GIB2, A NOVEL Gβ-LIKE/RACK1 HOMOLOG, FUNCTIONS AS A Gβ SUBUNIT IN cAMP SIGNALING AND IS ESSENTIAL IN CRYPTOCOCCUS NEOFORMANS*

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Running title: Novel function of Cryptococcus neoformans Gib2

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Canonical G proteins are heterotrimeric, consisting of α, β, and γ subunits. Despite multiple Gα subunits functioning in fungi, only a single Gβ subunit per species has been identified, suggesting that non-conventional G protein signaling exists in this diverse group of eukaryotic organisms. Using the Gα subunit Gpa1 that functions in cAMP signaling as bait in a two-hybrid screen, we have identified a novel Gβ-like/RACK1 protein homolog, Gib2, from the human pathogenic fungus Cryptococcus neoformans. Gib2 contains a seven WD-40 repeat motif and is predicted to form a seven-bladed β-propeller structure characteristic of β transducins. Gib2 is also shown to interact respectively with two Gγ subunit homologs, Gpg1 and Gpg2, similar to the conventional Gβ subunit Gpb1. In contrast to Gpb1 whose over-expression promotes mating response, over-production of Gib2 suppresses defects of gpa1 mutation in both melanization and capsule formation, the phenotypes regulated by cAMP signaling and associated with virulence. Furthermore, depletion of Gib2 by antisense suppression results in a severe growth defect, suggesting that Gib2 is essential. Finally, Gib2 is shown to also physically interact with a down-stream target of Gpa1-cAMP signaling, Smg1, and the protein kinase C homolog Pkc1, indicating that Gib2 is also a multi-functional RACK1-like protein.

In eukaryotic organisms, GTP-binding proteins transduce external signals to regulate a wide array of cellular functions. In fungi, such regulatory functions include the control of cellular growth and differentiation, asexual and sexual reproduction, the production of secondary metabolites, and/or virulence (see review (1-3)). Whereas Gα subunits are known to exert most regulatory functions, free Gβ(γ) subunits also regulate activities of effectors either independently or in concert with Gαs (4,5). Gβ subunits contain a distinct seven-bladed propeller structure with each blade composed of a conserved core of approximately 40 amino acids flanked by Trp-Asp (WD) (6,7). Unlike the higher eukaryotes in which multiple Gβ subunits have been identified, only a single Gβ per species has been found in fungi that regulates functions such as mating (8,9), growth and sporulation (10-12), infection-related morphogenesis (13), and virulence (10).

Like the Gβ subunit, the Gβ-like/receptor for activated C-kinase 1 (RACK1) protein also contains the seven WD-40 repeat motif. RACK1 was first found to function as a scaffold protein localizing the activated protein kinase C (PKC) to the insoluble cell fraction (plasma membranes) (14-16) and the RACK1 protein interacts with many signal proteins, including the Src tyrosine kinase (17,18), integrin β subunit (19), phosphodiesterase Pde4D5 (20), and G protein heterotrimeric α(t)βγ and heterodimeric βγ subunits (21-23). The Saccharomyces cerevisiae Gβ-like/RACK1 homolog Asc1/Cpc2 (cross-pathway-control) and mammalian RACK1 proteins were also found to be core 40S ribosomal proteins that repress gene expression (24,25).
Similar to other fungi, there exists an elaborate G protein-signaling network in *C. neoformans* that senses the environmental and host-imposed cues to regulate growth, differentiation, and virulence (26-28). *C. neoformans* is an encapsulated yeast-like basidiomycetous fungus and the main cause of meningoencephalitis in individuals with a compromised immune system (29). Previous studies have shown that the Gα subunit Gpa1 functions in a conserved cAMP-dependent signaling pathway and this pathway regulates a variety of cellular functions, including specialized processes such as the production of the antioxidant melanin pigment and the antiphagocytic capsule, two well-established virulence factors in this pathogen (30-32).

In contrast to Gpa1, the Gβ subunit Gpb1 functions to regulate pheromone-responsive mating and haploid differentiation upstream of a conserved MAP kinase cascade, and Gpb1 is not involved in virulence (9). The completed *C. neoformans* genome project has revealed two additional Gα subunits, Gpa2 and Gpa3, whose functions have not yet been elucidated (33). Whereas either Gpa2 or Gpa3 could couple to Gpb1 and a Gγ subunit(s) to form a functional heterotrimeric G protein complex, no Gβ subunit that could couple to Gpa1 in cAMP signaling was found. Thus, answering whether G protein signaling is indeed unique and thereby explaining the presence of a single Gβ subunit would represent a significant achievement in the understanding of G protein signaling in this or other fungal organisms. We now report that a novel Gβ-like/RACK1 protein, Gib2, functions as an atypical Gβ in Gpa1-cAMP signaling. We also show that Gib2 encodes RACK1-like multi-functions, including an essential one in *C. neoformans*. Our studies suggest that a similar signaling mechanism could exist in other organisms where multiple Gα subunits but only a single Gβ subunit has been found.

**Experimental Procedures**

*Strains and media-C. neoformans* var. *grubii* (serotype A) MATα strains H99 and F99 (H99 ura5), MATa strain KN99a and F99a (KN99a ura5), var. *neoformans* (serotype D) MATα strains JEC21 and JEC43 (JEC21 ura5), MATa strains JEC20, JEC34 (JEC20 ura5), BAC20-1 (gpa1 ura5), and a laboratory derived diploid strain, RAS009 (a/α ade2+/+ ura5/ura5 lys1/+ lys2+/+), were from the laboratory of J. Heitman at DUMC (9,34,35).

Standard yeast extract-peptone-dextrose (YPD), yeast minimal medium (YNB), synthetic medium (SD), 10% V8 agar (pH 5.0), 5-FOA, Niger seed agar, and filament agar were prepared as previously described (9). Southern, Northern, and Western blotting analysis were performed according to standard protocols (36). The total cellular cAMP level was measured as previously described (32,37).

Two-hybrid interaction-For the yeast two-hybrid screen, GPA1 cDNA was synthesized using primers: PW99 (5’-AAG GAA TTC ATG GGC GGC TGT ATG TCT-3’) and PW100 (5’-GAA CTG CAG CCT TAT AAG ATA CCA GAG TC-3’) and cloned into pGBKT7 (Clontech) at the EcoRI-PstI sites to create pGBKT7-GPA1, expressing the fusion protein GAL4 (BD)-Gpa1. pGBKT7-GPA1 was used to screen a cDNA library of H99 using the yeast AH109 strain. Candidate colonies expressing interacting proteins were screened by plating on SD-Leu–Trp–His–Ade plus X-α-Gal. DNA plasmids were purified from positive yeast cultures, recovered through transformation of *E. coli*, and inserts were sequenced.

The GPA1Q284L was synthesized using primers: PW99, PW100, JH12499 (5'-CAT TCA TAT GTT CGA TGT CGG TGG ACT GAG AAG CGA GAG AAA GAA GTG G-3') and JH12500 (5'-CCA CTT CTT TCT CTC GCT TCT CAG TCC ACC GAC ATC GAA CAT ATG AAT G-3'), inserted into pGBKT7, and verified by sequencing.

**GPA2** (Genbank #AY357297) and GPA3 (#AY371698) cDNA encoding Gα subunits Gpa2 and Gpa3 were also synthesized with primers: PW138 (5’-AAG GAT CAT GGG CCG GTG ACT GAG CAG AAA GAA GTG G-3’) and JH12500 (5’-CCA CTT CTT TCT CTC GCT TCT CAG TCC ACC GAC ATC GAA CAT ATG AAT G-3’), inserted into pGBKT7, and verified by sequencing.

GW138 (5’-AAG GAT CCC CAT GGG CGG GTG ACT GAG CAG AAA GAA GTG G-3’) and PW139 (5’-AAC TGC AGG GAT TAG AGA AGA CCG CAG TC-3’), and PW140 (5’-AAG GAT CCC CAT GGG CGG ATG TAT GTC
TTC G-3’) and PW141 (5’-AAC TGC AGT TAT AAG ATG GCC ATA TCT CTC-3’), cloned into pGBK7 at the BamHI-PstI sites respectively, and were verified by sequencing. The full-length GI2 ORF encoding Gib2 was identified from the genome of the prototypic strain H99 (http://cne0.genetics.duke.edu/) using the cDNA clone as trace and was obtained by PCR amplification with primers: PW168 (5’-AAG AAT TCA TGG CCG AGC ACC TCG TCA TCA-3’) and PW169 (5’-AAC TGC AGC TAA GCA ACG ACA GCC CAG ACT-3’). Transcription initiation sites were determined by 5’-RACE (Invitrogen). GI2 cDNA was also cloned into pGAD424 at the BamHI-PstI sites and pGADT7 at the BamHI site to create plasmids pGAD424-GI2 and pGADT7-GI2. pGBKT7-GI2, pGADT7-GPA1, and pGADT7-GPB1 were constructed similarly.

Two putative Gγ subunits, Gpg1 (Genbank #AY907677) and Gpg2 (AY907678), were identified from the H99 genome and cDNA was amplified with primers: PW237 (5’-AAG AAT TCA TGT CCA TAC GCA CAA CAA AGG-3’) and PW238 (5’-GAC TGC AGT CAC ATG ACG GAA CAG CAG ACA GCT-3’), and PW241 (5’-AAG AAT TCA TGT CCC ATC TCG CCC CCG CTT-3’) and PW242 (5’-GAC TGC AGC TAC ATG ATG GTG CAG CAC CCA G-3’). cDNA was then cloned into pGBK7 at the EcoRI-PstI sites to create pGBK7-GPG1 and pGBK7-GPG2. Standard yeast transformation protocols were performed (Clontech). The β-galactosidase (β-Gal) activity was also measured as described previously (38).

pGBK7-GI2 was used to screen the H99 cDNA library by the same method described above. Full-length SMG1 cDNA was identified from the H99 database using positive cDNA as trace and amplified by PCR with primers: PW357 (5’-AAG AAT TCA TGG TTC ACG CTG CTA CTC ACC CC-3’) and PW358 (5’-AAG GAT CCT TAC TTT TCT TTG TAA AGG TC-3’). The sequence was cloned into pGADT7 at EcoRI-PstI sites, creating pGADT7-SMG1.

Protein structures modeling- The protein-structure modeling tool SWISS-MODEL (http://swissmodel.expasy.org) was used to model the structures of Gpb1 and Gib2. Gpb1 and the partial Gib2-1 clone containing five C terminus WD-repeats were analyzed through the “First Approach Mode” using bovine Gβ as the model template (39), whereas the full-length Gib2 was analyzed by comparative modeling using Schizosaccharomyces pombe Gt5 (Genbank #AAD09020) and Candida albicans Tup1 (#AAB63195) as additional templates (40-42). Protein structures were colored according to B-factor and displayed through the SWISS-PDB viewer, DeepView (http://www.expasy.org/spdbv) (43-45).

Disruption of the GI2 gene-Because of sequence divergence between serotype A and D strains, two serotype-specific gib2::URA5 gene knockout alleles were constructed by a two-step process. For serotype A, a 1.6 kb region encompassing the GI2 gene was first amplified from H99 by PCR into two partially overlapping fragments that were each approximately 0.8 kb in length. The first fragment was amplified using primers: PW166 (5’-TTT GTG CTT TCT TTC ACT GTT C-3’) and PW226 (5’-GGA GAT CTA GTG CTA TGG TTC TCA CCC CTC T-3’), and the second fragment was amplified using primers: PW225 (5’-GGG TGG AAG CAG ACC CGG GAT GAG ACC AAC TTT TTC TCA CCC CTC T-3’), and PW241 (5’-AAG AAT TCA TGG CCC ATC TCG CCC CCG CTT-3’) and PW242 (5’-GAC TGC AGC TAC ATG AGC GAA CAG CAG ACA GCT-3’). cDNA was then cloned into pGBK7 at the EcoRI-PstI sites to create pGBK7-GPG1 and pGBK7-GPG2. Standard yeast transformation protocols were performed (Clontech). The β-galactosidase (β-Gal) activity was also measured as described previously (38).

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All resulting transformants were screened by PCR. Heterozygous RAS009 transformants (gib2/GIB2) were verified by Southern hybridization analysis before subjecting to ploidy reduction. The smg1::URA5 knockout allele was generated by overlapping PCR (47) and was used to generate smg1::URA5 mutant strains by transformation of both F99 and F99α strains.

Over-expression and suppression of Gib2 - The GIB2 gene was inserted into the pCnTel1 vector containing the C. neoformans galactose inducible GAL7 promoter (48) at the EcoRI site, and the resulting construct (sense orientation) was introduced into JEC43, JEC34, and BAC20-1 (gpa1 ura-) strains by biolistic transformation. The BAC20-1 strain was also transformed with the GAL7-GIB2 construct in which the orientation of GIB2 was reversed (antisense construct). Transformants were verified by PCR, diluted serially, and spotted on YPD, YNB, and YNB containing either 2% galactose or 2% glucose. Cells were grown on filament agar to promote conjugation tube formation and on Niger seed agar for melanin pigmentation. Cells grown on Niger seed medium were also collected and stained with India ink to observe the capsule. RNA and protein were extracted from cells first grown overnight in YPD and then re-suspended in minimal YNB medium containing 2% galactose or 2% glucose for up to four hours (36). RNA and protein were also extracted from strains grown continually in YNB containing 2% galactose, or otherwise as stated. The GIB2 transcript and Gib2 protein were detected by Northern and Western blot analysis accordingly (36).

A Gib2 specific oligopeptide, PW3 (N’-CPDFDGLSDKARKPE-C’), was synthesized and the affinity-purified Gib2-specific antibody was obtained through a commercial source (GenScript Corp., Piscataway, NJ).

Gib2 essentiality assessment - A serotype D GIB2/gib2::URA5 heterozygous transformant, #65, was incubated on 10% V8 mating agar at room temperature to allow basidiospore production and reversion into the haploid state through meiosis (35). After ten days of incubation, basidiospores were dissected using a micromanipulator-equipped microscope (Zeiss Axioskop 40 Tetrad) following published method (35). Surviving basidiospores were screened for the presence of the GIB2 gene using primers PW166 and PW167. Mating types were determined by genetic cross and verified by PCR with mating type-specific STE20α primers: PW394 (5’- GTG TCT CTG GAG GAC ATA CAA-3’) and PW395 (5’-CAC TAT CAA ACG ATG GCC GAA CA-3’), and STE20α primers: PW360 (5’-AAA TGG CTT TCA ATG GGT CAT CTC TC-3’) and PW361 (5’-AAA AGA AGG TGG ATT AGA TAG ATG AT-3’) (34).

Protein “pull-down” assays - The Gpa1 protein was expressed by an in-vitro transcription and translation kit using the TnT T7 coupled reticulocyte lysate system (Promega). The Gib2 protein was produced in E. coli (Rosetta DE3, Novagen) as a glutathione-S-transferase (GST) fusion protein using pET-41a (+) also from Novagen. Expression of the GST fusion protein was induced for 5 hrs at room temperature and was extracted in lysis buffer (50 mM HEPES pH 7.6, 150 mM NaCl, 10 mM MgCl₂, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 25 mM β-glycerophosphate, 100 µM Na₃VO₄, and protease inhibitor cocktail tablets [Roche] and 0.5 mM PMSF) as described (36, 49). The Gpa1 protein (10 µl) was conditioned in lysis buffer with 10 µM GDP for 1 hr at room temperature. Bacterial extracts containing GST-Gib2 were mixed with glutathione-sepharose resin (Pierce) for 1.5 hrs at 4°C. The gel was precipitated, washed three times with lysis buffer, and bound proteins were analyzed by SDS-PAGE and Western blotting using the anti-c-Myc peroxidase-conjugates (Roche, #1814150) and anti-GST antibody (Santa Cruz, SC-138).

The GPG2 gene was inserted in pET41a and the GST-Gpg2 fusion protein was expressed similarly as above. Gib2 was also expressed in S. cerevisiae using the pYES2NT vector (Invitrogen). The pYES2NT-GIB2 plasmid was transformed into the yeast strain...
RESULTS

Identification of Gib2-The completed genome of *C. neoformans* var. *neoformans* has confirmed the presence of three Ga subunits in *C. neoformans*: Gpa1, Gpa2, and Gpa3. While Gpa1 is known to function in a conserved cAMP signaling pathway, functions of Gpa2 and Gpa3 remain unreported. We have recently identified a regulator of G protein signaling (RGS) protein, Crg1, as one of the key regulators for mating and virulence in *C. neoformans* (51,52) and have shown that Crg1 functions as a GTPase activating protein (GAP) specific to Gpa2 (Li and Wang, unpublished observation). To expand our studies on regulators of G protein signaling, we performed a yeast two-hybrid screen using Gpa1 as bait.

Among 12 positive clones out of 1x10^8 yeast transformants, a single clone was found to contain, in pGADT7-REC, an insert of 743 nt predicted to encode a peptide of 227 amino acids (Genbank #AY954369). This clone, designated as *GIB2-1* for Gpa1-interacting-β, allowed interaction-dependent growth of the host strain AH109 only in the presence of Gpa1, but not Gpa2 or Gpa3 (Fig. 1A). Examination of the peptide sequence encoded by *GIB2-1* revealed five WD-repeats, and using the sequence to search *C. neoformans* sequence databases ([http://www.tigr.org/](http://www.tigr.org/) and [http://cneo.genetics.duke.edu/](http://cneo.genetics.duke.edu/)), an open reading frame (ORF) containing an additional 87 N-terminal amino acids, for a total of 314 amino acids, was identified. The deduced amino acid sequence of Gib2 contains seven WD-40 repeats and shares 78% amino acid identity with the Gβ subunit Gβ1 of Shiitake mushroom *Lentinula edodes* (AAP13580), 72% identity with *Neurospora crassa* Cpc2 protein (CAA57460), and 70% identity with mammalian Gβ-like/RACK1 protein GNB2L1 (#AAH32006). In comparison, Gib2 shares 25% amino acid sequence identity with Gpb1 and 27% with *S. p. Git5* (Supplementary Fig. 1). Apparently, Gib2 is more related to Gβ-like/RACK1 proteins than Gβ subunit proteins (Fig. 2).

We named the full-length gene *GIB2*. A single copy of the *GIB2* gene was found in both INVS.c1 (Invitrogen), transformants were selected on SD-ura agar plates, and grown in liquid SD-Ura medium containing 2% glucose for 24 hrs at 30°C. Cells were precipitated, washed with induction medium (SD-ura medium containing 2% galactose and 1% raffinose), and grown in the same medium overnight in a 30°C shaker, cells were then collected and stored at -80°C. To extract proteins, cells were resuspended in lysis buffer (50 mM Tris, pH7.5, 150 mM NaCl, 0.5% Nonidet P-40, and a protease inhibitor cocktail (Roche)) and homogenized in a Fast-Prep cell disrupter (Bio101) six times for 40 sec at 4°C, with an equal volume of acid-washed glass beads. Homogenized samples were centrifuged at 13,000 rpm for 30 min at 4°C, and supernatants were recovered. For protein purification, cell extracts were incubated with Co^2+ affinity resins (TALON, Clontech) for 1.5 hrs, washed, and proteins eluted with buffer containing 200mM imidazole. Proteins were then concentrated, buffer-exchanged using a centrifugal filter device (Millipore), and concentration determined by BCA protein assay (Pierce). The pull-down assay was carried out similarly as above (Gpa1-Gib2) in binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl\_2 0.2 % NP-40, 1 mM EDTA, and 1 mM PMSF). After precipitation, resin was washed five times and bound proteins were analyzed by SDS-PAGE and Western blotting using the anti-Gib2 or anti-GST antibodies.

The “pull-down” assay between the GST-Gib2 fusion protein and *C. neoformans* Pkc1 was carried out also similarly in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na\_3VO\_4, 1 mM NaF, and the protease inhibitor cocktail and 0.5 mM PMSF, with or without the PKC activator 200 mM 1-oleoyl-2-acetyl-sn-glycerol (OAG). The Pkc1 protein was expressed and affinity-purified from a yeast strain (KD9-3α) containing the pYES2/Gal1::Xpress::*PKC1* construct (50). The anti-Xpress-HRP conjugate was purchased from Invitrogen (R911-25).
serotype A (Genbank #AY907679) and D (#AY907680) strains that encodes the identical protein.

To confirm the Gib2-Gpa1 interaction, GIB2 cDNA was switched into the BD vector pGBT9 and co-transformed with pGAD424-GPA1. Only yeast cells carrying both genes were able to grow in SD medium lacking Leu, Trp, His, and Ade, plus 3-amino trizole (3-AT) that was added to suppress leaky HIS3 expression of the reporter strain (Fig. 1B). A GPA1Q284L mutant allele encoding a constitutively activated Gpa1 (30) was also tested for interaction with Gib2. A weak interaction was still observed in yeast cells expressing Gpa1Q284L and Gib2, as growth can occur on selective medium in the absence of 3-AT (data not shown).

To test Gib2-Gpa1 interaction in vitro, a protein ‘pull-down’ assay was performed. The c-Myc tagged Gpa1 (~48 kDa), produced by in vitro transcription and translation, was “pulled-down” by the GST-Gib2 fusion protein (~60.5 kDa) (Fig. 1C, left panel), but not by the GST protein alone (~26 kDa) (Fig. 1C, right panel).

Gib2 is constitutively expressed in serotype A cells grown in YPD, however, higher levels of expression were observed when cultures were switched to nutrient-limiting YNB minimal medium (Fig. 1D).

Gib2 binds to Gγ subunits Gpg1 and Gpg2- Conventional Gβ subunits exhibit a high affinity for Gγ subunits and function as Gβγ heterodimers to bind and stabilize GDP bound Gα subunits. In addition, a Gβ can associate with multiple individual Gγ subunits (4). To test whether Gib2 could physically associate with a Gγ subunit(s), two Gγ homologs were identified from C. neoformans by using the amino acid sequence of the mushroom fungus Lentinula edodes Gγ subunit homolog Gγ1 (Genbank #Q870G5) to search the H99 database. Two open reading frames each encoding a putative Gγ subunit were obtained and named Gpg1 (Genbank #AY907677) and Gpg2 (Genbank #AY907678). Gpg1 consists of 81 amino acids and shares 62% amino acid identity with L. edodes Gγ1, whereas Gpg2 consists of 87 amino acids and shares 59% sequence identity with Gγ1 (Supplementary Fig. 2). In comparison, Gpg1 and Gpg2 are less similar to S. cerevisiae Ste18 (28% and 30% identity, respectively) (Supplementary Fig. 2). Both Gpg1 and Gpg2 exhibit common characteristics of Gγ subunits, short peptides with the C-terminal CAAX (C=cysteine, A=aliphatic, X=cysteine, methionine, serine, etc) motif marked for protein prenylation, which is necessary for membrane association and Gγ function (54).

cDNA for GPG1 and GPG2 was obtained and inserted into pGBK7, creating pGBK7-GPG1 and pGBK7-GPG2. Using the yeast two-hybrid assay, both Gpg1 and Gpg2 were found to interact with Gib2 (Fig. 4A), suggesting that Gib2 forms heterodimers with Gpg1 or Gpg2.
The *gpa1* mutant strain is defective in cAMP signaling and mutant cells are unable to produce melanin and capsule. To determine whether Gib2 has a role in cAMP signaling, the *gpa1* cells with the *GAL7-GIB2* fusion gene construct were placed on melanin-inducing Niger seed agar. On medium containing 0.1% galactose, cells over-expressing Gib2 partially restored the production of melanin (Fig. 5B). The result is similar to that when cells were grown on medium supplemented with exogenous cAMP (5 mM final concentration, Fig. 5B). Intriguingly, colonies overexpressing Gib2 also exhibited a mucoid appearance indicating a restoration in capsule formation (Fig. 5B). Indeed, cells collected from the medium containing galactose showed capsules similar to or even larger than those of the wild type strain (Fig. 5C). No production of melanin or capsule was observed in the control *gpa1* mutant strain (Fig. 5B and 5C).

Consistent with the observation that supports a positive regulatory role of Gib2 in cAMP signaling, the levels of intracellular cAMP in the Gib2 over-expression strain were similar to that of the wild type strain, again in sharp contrast to the *gpa1* mutant strain (Fig. 5D).

Similarly, Gpg1 and Gpg2 interacted with Gpb1 when expressed, respectively, as pGBK7-*GPG1*, pGBK7-*GPG2*, and pGADT7-*GPB1*. Previously, such an interaction could not be detected when proteins were expressed using the low-level expression plasmids pGBT9 and pGAD424 (data not shown).

A β-galactosidase activity assay was performed to quantify binding between Gib2 and Gpg1/Gpg2, and also between Gpb1 and Gpg1/Gpg2. Gib2 exhibited the higher affinity for Gpg2 (106 ± 10 Miller units) than for Gpg1 (48 ± 9 Miller units), while binding affinities between Gpb1 and Gpg1/Gpg2 were 50 ± 0 and 39 ± 1 Miller units, respectively (Fig. 4B).

A protein “pull-down” assay was performed to verify the interaction between Gib2 and Gpg2. Gib2 was expressed as a bacterial GST-Gpg2 fusion protein and Gib2 was expressed in yeast as a His-tagged protein that was purified through immobilized metal affinity chromatography. Consistently, Gib2 bound to Gpg2 after five times of stringent buffer washes (Fig. 4C). No binding was detected between GST and Gib2 (Fig. 4C).

**Gib2 positively regulates cAMP signaling**—Previously, Gpb1 was found to play a positive role in mating and haploid differentiation, since the *gpb1* mutant strain was sterile and pheromone-induced conjugation tube formation, a response leading to mating, was diminished (9). In addition, over-production of Gpb1 induced conjugation tube formation in the absence of pheromone stimulation (9). A similar approach was used to test Gib2 function by fusing *GIB2* with the galactose inducible *GAL7* promoter (48) and by introducing the *GAL7-GIB2* fusion gene into JEC34 and JEC43 strains, as well as the BAC20-1 strain. When grown in filament agar medium containing 0.5% galactose, no conjugation tube formation was observed in strains transformed with the *GAL7-GIB2* construct (Fig. 5A, two left panels), in contrast to the same strain over-expressing Gpb1 (Fig. 5A, far right panel).

The *gpa1* mutant strain is defective in cAMP signaling and mutant cells are unable to produce melanin and capsule. To determine whether Gib2 has a role in cAMP signaling, the *gpa1* cells with the *GAL7-GIB2* fusion gene construct were placed on melanin-inducing Niger seed agar. On medium containing 0.1% galactose, cells over-expressing Gib2 partially restored the production of melanin (Fig. 5B). The result is similar to that when cells were grown on medium supplemented with exogenous cAMP (5 mM final concentration, Fig. 5B). Intriguingly, colonies overexpressing Gib2 also exhibited a mucoid appearance indicating a restoration in capsule formation (Fig. 5B). Indeed, cells collected from the medium containing galactose showed capsules similar to or even larger than those of the wild type strain (Fig. 5C). No production of melanin or capsule was observed in the control *gpa1* mutant strain (Fig. 5B and 5C).

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**Gib2 encodes an essential function**—To further dissect the function of Gib2, we employed an antisense suppression approach by transforming strains with a *GIB2* antisense construct driven by the same *GAL7* promoter. Modulation of gene expression by antisense suppression (or repression) occurs at the level of mRNA rather than at the level of DNA and this approach has been successfully employed in this organism previously (55). Suppression of *GIB2*, instead of gene disruption, provided us with an alternate experimental approach to assess Gib2 functions. In general, transformation of the BAC20-1 (*gpa1 ura-*) strain with the *GIB2* antisense construct yielded few transformants that were characterized by slow growth. The deleterious effect of Gib2 suppression was apparent in assays for melanin, capsule and cAMP (Fig. 5B, 5C, and 5D). Furthermore, the strains with the antisense construct exhibited a severe growth defect when cultured in YNB containing 2% galactose (Fig. 6A, right panel), in contrast to growth on...
nutrient rich YPD (Fig. 6A, left panel). Due to the observation that YNB induces Gib2 expression, as well as the leaky nature of the GAL7 promoter, strains with the GIB2 antisense construct also exhibited a partial growth defect on YNB (Fig. 6A, middle panel).

Suppression of Gib2 is synthetically lethal, indicating that Gib2 encodes an essential function in cellular growth. To test this hypothesis, serotype-specific gib2::URA5 mutant alleles were introduced into F99, JEC34, and JEC43 strains. Previous studies have shown that the knockout efficiency via biolistic transformation in either serotypes is 2-40% (47). However, no knockout strains were recovered from a total of 247 transformants, suggesting that Gib2 is required for cellular viability.

To confirm that Gib2 is essential, the laboratory derived serotype D diploid strain RAS009 (35) was employed. First, the gib2::URA5 allele was used by biolistic transformation to obtain gib2::URA5/GIB2 heterozygous transformants. Four such heterozygous transformants were obtained out of 198 transformants. Once heterozygocity was verified by Southern blot hybridization analysis (Fig. 6B), the transformant #65 was allowed to undergo meiosis and sporulation. Basidiospores were micro-dissected and genotypes of surviving progenies were examined. Among seven segregants recovered (2 MATα and 5 MATα), all contained the wild type GIB2 gene, confirming that Gib2 is essential in serotype D strains (Fig. 6C).

Gib2 interacts with Smg1, a downstream target of Gpa1-cAMP signaling, and PKC-Our studies have provided evidence suggesting that Gib2 functions as an atypical Gβ subunit in Gpa1-cAMP signaling, and that it also encodes an essential function. We reasoned that an effector(s)/target(s) of Gib2 might provide additional clues for revealing Gib2’s true identity and have thus performed a yeast two-hybrid screen using Gib2 as bait. A screen of 10⁶ yeast transformants yielded several positive clones, including a peptide containing an Src homology-3 (SH3) domain and a peptide that encodes a glucose-methanol-choline (GMC) oxidoreductase domain (Genbank #AY954370). While the prospect of Gib2 binding to an SH3 domain would support Gib2 being a Gβ- and RACK1-like protein since the mammalian RACK1 is known to negatively regulate the activity of the Src kinase through binding, we chose to examine the clone containing the GMC domain, as it represents an internal region of Smg1 (Genbank #AF349949), a protein previously identified as a multi-copy suppressor of gpa1 mutation. Smg1 has been implicated as one of the downstream targets of the Gpa1-cAMP signaling pathway in C. neoformans (56,57). Physical association between Gib2 and the full-length Smg1 was also confirmed (Fig. 7A).

Whereas a chromosome translocation and segmental duplication event resulted in two SMG1 alleles in the serotype D strain JEC21 (58), only a single SMG1 allele was found in serotype A strains H99 and KN99a (data not shown). smg1::URA5 mutant strains of both mating types were generated to test the role of Smg1 in melanin production. Surprisingly, all five (three MATα and two MATα) smg1::URA5 mutants exhibited levels of melanization similar to that of the wild type strain (data not shown), bringing the role of Smg1 in melanin formation and virulence into question.

Since the mammalian RACK1 protein was originally identified and named for its ability to bind PKC, a protein “pull-down” assay was performed to test for the physical interaction between Gib2 and Pkc1. Pkc1 is a diacylglycerol activated C-kinase homolog that is a part of the inositol phosphoryl ceramide (Ipc1)-diacylglycerol (DAG)-Pkc1 signaling pathway and this pathway is linked to the production of the virulence factor melanin in C. neoformans (50). Consistently, Gib2 binds to Pkc1 in vitro and the binding occurred even without the presence of the common PKC activator OAG (Fig. 7B). It is not known whether the heterologously expressed Pkc1 protein is already partially activated or whether Gib2 can bind to Pkc1 regardless of its activation state. The conserved nature of the interaction between these two proteins could suggest that Gib2 encodes a RACK1-like function important in PKC signaling of C. neoformans.
DISCUSSION

Gib2 is an atypical Gβ-The discovery of multiple Ga subunits, but only a single Gβ subunit, suggests that G protein signaling is unique in fungi: either certain Gαs could function as monomeric proteins independent of Gβγ heterodimers, a single Gβ(γ) heterodimer could couple to multiple Gαs to transduce different signals, or certain proteins could substitute for functions of a conventional Gβ. While studies supporting the first two hypotheses are lacking, recent studies have shown that S. cerevisiae proteins Gpb1 and Gpb2 could couple to Gpa2 in cAMP signaling (49,59). The yeast Gpb1 and Gpb2 contain a seven-Kelch repeat motif mimicking the structure of the β transducin. No similar proteins have been found in C. neoformans (33) or any other fungi, however.

The C. neoformans cAMP signaling pathway consists of several conserved components such as Gpa1, Cac1, Aca1, Pka1, and Pkr1 (30-32,60). Recently, a GPCR homologue, Gpr4, has been found to activate Gpa1 by sensing ligands such as methionine (61), further highlighting the conserved mechanism of this pathway. Without a conventional Gβ subunit as a Gβγ heterodimer, how Gpa1 accomplishes such a signaling role remains elusive.

The observation that Gib2 binds to Gpa1 may enable us to elucidate the mechanism by which Gpa1 functions as a Ga. Several lines of evidence suggest that Gib2 functions as an atypical Gβ by coupling to Gpa1. First, Gib2 was identified by the virtue of its binding to Gpa1, which appeared to be specific, as Gib2 did not physically associate with Gpa2 or Gpa3. Second, the sequence of Gib2 is compatible to that of the β transducin in forming a seven-bladed propeller-like structure. Third, Gib2 bound to Gpg1 and Gpg2 in the yeast two-hybrid and protein “pull-down” (Gpg2) assays. Fourth, over-expression of Gib2 restored cAMP signaling by suppressing defects in melanin and capsule formation due to gpa1 mutation. Finally, Gib2 interacts with Smg1, a multicopy suppressor of gpa1 mutation and a presumptive down-stream target of cAMP signaling. Despite previous studies reporting that the mammalian RACK1 protein can bind to Ga(α(γ)βγ and Gβγ but not the Gα subunit alone (21-23), our study does support a model in which Gib2 binds directly to Gpa1 as a Gβ-like protein to likely stabilize Gpa1 and facilitate its activation and inactivation cycle, and to regulate cAMP signaling, in conjunction with Gpa1 (Fig. 8).

Gpg1 and Gpg2 are conserved Gγ subunits-Our studies have demonstrated that Gpg1 and Gpg2 are Gγ subunit homologs that bind to Gpb1 and Gib2 to form protein heterodimers. It has been suggested that the Gγ folds into two linked α-helical domains that interact with the Gβ (39). Indeed, a truncated form of Gpg1 retaining only the N-terminal 43 amino acid residues still exhibited binding with Gib2 (data not shown). Binding between Gpb1 and Gpg1/Gpg2 could not be established initially using the low protein expression plasmids pGAD424 and pGBT9, but were possible with plasmids pGADT7 and pGBK7 that allow a high level of protein expression.

Our studies have indicated that Gpg1 and Gpg2 could bind to both Gib2 and Gpb1, which is not surprising since a single Gγ could interact with various Gβ and different Gβγ combinations often confer different signal strength and specificity (62). It is interesting to note that mutant strains of gpg1 and gpg2 each exhibited distinct dysfunction in mating and that gpg1 gpg2 mutants were nonviable (Zhang and Wang, unpublished observation). On-going studies should provide further understanding for any signaling roles of Gpg1 and Gpg2 in mating and cAMP signaling.

Gib2 is a multi-functional protein-The S. cerevisiae Asc1/Cpc2 protein is a core 40S ribosomal protein that represses gene expression (24,25). The S. pombe Cpc2/RACK1 homolog is required for efficient translation, cell cycle progression, and meiotic development of the cell (63,64). The high sequence similarity shared between Gib2 and Asc1/Cpc2/RACK1 could suggest that Gib2 has functions similar to these proteins. Indeed, cells grown, although poorly, under the
condition for depletion of Gib2, appeared to have a higher protein content (data not shown), analogous to the asc1 null mutant strain (25).

Gib2 is shown to interact with Pkc1 and Smg1, and one of the positive two-hybrid clones also contains a SH3 domain that is known to interact with the RACK1 protein. In addition, Gib2 is a unique protein. Unlike RACK1 that binds to heterodimer Gβγ or heterotrimeric Gaβγ (21-23), Gib2 appears to bind to Gpa1 directly and it also positively regulates cAMP signaling. Finally, Gib2 appears to encode an essential function whereas Asc1/Cpc2/RACK1 does not.

We took into consideration the possibility that yeast Gβγ (Ste4/Ste18) might mediate the interaction between Gib2 and Gpa1 or Gib2 and Gpg1/Gpg2. In addition to protein “pull-down” assays indicating direct bindings, we also reasoned that if Ste4 mediated the binding, Gpa3 or Gpa2 would be identifiable from the yeast screen, not Gpa1, since Gpa3 shared the highest sequence homology with the yeast Gpa1 (40%) that form a heterotrimeric G protein with Ste4 and Ste18, and Gpa2 that is functionally analogous to yeast Gpa1 couples to Gpb1 in mating (Li and Wang, unpublished data). Finally, we failed to demonstrate any direct interactions between Ste4 and Gib2 using the yeast assay (data not shown).

Gib2 is involved in fungal virulence—Gib2 plays a positive role in cAMP signaling of C. neoformans, in contrast to S. cerevisiae Gpb1 and Gpb2 that play a negative role. Gpb1 and Gpb2 were suggested to have a target(s) either upstream of the cAMP-dependent protein kinase (PKA) signaling pathway or downstream of a pathway that requires PKA for function (49,65). The mammalian RACK1 has been shown to modulate the level of cAMP through its interaction with the phosphodiesterase Pde4D5 (20) via a N-terminal RACK1-interacting-domain (RAID1); however, yeast phosphodiesterases, including C. neoformans var. grubii Pde1 and Pde2 homologs (66), do not appear to contain the unique N-terminal RAID1 region. Interestingly, a phosphodiesterase ORF (Cnb01170) with a putative RAID1 was identified from C. neoformans var. neoformans whose study may reveal the mechanism by which over-expressing Gib2 increased the level of cAMP in BAC20-1, a var. neoformans gpa1 mutant strain.

To find a target(s) of Gib2, we have resorted to the interaction cloning strategy and have identified Smg1. This is rather intriguing because Smg1 was previously identified as a multi-copy suppressor of gpa1 mutation for melanin deficiency and is implicated in melanin formation and virulence (56,57). Results of our gene disruption studies have contradicted this proposition, since smg1 mutant strains did not exhibit any altered levels of melanin pigmentation. It is feasible, however, to speculate that Smg1 may play certain role in melanin biosynthesis and that Gib2 could recruit additional targets to modulate the Gpa1-cAMP signaling pathway that affects melanin production. Conversely, the interaction between Gib2 and Smg1 could be mediated through PKC signaling, independent of the Gpa1-cAMP pathway, as Pkc1 has been demonstrated to also play a regulatory role in melanin formation and virulence (50). Therefore, further examination of interactions between these proteins and their resulting biological significance would provide meaningful insights into functions of Gib2, either as an atypical Gβ or a RACK1 protein, in virulence.

Taken together, our studies have identified the novel Gβ-like/RACK1 protein homolog Gib2 that functions as an atypical Gβ in cAMP signaling, in conjunction with Gpa1. Additionally, Gib2 is a multi-functional protein that encodes essential functions for survival. Our studies suggest that fungal Gβ-like/RACK1 protein homologs may have diverged from their mammalian counterparts in evolution by adapting a role as an atypical Gβ in G protein signaling while maintaining a RACK1-like function. Given the remarkable conservation in signaling mechanisms among fungal and other eukaryotic organisms, similar proteins may also exist in those systems that constitute unique signaling networks controlling cellular growth, differentiation, and/or virulence.
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**FOOTNOTES**

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1The abbreviations used are: RACK1, receptor for activated C-kinase; PKC, activated C-kinase; CPC, cross-pathway-control; X-α-Gal, 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside; GST, glutathione-S-transferase; PMSF, phenyl-methyl-sulphonyl-fluoride; RGS, regulator of G protein signaling; GAP, GTPase activating protein; SH3, Src homology-3; GMC, glucose methanol choline; OAG, 1-oleoyl-2-acetyl-sn-glycerol; kb, kilobases; kDa, kilodaltons.

2D.A.P. and J.K.T. contributed equally to this work.

**FIGURE LEGENDS**

**Fig. 1.** *C. neoformans* Gib2 couples to Gpa1. A, Two-hybrid assay showing the interaction between Gib2-1 and Gpa1. The DNA-binding domain (BD) vector pGADT7-REC has an insert of *GIB2-1* encoding the C-terminal partial sequence and the construct was recovered from the original yeast screen. *GPA1, GPA2,* and *GPA3* were cloned into the DNA-binding domain (BD) vector pGBK7T7. The AD and BD plasmids were co-transformed into AH109 and yeast transformants were patched on SD-Leu-Trp for plasmid-dependent growth (three days) and transferred to SD-Leu-Trp-His-Ade plus 3-AT for protein interaction-dependent growth (three days). B, Two-hybrid assay confirming the positive interaction between Gib2 and Gpa1. The full-length cDNA (*GIB2*) was cloned into pGBK7T7 and *GPA1* cDNA was cloned into pGAD424. C, Gib2 interacts with Gpa1 in vitro. The Gib2-GST fusion and GST proteins were absorbed to glutathione gel, washed, and mixed with the Gpa1 protein. Bound proteins were separated by SDS-PAGE in duplicate and analyzed by Western blotting. Blotted membranes were incubated with either anti-c-Myc-peroxidase conjugate (left panel) or anti-GST monoclonal antibody (right panel). D, Gib2 is constitutively expressed in *C. neoformans* serotype A strains and high levels can be induced by switching cells to nutrient-limiting media such as YNB. The Gib2 protein is detected using an affinity purified Gib2 specific antibody as described in text.

**Fig. 2.** Phylogenetic analysis of Gib2, Gβ and Gβ-like/RACK1 proteins. Amino acid sequences were aligned with a ClustalW multiple sequence alignment program (MAFFT version 5.8) with the gap open penalty set at 1.53 and the phylogenetic tree was constructed with bootstrap values shown. Organism sources and NCBI accession numbers are *L. edodes* Gβ1 (AAP13580), *C. neoformans* Gib2 (AY907679), *D. melanogaster* RACK1 (NP_477269), human GNB2L1.
Fig. 3. Gib2 is shown through modeling to show a seven-bladed β-propeller structure compatible to those of β transducins and Gpb1. Ribbon diagrams of Gib2-1 (#AY954369), Gib2 (#AY907679), and Gpb1 (#AAD03596) are shown with WD-40 repeats numbered as β1 through β7 (β3-β7 for Gib2-1). Structures of Gpb1 and Gib2-1 were modeled via SWISS-MODEL by First Approach Mode, colored according to B-factor, and visualized through the SWISS PDB viewer “DeepView”. The structure of Gib2 was made by comparative modeling based on those of Gib2-1, Git5, and Tup1.

Fig. 4. Gib2 associates with Gγ proteins Gpg1 and Gpg2. A, Gpg1 and Gpg2 interact with Gib2 in a yeast two-hybrid assay. Gib2 cDNA was cloned into pGADT7 (AD) and GPG1 and GPG2 cDNA was cloned into pGBK7 (BD). Yeast transformation, selection of positive interaction transformants, and incubation conditions were the same as described in Fig. 1. B, β-galactosidase activities were measured to quantify bindings between Gib2 and Gγ-like proteins and Gγ subunits. The assay was performed in duplicate and data were expressed as the mean ± SE of two independent experiments. C, Gib2 interacts with Gpg2 in vitro. Gib2 was expressed in yeast and purified by chromatography. Gpg2 was expressed as a GST fusion protein in bacteria. Bound proteins were analyzed with the anti-Gib2 antibody (top panel). GST and GST-Gpg2 were visualized using the anti-GST monoclonal antibody (lower panel). Gib2 protein input (20 µl) is shown in the first lane (top panel), whereas 300 µl purified proteins were used in each immunoprecipitation experiment.

Fig. 5. Gib2 regulates cAMP signaling. A, Gib2 does not play a role in pheromone responsive mating. JEC34 transformants containing GAL7-GPB1 and GAL7-GIB2 constructs were patched on filament agar containing either 0.5% galactose or 0.5% glucose to induce or suppress the expression of Gpb1 or Gib2. Plates were incubated at room temperature (25°C) for two days before being photographed (25x magnification). B, Over-expression of Gib2 suppresses melanization defect of gpa1 mutation. The gpa1 ura- strain was transformed with forward (sense) and reverse (antisense) GAL7-GIB2 constructs respectively. Cells were spotted on Niger seed agar containing 0.1% glucose (Suppression), 0.1% galactose plus 0.1% raffinose (Induction), or 0.1% glucose with cAMP. Plates were incubated at 30°C for three days before being photographed. C, Over-expression of Gib2 suppresses the capsule defect of gpa1 mutation. Cells were collected from colonies on Niger seed agar with galactose (shown in Fig. 4B, above), washed in sterile water, and stained with India ink. Imagines were documented using an Olympus microscope (BX51) equipped with a digital camera. Scales of magnification are as indicated. D, Gib2 positively regulates intracellular cAMP. Cells were grown in YNB (with 0.2% galactose) overnight before being subjected to glucose starvation. Because of poor growth in medium containing galactose, 0.2% raffinose was added to cells with the GAL7-GIB2 antisense construct to improve growth. Each sample was assayed in quadruplets and mean values were plotted.

Fig. 6. Gib2 is essential in serotype D strains. A, The BAC20-1 transformant strains with sense and antisense GAL7-GIB2 constructs were serially diluted and spotted onto YPD, YNB, and YNB with 2% galactose. Plates were incubated at 30°C for two days. B, Southern blot hybridization indicates the presence of a heterozygous GIB2/gib2::URA5 allele in diploid transformant #65. Genomic DNA was digested with XbaI and XmnI, and the full length GIB2 was used as the probe. The presence of the wild type GIB2 gene is indicated by a 1.6 kb DNA fragment, whereas the mutant gib2::URA5 allele is indicated by a 3.5 kb fragment. Lane 1, wild type JEC21; 2, wild
type diploid RAS009; 3, heterozygous diploid #65. C, Genotype analysis of basidiospore progenies. Lane 1, DNA ladder; 2, RAS009; 3, JEC20; 4, JEC21; 5-9, MATa progenies; 10-11, MATα progenies. The wild type GIB2 gene is indicated by a 1.6 kb fragment (upper panel), whereas STE20α is represented by a 0.5 kb fragment (middle panel) and STE20α by a 1.0 kb fragment (lower panel).

Fig. 7. Gib2 interacts with multiple proteins. A, Smg1 was identified from a yeast two-hybrid screen using Gib2 as bait. pGADT7-REC-SMG1-1 was recovered from a positive yeast culture and full-length SMG1 cDNA was cloned into pGADT7. Yeast transformation, selection of transformants, and incubation conditions were the same as described in Figure 1, except that the plate was incubated 5 days for interaction-dependent growth. B, In vitro GST “pull-down” assay showing the interaction between Gib2 and Pkc1. GST and GST-Gib2 fusion proteins were absorbed to glutathione gel, washed, and mixed with Pkc1. Bound proteins were separated by SDS-PAGE and filter membranes were blotted either with an anti-Xpress antibody (upper panel) or an anti-GST antibody (lower panel).

Fig. 8. Two conserved G protein signal transduction pathways functioning in C. neoformans. Crg1 and Gpb1 are two key regulators of the pheromone-responsive mating pathway, whereas Gpa1 and Gib2 in conjunction govern the cAMP-signaling pathway to regulate the production of two key virulence factors melanin and capsule formation. Gpa2 is the cognate Gα for Gpb1 (Lei et al., unpublished data), whereas Gpr4 is a G protein coupled receptor homolog recently implicated in Gpa1-cAMP signaling (61).
Figure 1 (Palmer et al.)

A.  

| AD | Gib2-1 |
|----|--------|
| BD |        |
| Gpa1 |    |
| Gpa2 |    |
| Gpa3 |    |

SD-Leu-Trp  SD-Leu-Trp-His-Ade (1mM 3-AT)

B.  

| AD | Gpa1 |
|----|------|
| BD |      |
| Gib2 |   |

SD-Leu-Trp  SD-Leu-Trp-His-Ade (2mM 3-AT)

C.  

Gpa1  +  -  +  +  -  +  
GST  +  -  -  +  -  -  
GST-Gib2  -  +  +  -  +  +  

cMyc-Gpa1  →
GST-Gib2  ↓
GST  ↓

D.  

(kDa)  0  30'  1h  2h  4h  0  30'  1h  2h  4h

WT  gpa1
Figure 3 (Palmer et al.)
Figure 4 (Palmer et al.)

A.

AD Gib2
BD
Gpg1
Gpg2
SD-Leu-Trp
SD-Leu-Trp-His-Ade (1mM 3-AT)

B.

β-gal activity (Miller units)

C.

+  +  +  Gib2
-  +  GST
+  -  GST-Gpg2

GST-Gpg2  →  Gib2

Gib2     GST-Gpg2

GSP-Gpg2  →  GST

Gpa1  Gpg1  Gpg2  Gpg1  Gpg2

20  48  106  50  39
A. 

\( \text{P}_{\text{Gal7}} \)-GIB2 \hspace{1cm} \text{P}_{\text{Gal7}} \)-GPB1

Glucose \hspace{1cm} \text{Galactose} \hspace{1cm} \text{Glucose} \hspace{1cm} \text{Galactose}

B. 

WT \hspace{0.5cm} \text{gpa1} \hspace{0.5cm} \text{gpa1}+ \text{P}_{\text{Gal7}} \)-GIB2

\#3 \hspace{0.2cm} \#4 \hspace{0.2cm} \#1 \hspace{0.2cm} \#2

0.1% Glucose

0.1% Galactose

5 mM cAMP

forward \hspace{0.5cm} reverse

C.

WT \hspace{1cm} \text{gpa1} \hspace{1cm} \text{gpa1}+ \text{P}_{\text{Gal7}} \)-GIB2

\#3 \hspace{0.2cm} \#4 \hspace{0.2cm} \#1 \hspace{0.2cm} \#2

10 \mu m \hspace{0.5cm} 10 \mu m \hspace{0.5cm} 10 \mu m \hspace{0.5cm} 10 \mu m

forward \hspace{0.5cm} reverse

D. 

\text{cAMP (fmol/6x10^5 cells)}

\text{WT} \hspace{1cm} \text{gpa1} \hspace{1cm} \text{forward #3} \hspace{1cm} \text{reverse #1}

Time (minutes)

0 \hspace{0.5cm} 0.5 \hspace{0.5cm} 1 \hspace{0.5cm} 2 \hspace{0.5cm} 3
Figure 6 (Palmer et al.)

A. YPD YNB (Glu) YNB (Gal)

WT

\[ P_{\text{Gal7}} \text{-GIB2 for. #3} \]

\[ P_{\text{Gal7}} \text{-GIB2 for. #4} \]

\[ P_{\text{Gal7}} \text{-GIB2 rev. #1} \]

\[ P_{\text{Gal7}} \text{-GIB2 rev. #2} \]

B. JEC21

\[ \text{a progeny} \]

\[ \alpha \text{ progeny} \]

\[ \text{gib2::URA5} \]

\[ \text{GIB2} \]

C. Marker

\[ \text{RAS009} \]

\[ \text{JEC20} \]

\[ \text{JEC21} \]

\[ \text{s19} \]

\[ \text{s20} \]

\[ \text{s21} \]

\[ \text{s22} \]

\[ \text{s38} \]

\[ \text{s35} \]

\[ \text{s30} \]

\[ \text{GIB2} \]

\[ \text{STE20} \alpha \]

\[ \text{STE20} \alpha \]
Figure 7 (Palmer et al.)

A.  

|        | AD Smg1-1 | Smg1 | AD Smg1-1 | Smg1 |
|--------|-----------|------|-----------|------|
| BD     |           |      |           |      |
| Gib2   |           |      |           |      |
|        | SD-Leu-Trp|      | SD-Leu-Trp-His-Ade (1 mM 3-AT) |  

B.  

|        | OAG | GST | Xp-Pkc1 | GST-Gib2 |
|--------|-----|-----|---------|----------|
|        | -   | +   | +       | +        |

- Pkc1
- GST-Gib2
Figure 8 (Palmer et al.)

Mf$\alpha$/$\alpha$ Gpb1 ($\beta$)
Gpa2 ($\alpha$) Gpg1, 2 ($\gamma$)
Ste3, Cpr2

nutrients
Gpr4

Gpa1 ($\alpha$) Gib2 Gpg1, 2 ($\gamma$)

Crg1
Gpa2 ($\alpha$) Gpb1 ($\beta$)
Ste20$\alpha$/$\alpha$

MAP kinases

Ste12$\alpha$/$\alpha$

Mating, melanin, capsule, and virulence

Ste20$\alpha$/$\alpha$

Cac1
cAMP

Pka1

Gpa1 ($\alpha$) Gpg1, 2 ($\gamma$)
Gib2, a novel G\(\beta\)-like/RACK1 homolog, functions as a G\(\beta\) subunit in cAMP signaling and is essential in Cryptococcus neoformans
Daniel A Palmer, Jill K Thompson, Lie Li, Ashton Prat and Ping Wang

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