1* has lost all of these functions, whereas *Pka2* retained all three functions. These findings reveal rapid and plastic rewiring of signaling cascades controlling virulence and development during fungal speciation.

### TABLE 1. Strains used in this study

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* FOA', 5-fluoroorotic acid resistant.

### MATERIALS AND METHODS

*C. neoformans* and *C. gattii* strains and media. All strains used in this study are listed in Table 1. *C. neoformans* (serotypes A and D) and *C. gattii* (serotypes B and C) strains were grown on standard *S. cerevisiae* media (26). The selective medium for biolistic transformation, proline medium for serotype AD hybrid selection, Niger seed medium, V8 medium, and Dulbecco’s modified Eagle’s (DME) medium were prepared as described previously (1, 8, 12, 14, 27).

Disruption of the *C. gattii* and *C. neoformans* serotype D *PKA1* and *PKA2* genes. A prototrophic serotype D wild-type strain (R265) was bioartistically transformed with the gel-extracted *PKA1* and *PKA2* disruption alleles as described previously (5) to create strains JKH290 and JKH293, respectively. Details on the construction of the overlap constructs can be found in the supplemental material. An auxotrophic serotype D strain containing a *ura5* mutation (JEC34) was transformed with the *PKA1D* disruption allele to create strain JKH313. To
obtain pka1Δ pka2Δ double mutants of serotypes B and D, the pka1BΔ::NAT strain (JKH290) and the pka1DaΔ::NEO strain (JKH313) were transformed with pka2BΔ::NEO and pka2DΔ::NAT disruption alleles to create strains JKH317 and JKH314, respectively. The pka1a and pka2a strains were screened by diagnostic PCR for the 5' junction and confirmed by Southern blot analysis using specific probes generated by PCR (data not shown).

**Complementation experiments.** Complementation of the pka2ΔA mutant with the serotype A PKA genes was tested by transforming strain JKH19 (serotype D pka2ΔA) (11) with plasmid pH80 (containing the wild-type PKA1A gene) and pH167 (containing the wild-type PKA2A gene) by using a bioplastic apparatus (27) to create strains JKH287 and JKH247, respectively. Construction details of pH80 and pH167, as well as the other plasmids in the complementation analysis, are provided in the supplemental material.

Complementation of the pka1ΔA strain with the serotype D PKA genes was tested by transforming strain JKH165 with pH26 (PKA2D) and pH166 (PKA1D) to create strains JKH176 and JKH242, respectively.

Complementation of the pka1a and pka2ΔA mutants with the serotype B PKA1B and PKA2B genes was tested by transforming strain JKH47 (serotype A pka1AΔ) with pΗ235 (PKA1B) and pH240 (PKA2B) to create strains JKH306 and JKH297, respectively, and strain CDC18 (serotype D pka2Δa) was transformed with pΗ235 and pH240 to create JKH315 and JKH292, respectively.

The serotype B pka2ΔB mutant (JKH293) was also transformed with pH240 to create strain JKH311. RNA analysis was performed on all transformants to confirm the expression of the heterologous gene.

**Serotype A and D hybrid assays.** Hybrid assays were accomplished by mixing 1 × 10³ cells/ml of each of the mating partners and then plating 20 μl of the mixed cell suspension onto V8 medium (pH 7.0). After 2 days of growth in the dark, the cells were scraped off the V8 medium (pH 7.0) and replated on selective medium containing either G418 (JKH96 × JKH313 and JKH4 × JKH313) or G418 and nourseothricin (JKH96 × JKH314 and JKH4 × JKH314). The plates were then incubated at 37°C for 4 days. Fusion products were then examined for melanin and capsule production. The test crosses were as follows: JKH324, MATa PKA1A pka2ΔΔ::URA5 (JKH4) × MATa pka1DaΔ::NEO pka2ΔD::NAT ura5 (JKH314); JKH321, MATa pka1ΔA::URA5 pka2ΔA::URA5 (JKH96) × MATa pka1DaΔ::NEO pka2ΔD::URA5 ura5 (JKH313); JKH323, MATa PKA1A pka2ΔA::URA5 (JKH4) × MATa pka1DaΔ::NEO pka2ΔD::URA5 ura5 (JKH313); JKH322, MATa pka1ΔA::URA5 pka2ΔA::URA5 (JKH96) × MATa pka1DaΔ::NEO pka2ΔD::NAT ura5 (JKH314). PCR reactions with serotype A MATa- and MATa-specific primers (7270/7271 and 7286/7286, respectively) and serotype D MATa- and MATa-specific primers (7273/7274 and 7287/7287, respectively) were utilized to confirm the hybrid diploid status of the fusion products.

**Mating assays.** α and a strains were cocultured on V8 medium (pH 7) and incubated at room temperature for 4 days in the dark prior to photography (×100 magnification). These strains included wild-type VGII α × wild-type VGII a (R265 × B454d); pka1Bα VGII α × VGII a (JKH290 × B454d); pka2Bα VGII α × VGII a (JKH293 × B454d); and pka1Bα pka2Bα VGII α × VGII a (JKH314 × B454d).

**Expression analysis.** Fungal strains were inoculated into 5 ml YPD medium and grown overnight at 30°C. Fifty-milliliter amounts of YPD medium in 125-ml flasks were inoculated with 500 μl of the overnight cultures and grown at 250 rpm and 30°C for 5 h prior to harvesting. RNA was isolated from the harvested cultures with TRizol (Gibco-BRL) following the manufacturer’s instructions. Fifteen micrograms (based on spectrophotometric measurement) of RNA was separated on a denaturing gel and transferred to nylon membrane. The resulting blots were probed with PKA1B, PKA1D, PKA2B, or PKA2D gene-specific probes and with actin as a loading control.

**Microscopy.** All images of mating and confrontation assays were captured with a Nikon Eclipse E400 microscope equipped with a Nikon DXM1200F camera. Images of melanized colonies were captured with a Nikon CoolPix digital camera. Differential interference microscopy images were taken with the ×1,000 objective of a Zeiss Axioscope 2 Plus fluorescence microscope equipped with an AxioCam MRM digital camera.

**RESULTS**

**Identification of the C. gattii serotype B Pka1 and Pka2.** We have previously shown that, despite sharing only 35% identity at the amino acid level, the serotype A Pka1 and serotype D Pka2 catalytic subunits share similar roles in regulating melanin and capsule production and mating in their respective serotypes (11). To better understand the functional divergence of Pka1 and Pka2, we identified the Pka1 and Pka2 catalytic subunits from the genomic sequence of the R265 strain (molecular group VGII (6) of C. gattii, a species closely related to C. neoformans (32) and the cause of an outbreak on Vancouver Island (6, 16). At the amino acid level, the Pka1 protein from C. gattii (Pka1B) shares 86% identity with the serotype A Pka1 subunit (Pka1A) and 84% identity with the serotype D Pka1 subunit (Pka1D); Pka1A and Pka1D are 93% identical. The C. gattii Pka2 subunit (Pka2B) shares 87% identity with the serotype A Pka2 subunit (Pka2A) and 88% identity with the serotype D Pka2 subunit (Pka2D), similar to the 93% identity shared between Pka2A and Pka2D. In contrast, the Pka1B subunit shares only 34% identity with the Pka2B subunit. This is similar to the identity comparison of Pka1A and Pka2A (35%) and that of Pka1D and Pka2D (35%).

**Complementation of pka1ΔA and pka2ΔA mutations with the PKA1 and PKA2 genes.** We were interested in determining whether the wild-type PKA1A, PKA2B, or PKA2D gene could complement the pka2ΔA and pka1ΔA mutations in serotypes D and A, respectively. To test this, wild-type PKA1A, PKA1B, PKA2A, PKA1D, and PKA2D genes were ligated into transformation vectors (plasmids pH80, pH167, pH1240, pH166, and pH126, respectively) and biologically transformed into pka2ΔA and pka1ΔA mutant strains. The resulting transformants were examined for melanin and capsule production. The wild-type PKA1A gene, but not PKA2A, complemented the melanin and capsule defects of a pka2ΔD mutant strain (Fig. 1, lanes 3 and 4), while the wild-type PKA2D gene, but not the PKA1D gene, complemented the melanin and capsule defects of a pka1ΔA mutant strain (Fig. 1, lanes 1 and 2). The PKA2B gene, but not the PKA1B gene, complemented the capsule and melanin defect in both pka1ΔA and pka2ΔD mutant strains (Fig. 1). In control experiments, when the pka1ΔA, pka2ΔD, and pka2ΔD mutant strains were transformed with the plasmids containing the wild-type PKA1A (pH80), PKA2D (pH126), and PKA2B (pH1240) genes, respectively, the melanin and capsule defects of the resulting transformants were complemented (data not shown). Expression analysis by Northern blot analysis confirmed that all heterologous genes were expressed (data not shown).

To independently confirm these findings by a complementary approach, we isolated a series of serotype AD hybrid diploid strains that contained combinations of the pka1ΔA, pka2ΔA, pka1ΔD, and pka2ΔD mutations and determined which PKA catalytic subunit was functional in the hybrid by examining melanin and capsule production. AD hybrids were isolated following cell-cell fusions, and PCR analysis using both serotype A- and serotype D-specific primers confirmed the presence of both the serotype A MATa mating type and the serotype D MATa mating type in each hybrid. Melanin and capsule were produced by pka2ΔΔ pka1ΔΔ pka2ΔD (JKH324), pka1ΔΔ pka2ΔA pka1ΔD (JKH321), and pka2ΔΔ pka1ΔΔ (JKH323) hybrids, all of which express PKA1A, PKA2D, or both. Only the hybrid with no functional PKA catalytic subunits (pka1ΔΔ pka2ΔA pka1ΔΔ pka2ΔD; JKH322) was unable to produce melanin and capsule (Fig. 2). These results confirm that Pka1A and Pka2D are able to cross-complement the pka2ΔD and pka1ΔΔ mutations in serotypes D and A, respectively.
The serotype B Pka2B subunit is responsible for melanin production, while both Pka1B and Pka2B play roles in capsule production. To determine which of the C. gattii PKA catalytic subunits, Pka1B or Pka2B, was responsible for melanin production in C. gattii, we deleted the entire open reading frames of both the PKA1B and the PKA2B genes. The deletions were confirmed by Southern blot and Northern analyses. The deletion strains were then grown on L-DOPA (L-, 4-dihydroxyphenylalanine) and Niger seed medium and incubated at 37°C for 12 to 14 h. The pka2BΔ strain exhibited a severe defect in melanin production, whereas melanin production by the pka1BΔ strain was indistinguishable from that of the serotype B wild-type strain (Fig. 3, lane 1). These findings indicate that the Pka2 catalytic subunit is responsible for positively regulating melanin production in serotype B.

In contrast, neither the pka1BΔ mutant strain nor the pka2BΔ mutant strain exhibited a demonstrable capsule defect (Fig. 3, lane 2). We hypothesized that Pka1B and Pka2B might...
play a shared role in regulating capsule production. To test this, a pka1BΔ pka2BΔ double-mutant strain was created via transformation of the pka1BΔ mutant with a pka2BΔ disruption allele. As with the pka2BΔ single mutant, the double mutant had a severe defect in melanin production (Fig. 3, lane 1). In addition, several independent double-mutant strains also exhibited a profound capsule defect that was not observed in either single-mutant strain, indicating that Pka1B and Pka2B play a redundant role in capsule production (Fig. 3, lane 2).

**Pka1B and Pka2B have degenerate roles in mating.** To examine the roles of Pka1B and Pka2B in mating, the pka1BΔ and pka2BΔ single-mutant strains and the pka1BΔ pka2BΔ double-mutant strain were tested as mating partners with the C. gattii MATa strain B4546 (molecular group VGIII) (6, 7). After 4 days of growth on V8 medium (pH 7.0), profuse filamentation and basidial formation was observed in the mating between the pka2BΔ mutant and wild-type strain B4546. This was in comparison to the mating between the wild-type strains (R265 × B4546) and the mating between the pka1BΔ mutant and wild-type strain B4546, in which only minimal filamentation and basidial formation were observed. With the pka1BΔ pka2BΔ double mutant crossed to the wild-type strain, no filamentation or basidial formation was observed (Fig. 4). These data provide evidence that the PKA catalytic subunits in C. gattii have overlapping roles and at least one functional PKA catalytic subunit is required for mating. In addition, the Pka2 catalytic subunit appears to play an additional role in repressing mating in C. gattii.

**Protein kinase A catalytic and regulatory subunits interact in the two-hybrid system.** To begin to address the mechanistic basis for the functional differences between the Pka1 and Pka2 catalytic subunits in divergent varieties and species, the yeast two-hybrid system was employed to assess interactions between the subunits. First, we found that the protein kinase A regulatory subunit Pkr1 was capable of interacting with itself when fused to both the Gal4 DNA binding domain and the activation domain, and the magnitude of this interaction was reduced by 10 mM cAMP (data not shown). Additionally, we found that the serotype A Pka1 and Pka2 subunits expressing Gal4 domains fused to Pkr1 and Pka1 or Pka2 from serotype A or D as indicated were tested for β-galactosidase expression in the presence (+) and absence (−) of 10 mM exogenous cAMP. Samples were assayed in triplicate, and the standard errors of the means are presented as error bars.
interacted to a more modest extent with the Pkr1 regulatory subunit, and the provision of cAMP reduced the Pkr1-Pka2 interaction but not the Pkr1-Pka1 interaction (Fig. 5). Taken together, these findings suggest that differences in the interaction of the catalytic and regulatory subunits of protein kinase A or in the cAMP responsiveness of the complex could contribute to functional differences between subunits.

**DISCUSSION**

We have taken advantage of the availability of the total genome sequences from three Cryptococcus serotypes (serotype A, C. neoformans var. grubii; serotype D, C. neoformans var. neoformans; and serotype B, C. gattii) to examine the evolution of two PKA catalytic subunits, Pka1 and Pka2. C. gattii diverged from C. neoformans approximately 38.5 mya, while the two C. neoformans varieties (C. neoformans var. grubii and C. neoformans var. neoformans) diverged from each other around 18.5 mya (32).

An examination of the C. gattii serotype B strain R265 genome revealed that C. gattii also has two catalytic subunits that share 84% (Pka1) and 87% (Pka2) identity with their serotype A and D counterparts. The presence of two catalytic subunits in C. gattii suggests that a gene duplication event occurred over 38.5 mya, prior to the divergence of C. gattii and C. neoformans, resulting in two catalytic subunits in both species. We were interested in characterizing the C. gattii Pka1 and Pka2 subunits to determine how the roles in regulating melanin and capsule production and mating were allocated between the two subunits in this more divergent species. The deletion of both the PKA1B and PKA2B genes revealed that solely Pka2B is responsible for regulating melanin production (Fig. 3, lane 1). However, a defect in capsule production was observed only when both the PKA1B and PKA2B genes were deleted (Fig. 3, lane 2), indicating that Pka1B and Pka2B share redundant roles in regulating capsule production.

Similar to the opposing roles played by Tpk1/3 and Tpk2 in regulating pseudohyphal growth in Saccharomyces cerevisiae (22, 24), the diverse roles of the PKA catalytic subunits in mating in Cryptococcus serotypes A, D, and B are consummate examples of the complexity of the PKA signaling pathway. In serotype A, the deletion of the PKA1 gene (but not PKA2) in either parental strain results in loss of mating. The situation is more complex in serotype D, wherein a mating defect is detected only when both parental strains harbor a pka2ΔD mutation. Furthermore, unlike serotype A pka1AΔ mutants, pka2DΔ mutants (but not pka1DΔ mutants) have defects in cell and nuclear fusion, leading to production of aberrant filaments (11). In C. gattii, the situation is different than that in the lineage of either serotype A or D because both the Pka1B and Pka2B subunits are required for mating, as indicated by the observation that mating still occurs when either the PKA1B or PKA2B gene was individually deleted but no mating was observed when both the PKA1B and PKA2B genes were deleted (Fig. 4). In addition to playing a redundant role that it shares with Pka1B in activating mating, Pka2B may play a role in repressing mating, as suggested by the observation that the mating of the pka2DΔ mutant with a tester strain resulted in more robust filamentation than was seen with the wild type (Fig. 4).

Our heterologous complementation studies, as well as our serotype AD hybrid diploid studies, showed that the wild-type PKA2D gene complemented the melanin and capsule defects resulting from a pka1AΔ mutant. Similarly, a wild-type PKA1A gene complemented the melanin and capsule defects of a pka2DΔ mutant. The wild-type PKA2B gene, but not PKA1B, was able to complement the capsule defect of the pka1AΔ and pka2DΔ mutants. Interestingly, even though our data show that both the C. gattii Pka1B and Pka2B subunits have roles in capsule production, only the wild-type PKA2B gene, and not PKA1B, was able to complement the capsule defect of the pka1AΔ and pka2DΔ mutants. The failure of the PKA1B gene to complement was not due to a defect in expression or attributable to a mutation in the complementation allele, based on sequence analysis. One remaining possibility is that the lack of complementation of the capsule defect of the pka1AΔ and the pka2DΔ mutant by the PKA1B gene is the result of the Pka1B subunit having, over time, lost its ability to promote capsule production in the divergent C. neoformans serotype A and D lineages.

Based on the data presented here and our previous data showing that the serotype A Pka1A subunit and the serotype D Pka2D subunit have the same function despite having only 35% identity at the amino acid level (11), we propose a model in which the C. gattii Pka1B and Pka2B subunits are representative of the PKA catalytic subunits shortly after the gene duplication event, when the two subunits both still retained ancestral shared functions (Fig. 5). Over time, the serotype A Pka2A subunit lost many of its original functions, with the exception of a minor role in melanin production, whereas the Pka1A subunit retained all of its original functions. On the contrary, in serotype D, the Pka1D subunit lost most of its original functions, whereas the Pka2D subunit retained its original functions (Fig. 6).

Our model is consistent with the neofunctionalization model proposed by Ohno (21). In this model, after gene duplication, one gene retains the ancestral function while selection pressure on the other gene is reduced, allowing it to lose the original functions and possibly gain new ones. Although neither Pka2A nor Pka1D showed any obvious new functions, as is predicted by Ohno’s model, this could be either because not enough time has elapsed since the gene duplication for these functions to have evolved or because we have not tested enough conditions to reveal novel functions. Alternatively, it is possible that instead of the subunits gaining functions, the Pka2A and Pka1D subunit genes may be in the process of becoming pseudogenes and will eventually be completely lost, leaving only one recognizable PKA catalytic subunit, Pka1A and Pka2D, in the respective serotypes.

The mechanism for the differential actions of Pka1 and Pka2 within each of the three serotypes is unclear. One possibility is that the Pka1 and Pka2 proteins interact differently with the regulatory subunit, Pkr1. Pkr1 is highly conserved among the serotypes (Pkr1B shares 89% amino acid identity with both Pkr1A and Pkr1D, and Pkr1A shares 95% amino acid identity with Pkr1D). Our yeast two-hybrid system data (Fig. 5) indicate that Pka1A, but not Pka2A, interacts strongly with Pkr1A. One explanation for this may lie in the amino acid residues in Pka1 and Pka2 that occur at positions in the catalytic subunit critical for binding to the regulatory subunit. Kim et al. (17)
have identified several residues in the PKA catalytic subunit that are necessary for interactions with the regulatory subunit. A comparison of the PKA catalytic subunit protein sequence analyzed by Kim et al. and the protein sequences of the Pka1 and Pka2 catalytic subunits from serotypes A, B, and D reveals that at least four of the critical amino acids (at amino acid positions 379, 381, 396, and 398) are substantially different between the Pka1 subunit (isoleucine, tryptophan, glutamine, and lysine at the respective positions) and the Pka2 subunit (arginine, phenylalanine, leucine, and glutamine at the respective positions), although the residues are conserved within the Pka1 and Pka2 proteins of the respective serotypes. Another possibility is that Pka1 and Pka2 interact with different substrates or interact differentially with the same substrates. Finally, previous studies have indicated that the catalytic subunits of PKA have targets both in the cytoplasm and in the nucleus (9). Thus, it is possible that the Pka1 and Pka2 proteins are localized differently within the cell and may either act on different targets depending on their location or, alternatively, be functional only if they are localized in one organelle or another. Further studies will be necessary to address these and other models.

Studies have utilized comparative approaches for closely related species to examine gene duplication and to test models of gene duplication. This is especially true for the well-characterized yeast Saccharomyces cerevisiae, in which a whole-genome duplication occurred and was subsequently followed by extensive gene loss and gene specialization (15, 25, 31). The majority of these studies have focused on gene and protein structures (e.g., references 10 and 15), although some studies have advanced a functional approach (e.g., reference 28). In our study, we implemented a gene function approach rather than a gene structure approach by examining the roles of duplicated proteins in divergent, related species (C. gattii) to understand the origin of function in a more recently diverged pair of varieties (C. neoformans var. grubii and C. neoformans var. neoformans). Similarly, we utilized inter- and intraspecific complementation approaches, including the isolation and analysis of hybrid diploid strains, to decipher the functions of these signaling cascade genes. Both of these techniques may, in combination with the more commonly used gene structure comparison approach, have applications for determining gene function and examining the results of gene duplication events in other fungi, such as the sensu stricto strains of Saccharomyces.

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