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G-Protein Signaling Mediates Asexual Development at 25°C but Has No Effect on Yeast-Like Growth at 37°C in the Dimorphic Fungus *Penicillium marneffei*

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The ascomycete *Penicillium marneffei* is an opportunistic human pathogen exhibiting a temperature-dependent dimorphic switch. At 25°C, *P. marneffei* grows as filamentous multinucleate hyphae and undergoes asexual development, producing uninucleate spores. At 37°C, it forms uninucleate yeast cells which divide by fission. We have cloned a gene encoding a Gα subunit of a heterotrimeric G protein from *P. marneffei* named *gasA* with high similarity to *fadA* in *Aspergillus nidulans*. Through the characterization of a Δ*gasA* strain and mutants carrying a dominant activating or a dominant interfering *gasA* allele, we show that GasA is a key regulator of asexual development but seems to play no role in the regulation of growth. A dominant activating *gasA* mutant whose mutation results in a G42-to-R change (*gasA*^{G42R}) does not express *brlA*, the conidiation-specific regulatory gene, and is locked in vegetative growth, while a dominant interfering *gasA*^{G203R} mutant shows inappropriate *brlA* expression and conidiation. Interestingly, the *gasA* mutants have no apparent defect in dimorphic switching or yeast-like growth at 37°C. Growth tests on dibutyryl cyclic AMP (dbcAMP) and theophylline suggest that a cAMP-protein kinase A cascade may be involved in the GasA signaling pathway.

Environmental signals leading to a variety of developmental processes and physiological responses are sensed and transduced to the cell interior through complex interactions at the cell membrane. Heterotrimeric guanine nucleotide-binding proteins (G proteins) act as signal transducers that couple cell surface receptors to cytoplasmic effector proteins in eukaryotes. Upon binding of the agonist with the corresponding serpentine transmembrane receptor, the Gα subunit becomes activated, exchanging GDP for GTP and dissociating from the βγ complex. Gα and Gβγ subunits can then interact with appropriate targets such as phosphodiesterases, protein kinases, adenylyl cyclases, phospholipases, and ion channels to trigger downstream signaling pathways (20, 27, 37, 44).

In fungi, G proteins affect a number of developmental and morphogenic processes and play essential roles during pathogenic and sexual development programs (4). The best-characterized fungal Gα proteins are *Saccharomyces cerevisiae* Gpa1 and Gpa2. Gpa1 is linked to cell-type-specific pheromone receptors encoded by the *STE2* and *STE3* genes and plays a negative role in the mitogen-activated protein kinase pheromone signaling pathway that leads to mating (32). Gpa2 is required for the induction of pseudohyphal growth in diploid yeast cells under nitrogen starvation and signals through a cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway (30, 35). Interestingly, Gpa2 is coupled to a G-protein-coupled receptor, Gpr1, which appears to be a glucose sensor (28, 33, 36). Gα subunits from other fungi with high homology to Gpa2 have also been implicated in morphogenesis through cAMP-PKA signaling. The corn smut fungus *Ustilago maydis* displays a dimorphic switch between a haploid yeast-like form and a pathogenic filamentous dikaryon. Haploid strains with the Gα subunit-encoding *gpa3* gene deleted show a filamentous phenotype and are sterile; these phenotypes can be rescued by exogenous cAMP (19, 29, 38).

The *gna1* and *gna2* genes of *Neurospora crassa* were the first filamentous-fungal Gα-encoding genes identified. Based on sequence comparison and the ability to serve as a substrate for pertussis toxin, GNA1 belongs to the mammalian $G\alpha_i$ superfamily, whose members regulate various effectors, such as adenylyl cyclase, ion channels, and cyclic-GMP phosphodiesterase (27, 37, 45). GNA1 signaling mediates several cellular processes, including apical growth and the development of aerial hyphae. In addition, Δ*gna1* strains have lower intracellular cAMP levels than the wild type (24, 46). Analysis of other fungal $G\alpha_i$ proteins has further demonstrated the importance of this group of Gα proteins in development and morphogenesis. Disruption and site-directed mutagenesis of *magB*, a gene encoding a $G\alpha_i$ subunit in the rice blast fungus *Magnaporthe grisea*, affect vegetative growth, conidiation, and appressorium formation. Furthermore, the addition of cAMP induces appressorium formation in *magB* loss-of-function mutants (15, 34). The CPG1 protein of the chestnut blight fungus *Cryphonectria parasitica* shows high homology to the *N. crassa* GNA1 protein. Disruption of *cpg1* greatly reduces virulence, growth, and conidiation and leads to elevated cAMP levels, suggesting that CPG1 functions as a negative regulator of adenylyl cyclase (10, 16).

In *Aspergillus nidulans*, a $G\alpha_i$ protein and a regulator of G-protein signaling domain protein, encoded by *fadA* and *flbA*, respectively, regulate the decision between growth and asexual development as well as the production of secondary metabolites such as the polyketide sterigmatocystin (21, 48). Asexual development involves the elaboration of a conidiophore through the differentiation of a number of specialized cell

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types (7, 11). BrlA and AbaA comprise the key transcriptional regulators controlling this process, and the induction of BrlA is sufficient to drive rudimentary conidiation (1, 2). AflR, on the other hand, has been shown to be a sterigmatocystin biosynthesis pathway-specific transcription factor (47). Both *brlA* and *aflR* require FlbA and FadA for their normal expression. The signal transduction pathway connecting FadA with its downstream effectors BrlA and AflR has recently been shown to be partially mediated by PkaA, the catalytic subunit of a PKA (43).

The ascomycete *Penicillium marneffei* is an opportunistic human pathogen endemic in Asia that exhibits a temperature-dependent dimorphic switch (12, 17, 23). At 25°C, multinucleate, septate hyphae are produced by apical growth and lateral branching. Exposure of hyphae to an air interface induces asexual development at this temperature to produce asexual spores on conidiophores similar to those of *A. nidulans*. It has been shown that both a *brlA* and an *abaA* homologue are required for asexual development in *P. marneffei* (A. R. Borneman, M. J. Hynes, and A. Andrianopoulos, unpublished data, and reference 5). At 37°C, *P. marneffei* hyphae undergo a process known as arthroconidiation, in which septation and nuclear division become coupled. The hyphae lay down double septa, which consequently allow cell separation to produce single uninucleate yeast cells, which divide by fission (8). These two developmental programs in *P. marneffei* have been shown to share elements controlling morphogenesis. At 25°C, deletion of the *abaA* gene leads to defects in conidial development and a failure to produce conidia. At 37°C, deletion of the *abaA* gene results in uncoupling of the nuclear and cell division cycles such that the yeast cells contain more than one nucleus and have aberrant morphology (5). Therefore, AbaA plays a role in both of these distinct developmental programs, and this suggests that upstream regulators of the conidiation pathway may also be important regulators of dimorphic switching.

This work describes the cloning of the *P. marneffei gasA* gene encoding a Gα subunit that is highly similar to *A. nidulans* FadA. We have investigated the role of GasA in the hyphal growth, asexual development, and yeast-like growth of *P. marneffei*. Through the characterization of a Δ*gasA* strain and strains carrying a dominant activating or a dominant interfering mutation, we show that GasA plays a key role in the regulation of asexual development. In contrast, *gasA* mutants have no apparent defect in dimorphic switching or yeast-like growth. We have also investigated possible signal transduction pathways connecting GasA and its downstream effectors BrlA and AbaA and show that in *P. marneffei*, a cAMP-PKA pathway may be involved in the GasA signaling pathway.

MATERIALS AND METHODS

Fungal strains, media, and growth conditions. The *P. marneffei* wild-type strain FRR2161 was obtained from J. Pitt (CSIRO Food Industries, Sydney, Australia). Transformation of the SPM4 strain, which is unable to grow on nitrate as a sole nitrogen source and is a uracil auxotroph, was performed by the previously described protoplast method (6). The *gasA*^{G42R} (resulting in a G42-to-R change) and *gasA*^{G203R} strains (TS-24-5-4 and TS28-4-8, respectively) were generated by transformation of strain SPM4 with pSZ5030 and pSZ5089, respectively, and selection for *pyrG*$^{+}$; TS-24-5-4 contains three copies of pSZ5030 and TS28-4-8 contains five copies of pSZ5089. In the same way, pSZ5029, which contains the *gasA* wild-type allele, was used to create *gasA*$^{+}$ strains containing from one to six copies of pSZ5029. The Δ*gasA* strain (TS27-3-29) was generated by transformation of strain SPM4 with 500 ng of a gel-purified *Xho*I/*Sph*I fragment from pSZ5031 and selection for *pyrG*$^{+}$.

P. marneffei strains were grown on *Aspergillus* nitrogen-free medium (ANM) supplemented with 10 mM gamma-aminobutyric acid (GABA) at 25°C and on *S. cerevisiae* synthetic-dextrose (SD) medium at 37°C (3, 13). These media were supplemented with 0.1 mM dibutyryl cAMP (dbcAMP; Sigma) and 10 mM theophylline, as required. Where appropriate, the strains were tested on media containing 10 mM uracil to confirm that the observed phenotypes were unrelated to poor expression of the *pyrG* selectable marker. For microscopic analysis, *P. marneffei* strains were grown at 25°C for 3 days on SD medium containing 0.1% glucose to induce conidiation and at 37°C for 4 days on brain heart infusion (BHI) broth (Oxoid) to favor the formation of single yeast cells.

Due to the lack of conidiation in the *gasA*^{G42R} strain, yeast cells grown for 4 days on SD plates at 37°C were used as an inoculum for all strains. Vegetative hyphal samples were grown in SD medium with shaking for 2 or 3 days at 25°C as stated for each individual experiment. Asexually developing cultures were prepared by growing the 2-day vegetative cultures on ANM-GABA plates containing 0.1% glucose for an additional 4 days to allow conidiation. Yeast cultures were grown in BHI liquid medium for 6 days as previously described (6).

Molecular techniques. Plasmid DNA was isolated by using a high-purity plasmid kit (Roche Diagnostics). Genomic DNA from *P. marneffei* was isolated as previously described (6). RNA was prepared by using a FastRNA kit (Bio 101, Inc.) as previously described (6). Southern and Northern blotting were performed with Amersham Hybond N+ membranes according to the manufacturer's instructions. Filters were hybridized with [α-^{32}P]dATP-labeled probes by standard methods (40). Northern blots were probed with a histone H3 gene probe as a loading control (14).

Degenerate PCR was performed on genomic DNA of strain FRR2161 with the sense primer GAGES (5′CGGGATCCGGNGCNGGNGARWSNGGNAA3′) and the antisense primer GαPCR3 (5′GAGGTCACGTTCTCGAAGCART RDAWCCA3′), which correspond to highly conserved domains in G-protein α subunits (27). PCR conditions consisted of an initial denaturation step of 94°C for 2 min, after which *Taq* DNA polymerase was added and DNA was amplified for 35 cycles in a Mastercycler gradient thermocycler (Eppendorf). The cycling parameters were as follows: 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The products were cloned into pGEMTeasy (Promega). A 600-bp insert with high homology to *A. nidulans fadA* was used to probe a 5- to 6-kb *Bgl*II/*Sal*I size-selected FRR2161 genomic library in pBluescript II SK(+) (Stratagene). One positive clone (pSZ4809) was chosen for subcloning and sequencing. Sequencing was performed by the Australian Genome Research Facility and analyzed with Sequencher 3.1.1 (Genes Codes Corporation). Database searches and sequence comparisons were performed via the Australian National Genomic Information Service. The phylogenetic tree was generated by using the Dayhoff PAM matrix and the neighbor-joining method in the Phylogeny Inference package (version 3.57c; University of Washington, Seattle [http://evolution.genetics.washington.edu/phylip.html]). *U. maydis* Gpa4 was used as an outgroup (4).

A GeneEditor in vitro site-directed mutagenesis kit (Promega) was used to create the dominant activating *gasA*^{G42R} mutation. Mutagenesis was performed on an *Xho*I/*Sac*II pBluescript II SK(+) subclone (pSZ4904) with the mutagenic primer gasA.dom (5′CTCTAGGTGCTCGCGAATCTGGAAA3′; base mismatches are underlined) to generate pSZ4967. This product was sequenced and used to recreate a full-length *gasA* clone in pALX223 which contained the *pyrG* gene for direct selection in the *P. marneffei* strain SPM4 (A. Andrianopoulos, unpublished data), generating pSZ5030. The dominant interfering *gasA*^{G203R} mutation was generated by inverse PCR on an *Age*I subclone (pSZ4921) with the mutagenic primers gasA.domneg.up (5′CAACGATCCGAACGTAAGAAGTG G3′) and gasA.domneg.lo (5′TCGACCAACGTCAAACATGCGGTAG3′) to yield pSZ5088. This fragment was sequenced and used to recreate a full-length *gasA* clone in pALX223, generating pSZ5089. Additionally, the full-length *gasA* wild-type allele was cloned into pALX223, generating pSZ5029. The dominant activating *fadA*^{G42R} and dominant interfering *fadA*^{G203R} alleles, kindly provided by T. H. Adams (48), were subcloned into pALX223 to generate pSZ5027 and pSZ5028, respectively. To disrupt *gasA*, a 2.2-kb *Eco*RV/*Sac*II fragment containing the *pyrG* cassette of pAB4626 (Borneman et al., unpublished) was cloned between the internal *Sac*II site and the *Hin*dIII end-filled site of pSZ4809, generating plasmid pSZ5031.

Microscopy. *P. marneffei* strains were grown on slides covered with thin layers of solid medium resting in liquid medium (5). The slides were mounted with the addition of 500 μg of 4′,6′-diamidino-2-phenylindole (DAPI)/μl and viewed by using either differential interference contrast or epifluorescence optics on a Reichart Jung Polyvar II microscope. Images were captured digitally with a

SPOT charge-coupled-device camera (Diagnostic Instruments) and processed with Adobe Photoshop software.

Nucleotide sequence accession number. The GenBank accession number of the *P. marneffei gasA* gene is AF448796.

RESULTS

***P. marneffei gasA* and *A. nidulans fadA* genes are highly conserved.** To isolate genes encoding Gα subunits from *P. marneffei*, we used a degenerate-primer PCR-based approach. Sequence analysis of one class of PCR products showed significant homology to *fadA* of *A. nidulans*. Southern blot analysis with the amplification product as a probe indicated a gene present as a single copy in *P. marneffei* (data not shown). This gene was designated *gasA* (Gα subunit), and the amplification product was used to screen a partial genomic DNA library. A 5.5-kb *Bgl*II/*Sal*I fragment was isolated, and a 3.5-kb *Eco*RV subclone was sequenced (Fig. 1A). The gene structure was predicted by comparison with the genomic sequence of the *A. nidulans fadA* gene, and the gene contained three introns which had conserved positions in the two species. The *gasA* gene is therefore predicted to encode a 353-amino-acid (aa) protein with 96% identity with the Gα subunit FadA of *A. nidulans* and 93% identity with *Cochliobolus heterostrophus* CGA1 and *C. parasitica* CPG1. Like FadA, CGA1, and CPG1, GasA shows the characteristic Gα subdomains P (aa 27 to 50), G′ (aa 200 to 217), and G (aa 261 to 273) as well as the presence of both a potential myristoylation site (MGXXXS) at the N terminus and the consensus site CXXX for ADP ribosylation by pertussis toxin (27, 44). These features have been taken as evidence that members of this group are homologous to the mammalian $G\alpha_i$ subunits and show that *gasA* encodes an α subunit of a heterotrimeric G protein. Phylogenetic analysis of GasA and other fungal Gα proteins revealed three major subgroups within the family of Gα proteins, with GasA in the same group as FadA (Fig. 1B).

***gasA* is expressed differentially during different growth stages.** RNA from the wild-type strain was isolated from vegetative hyphal cells grown in liquid medium for 2 days at 25°C, from asexually developing (conidiating) cultures at 25°C, and from yeast cells at 37°C (see Materials and Methods). Northern blot analysis showed a single highly expressed *gasA* transcript of 1.3 kb during vegetative growth which was strongly downregulated during asexual development. In the yeast phase, two transcripts, of 1.1 and 1.3 kb, were detected (Fig. 2).

GasA-mediated signaling negatively affects asexual development. To examine the function of GasA, a number of mutant *gasA* alleles were generated. A dominant activated ($gasA^{G42R}$) mutant allele was produced by site-directed mutagenesis to convert glycine 42 to arginine, as this particular glycine is required for GTPase activity and is therefore predicted to result in a GTP-bound Gα constitutively activating signaling (27, 31). The plasmid containing $gasA^{G42R}$ (pSZ5030) was transformed into SPM4, and transformants were isolated by direct selection for $pyrG^{+}$ (Fig. 3A). The presence and the copy number of the plasmid were determined by Southern blot analysis of genomic DNA (data not shown). Four representative transformants of $gasA^{G42R}$, ranging in copy number from 1 to 20, were analyzed further. Dominant activated $gasA^{G42R}$ transformants displayed thick aerial hyphae and failed to differentiate conidiophore structures and therefore completely lacked conidia (Fig. 3B and C). No effect was observed in transformants containing multiple copies of the *gasA* wild-type allele (pSZ5029). This suggests that GasA-mediated signaling negatively affects asexual development. Additionally, the transformants showed an increased production of a red pigment

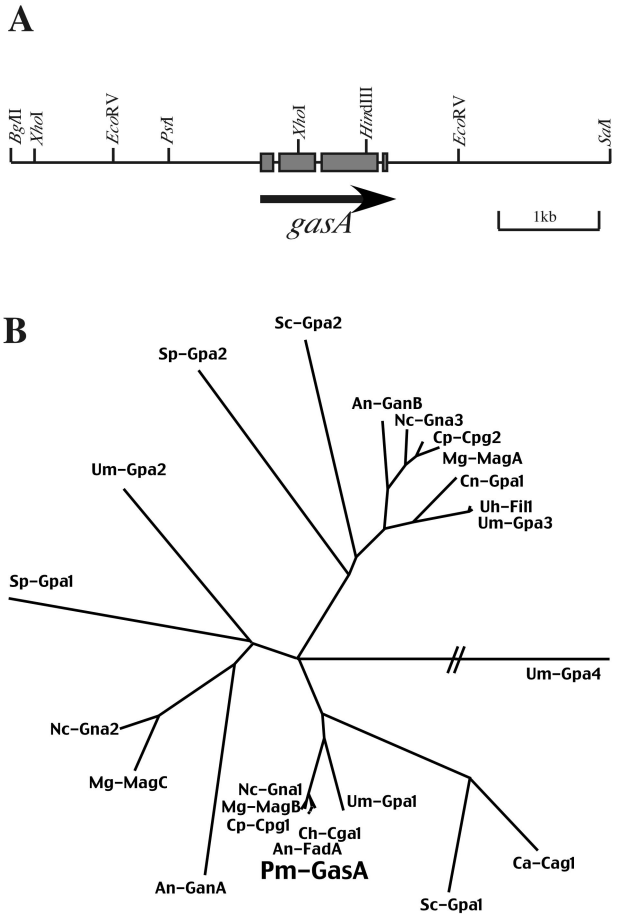

FIG. 1. The *gasA* gene of *P. marneffei* encodes a Gα subunit of a heterotrimeric G protein. (A) Gene structure and partial restriction map of the *gasA* gene corresponding to pSZ4809. The region predicted to encode the GasA protein is indicated by gray boxes representing the exons, interrupted by three introns. The solid black arrow shows the direction of transcription. (B) Relationship of fungal Gα subunits. The tree is based on amino acid sequence alignments and was constructed as described in Materials and Methods. Abbreviations of species and GenBank gene accession numbers are as follows: An-FadA, *A. nidulans* FadA (Q00743); An-GanA, *A. nidulans* GanA (AF142058); An-GanB, *A. nidulans* GanB (AF198116); Ca-Cag1, *Candida albicans* Cag1 (M88113); Ch-Cga1, *Cochliobolus heterostrophus* CGA1 (AF070446); Cn-Gpa1, *Cryptococcus neoformans* Gpa1 (AAD46575); Cp-Cpg1, *C. parasitica* CPG1 (L32176); Cp-Cpg2, *C. parasitica* CPG2 (L32177); Mg-MagA, *M. grisea* MagA (AF011340); Mg-MagB, *M. grisea* MagB (AF011341); Mg-MagC, *M. grisea* MagC (AF011342); Nc-Gna1, *N. crassa* GNA1 (L11453); Nc-Gna2, *N. crassa* GNA2 (L114532); Nc-Gna3, *N. crassa* GNA3 (AF281862); Pm-GasA, *P. marneffei* GasA (AF448796); Sc-Gpa1, *S. cerevisiae* Gpa1 (AAA34650); Sc-Gpa2, *S. cerevisiae* Gpa2 (AAA34651); Sp-Gpa1, *Schizosaccharomyces pombe* Gpa1 (M64268); Sp-Gpa2, *S. pombe* Gpa2 (D13366); Uh-Fil1, *Ustilago hordei* Fil1 (U76672); Um-Gpa1, *U. maydis* Gpa1 (U85775); Um-Gpa2, *U. maydis* Gpa2 (U857756); Um-Gpa3, *U. maydis* Gpa3 (U85777); Um-Gpa4, *U. maydis* Gpa4 (U85778n). Hatched lines indicate nonproportional length.

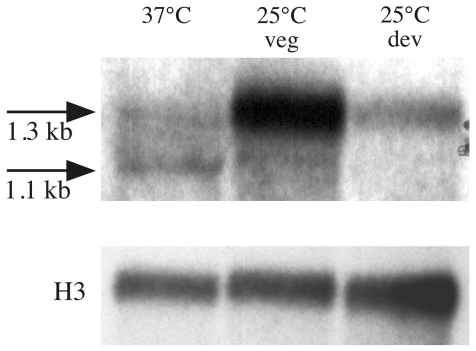

FIG. 2. Northern blot analysis of *gasA* expression. Total RNA from the *P. marneffei* wild-type strain (FRR2161) was isolated from yeast cultures (37°C), vegetative mycelia grown in liquid at 25°C for 2 days (25°C veg), and asexually developing cultures (25°C dev), as indicated in Material and Methods. RNA from each of the three growth stages (37°C, 25°C veg, and 25°C dev) was hybridized with probes specific for either *gasA* or histone H3 (H3), which was used as a loading control. Arrows indicate the two *gasA* transcripts of 1.1 and 1.3 kb.

which is characteristic of the vegetative filamentous form of *P. marneffei* (42; data not shown). We also transformed SPM4 with the plasmid containing the *A. nidulans fadA*^{G42R} allele (pSZ5027), isolating transformants by direct selection for *pyrG*$^{+}$. These transformants displayed the same phenotype as that of transformants carrying the *gasA*^{G42R} allele, suggesting functional interchangeability between GasA and FadA.

The *brlA* gene of *P. marneffei* is necessary for asexual development, as is *brlA* of *A. nidulans* (Borneman et al., unpublished, and reference 1). We examined the expression of *brlA* in the dominant activated *gasA*^{G42R} strain, which fails to conidiate. A *brlA* transcript was not detected in RNA from vegetative hyphal cells of either the wild type or the *gasA*^{G42R} mutant grown at 25°C (Fig. 3D). In developmental cultures, the *brlA* transcript was strongly expressed in the wild type but not in the *gasA*^{G42R} transformant (Fig. 3D).

A dominant interfering *gasA*^{G203R} mutation results in inappropriate asexual development. To further examine the role of GasA in the regulation of conidiation, a dominant interfering (*gasA*^{G203R}) mutant allele was generated. For this purpose, we converted glycine 203 to arginine by site-directed mutagenesis, which is predicted to prevent the conformational switch that accompanies GTP binding and is necessary for Gβγ release, thereby maintaining the association of the Gαβγ complex and leading to a dominant inactivation of signaling (27, 31). The plasmid containing *gasA*^{G203R} (pSZ5089) was transformed into SPM4, and transformants were isolated by direct selection for *pyrG*$^{+}$ (Fig. 3A). The presence and the copy number of the plasmid were determined by Southern blot analysis of genomic DNA (data not shown). Four representative *gasA*^{G203R} transformants, ranging in copy number from 3 to 10, were analyzed further. In addition, a construct in which part of the *gasA* coding sequence was replaced with the *A. nidulans pyrG* gene was used to transform SPM4 and inactivate the *gasA* gene by homologous gene replacement (Fig. 3A). In 5 transformants out of 65 screened by Southern blotting, the endogenous copy was deleted (data not shown).

At 25°C, the dominant interfering *gasA*^{G203R} transformants and the Δ*gasA* strain showed a colonial phenotype similar to that of the wild type. The radial growth rate was the same as that of the wild type; however, the *gasA*^{G203R} and Δ*gasA* strains were slower in producing aerial hyphae, leading to a slight delay in conidiation (Fig. 3B and C). Additionally, both mutants showed reductions in the production of the red pigment (42; data not shown).

If GasA negatively regulates asexual development, a *gasA*^{G203R} mutant would be expected to enter asexual development under conditions where the wild type does not. To test this hypothesis, we examined growth in liquid culture, where the wild type exhibits no conidiation. Unlike the wild type, the dominant interfering *gasA*^{G203R} mutant produced a large number of relatively normal conidiophore structures after 3 days at 25°C (Fig. 4A). The Δ*gasA* strain failed to produce conidiophores; however, the hyphal cells were larger in diameter than those of the wild type, resembling those observed in the dominant interfering *gasA*^{G203R} mutant (Fig. 4A). We also transformed SPM4 with the plasmid containing the *A. nidulans fadA*^{G203R} allele (pSZ5028), isolating transformants by direct selection for *pyrG*$^{+}$. In liquid culture, these transformants displayed the same phenotype as that of the transformants carrying the *gasA*^{G203R} allele, providing further evidence for the functional interchangeability of GasA and FadA (data not shown). To support the microscopic observations, RNAs from the wild-type, *gasA*^{G203R}, and Δ*gasA* strains were isolated from vegetative hyphal cells grown in liquid culture for 3 days at 25°C. No *brlA* mRNA was detected in the wild type. As expected, a *brlA* transcript was readily detectable in the *gasA*^{G203R} mutant. In the Δ*gasA* strain, a *brlA* transcript was detected but was clearly less abundant than in the *gasA*^{G203R} mutant (Fig. 4B).

GasA is not required for yeast-like growth at 37°C. In order to investigate if GasA plays a role in hyphal-yeast morphogenetic transition as well as the maintenance of yeast-like growth at 37°C, we first compared the colony morphology of the wild type with those of the dominant activated *gasA*^{G42R}, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. All of the strains formed normal yeast-like colonies; however, the Δ*gasA* strain produced colonies which were smaller in diameter. Upon examination of four additional Δ*gasA* strains, it was determined that the *pyrG* selectable marker which replaced the *gasA* coding region in these deletion strains was being poorly expressed, resulting in reduced growth. Therefore, it can be concluded that *gasA* does not appear to play a direct role in yeast-like growth.

In the wild type at 37°C, hyphal compartments became uninucleate, as nuclear and cell division became coupled and the cells of these filaments separated to form arthroconidial yeast cells that propagated by fission. This developmental pathway was not affected in any of the *gasA* mutants, which were capable of switching to yeast-like growth and producing morphologically wild-type yeast cells (Fig. 5B).

Expression of *brlA* and *abaA* in the *gasA* mutants at 37°C. *P. marneffei* does not express *brlA* during yeast-like growth at 37°C (Borneman et al., unpublished). The *abaA* gene, however, is expressed at 37°C, and deletion of *abaA* results in the misregulation of arthroconidiation such that the transitional hyphal cells and the yeast cells contain more than one nucleus and have aberrant morphology (5). As described above, the dominant interfering *gasA*^{G203R} mutant shows inappropriate

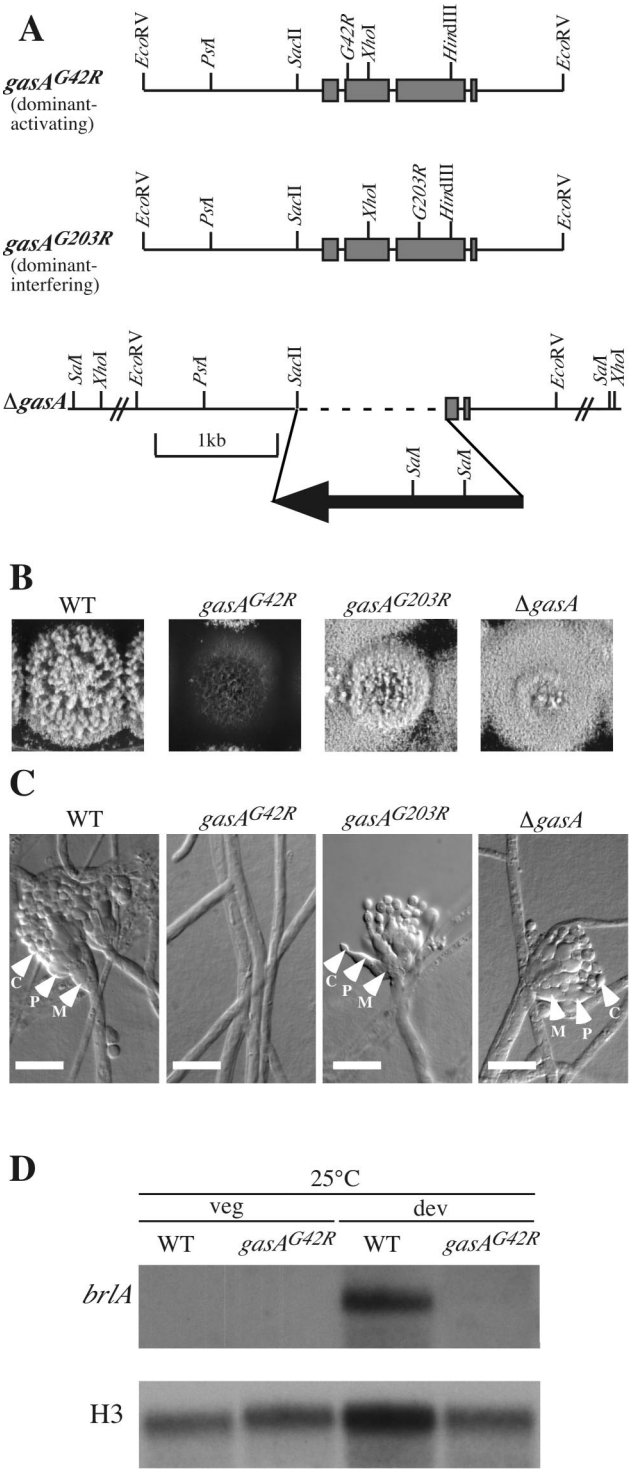

FIG. 3. Analysis of the *gasA*^{G42R}, *gasA*^{G203R}, and Δ*gasA* strains at 25°C. (A) Partial restriction map of the dominant activated *gasA*^{G42R} (pSZ5030) and dominant interfering *gasA*^{G203R} (pSZ5089) alleles used to generate the respective eponymous strains. The *gasA* locus in the Δ*gasA* strain is shown after insertion of the *gasA* deletion construct (pSZ5031). Hatched lines indicate nonproportional length. (B) Colonial morphologies of the *P. marneffei* wild-type (FRR2161), dominant activated *gasA*^{G42R}, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. The strains were grown on ANM-GABA for 14 days at 25°C. All strains conidiated normally except for the *gasA*^{G42R} mutant, which is aconidial and exhibited flat colony morphology with thick aerial hyphae. (C) Microscopic examination of the *P. marneffei* wild-type (FRR2161), dominant activated *gasA*^{G42R}, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. The strains were grown on SD containing 0.1% glucose for 3 days at 25°C. All strains exhibited wild-type conidiophores except for the *gasA*^{G42R} mutant, which showed no signs of asexual development. Scale bars, 20 μm. Arrowheads highlight the conidiophore structures (M, metulae; P, phialides; C, conidia). (D) Northern blot analysis of the *P. marneffei* wild-type strain (FRR2161) and the dominant activating *gasA*^{G42R} mutant. Total RNA was isolated from vegetative mycelia grown in liquid at 25°C for 2 days (veg) and from asexually developing cultures (dev). Northern blots were probed with either *brlA* or histone H3 (H3). WT, wild type.

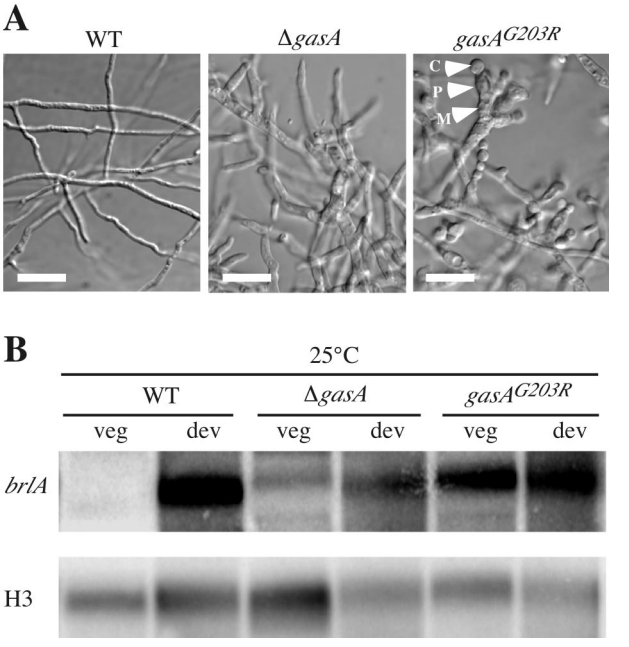

FIG. 4. Analysis of the *gasA*^{G203R} and Δ*gasA* strains in liquid cultures at 25°C. (A) Microscopic examination of the *P. marneffei* wild-type (FRR2161), dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. The strains were grown in liquid at 25°C for 3 days. Scale bars, 20 μm. Arrowheads highlight the conidiophore structures (M, metulae; P, phialides; C, conidia). (B) Northern blot analysis of the *P. marneffei* wild-type (FRR2161), dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. Total RNA was isolated from vegetative mycelia grown in liquid at 25°C for 3 days (veg) and from asexually developing cultures (dev). Northern blots were probed with either *brlA* or histone H3 (H3). WT, wild type.

brlA expression at 25°C in liquid culture. It was therefore of interest to determine whether any of the *gasA* mutants, especially the *gasA*^{G203R} mutant, showed altered *brlA* and *abaA* expression at 37°C. As for the wild type, the *brlA* transcript was not detected in any of the *gasA* mutants. The *abaA* transcript was detected in all the strains (Fig. 5C).

GasA in *P. marneffei* may signal through a cAMP-PKA cascade. We investigated the signal transduction pathways connecting GasA and its downstream effectors BrlA and AbaA. To test if a cAMP-PKA cascade mediates GasA signaling in *P. marneffei*, we assessed the phenotypes of the different mutants in the presence of 0.1 mM dbcAMP and 10 mM theophylline, a phosphodiesterase inhibitor. Growth on dbcAMP and theophylline inhibited conidiation and led to a fluffy phenotype

after 10 days at 25°C for the wild-type, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. The dominant activated *gasA*^{G42R} mutant did not show any additional phenotype in the presence of these compounds (data not shown). These observations suggest that GasA signals through a cAMP-PKA cascade to inhibit asexual development (Fig. 6).

DISCUSSION

The *P. marneffei gasA* gene encodes a Gα subunit of a heterotrimeric G protein. GasA has the highest level of amino acid identity with the Gα subunits FadA of *A. nidulans*, CGA1 of *Cochliobolus heterostrophus*, CPG1 of *C. parasitica*, MagB of *M. grisea*, and GNA1 of *N. crassa*. All of these fungal Gα subunits are homologous to members of the mammalian $G\alpha_i$ family and have been shown to regulate a number of developmental and morphological pathways (22, 24, 27, 34, 48). We have shown in this study that a *P. marneffei* mutant carrying a dominant activating *gasA*^{G42R} allele is locked in vegetative growth and cannot enter asexual development. The same phenotype has been described for an *A. nidulans* mutant carrying the corresponding *fadA*^{G42R} allele (48). This suggests that GasA signaling in *P. marneffei* at 25°C antagonizes conidiophore development and that GasA is a key regulator of asexual development in *P. marneffei*, as FadA is in *A. nidulans*. This hypothesis is further supported by the observation that a *P. marneffei* mutant carrying a dominant interfering *gasA*^{G203R} allele conidiates abundantly in liquid culture. Furthermore, *P. marneffei* mutants carrying a dominant activating *fadA*^{G42R} or a dominant interfering *fadA*^{G203R} mutation are phenotypically similar to mutants carrying the corresponding *gasA* mutations. This shows that the *P. marneffei gasA* gene encodes a G-protein α subunit which appears to be a functional homologue of the *A. nidulans fadA* gene. The facts that hyphal cells of the Δ*gasA* mutant show conidiophore-like characteristics but that this strain does not conidiate in liquid culture suggest that GasA acts together with a Gβγ subunit and that both the Gα subunit and the Gβγ subunit antagonize conidiophore development. It is only in a dominant interfering *gasA*^{G203R} mutant, where signaling of both the Gα and the Gβγ subunit is impaired, that inappropriate conidiation is evident. This finding is consistent with the observation that in liquid cultures, *brlA*, which codes for one of the key transcriptional regulators controlling asexual development, is strongly expressed in the dominant interfering *gasA*^{G203R} mutant, less abundantly expressed in the Δ*gasA* mutant, and not expressed in the wild type. Similar results have been reported for *A. nidulans*, in which a mutant carrying the dominant interfering *fadA*^{G203R} allele conidiates in submerged culture while the Δ*fadA* strain does not (48). Moreover, deletion of *sfaD*, which encodes a Gβ subunit, also leads to hyperactive sporulation (39). Signaling by the Gβγ subunit has also been suggested to occur in *M. grisea*, where both a mutant carrying a dominant activating *magB*^{G42R} allele and a *magB* loss-of-function mutant exhibit severe reductions in conidiation. In contrast, the *magB*^{G203R} mutation has no significant effect on conidiation. Thus, it is proposed that Gβγ in *M. grisea* remains active in both null and constitutively active *magB*^{G42R} mutants and may be responsible for negatively regulating development (15).

GasA signaling negatively regulates asexual development and must be turned off in order for *P. marneffei* to enter asexual development. Northern blot analysis showed that the level of *gasA* transcription was downregulated during asexual development compared to that observed during vegetative hyphal growth, and

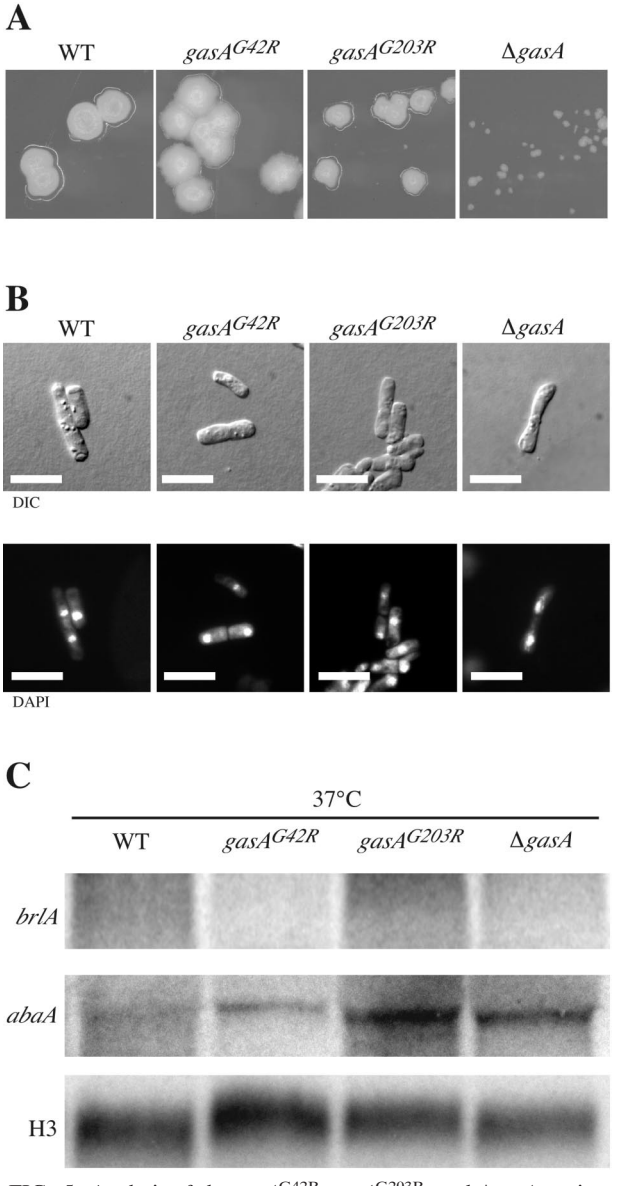

FIG. 5. Analysis of the *gasA*^{G42R}, *gasA*^{G203R}, and Δ*gasA* strains at 37°C. (A) Colonial morphologies of the *P. marneffei* wild-type (FRR2161), dominant activated *gasA*^{G42R}, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. The strains were grown on SD medium at 37°C for 4 days. All strains exhibited normal yeast-like colonies except for the Δ*gasA* strain, which showed smaller colonies. This phenotype was found not to result from the inactivation of *gasA* (see the text). (B) Microscopic examination of the *P. marneffei* wild-type (FRR2161), dominant activated *gasA*^{G42R}, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. The strains were grown on BHI broth at 37°C for 4 days. All strains exhibited wild-type yeast cells. Scale bars, 20 μm. Differential interference contrast (DIC) and DAPI-stained epifluorescence of nuclei (DAPI) are shown. (C) Northern blot analysis of the *P. marneffei* wild-type (FRR2161), dominant activating *gasA*^{G42R}, dominant interfering *gasA*^{G203R}, and Δ*gasA* strains. Total RNA was isolated from yeast cultures grown in liquid at 37°C for 6 days. Northern blots were probed with either *brlA*, *abaA*, or histone H3 (H3). WT, wild type.

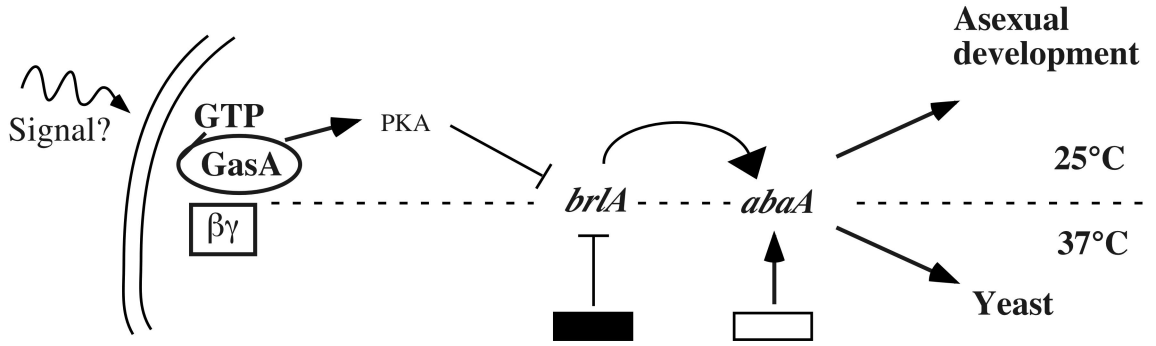

FIG. 6. Proposed model for signal transduction pathways regulating development and growth in *P. marneffei*. GasA signaling at 25°C involves a cAMP-PKA pathway in the regulation of asexual development and the expression of the core regulatory factors BrlA and AbaA. GasA has no apparent role during yeast cell morphogenesis at 37°C. Therefore, we hypothesize the presence of a *brlA* repressor (black box) as well as an *abaA* activator (white box) which are independent of GasA signaling at this temperature.

this appears to be part of the mechanism by which the level of GasA signaling is controlled. However, in *A. nidulans*, both the mRNA and the protein encoded by *fadA* can be detected at relatively constant levels throughout the *A. nidulans* life cycle (48). These findings suggest that a mechanism regulating the expression of *gasA* in *P. marneffei* does not operate for *fadA* in *A. nidulans*.

FadA signaling in *A. nidulans* not only antagonizes conidiophore development but also plays a key role in the regulation of hyphal proliferation. Activation of FadA is thought to be initiated by the binding of a growth factor ligand to a putative G-protein-coupled receptor (48). A dominant activating *fadA*^{G42R} mutation stimulates growth and leads to colonies which eventually autolyze. The Δ*fadA* mutant produces smaller colonies than those of the wild type, and the dominant interfering *fadA*^{G203R} mutant shows a more severe growth defect than that of the Δ*fadA* strain, suggesting that the Gα subunit FadA is a positive regulator of growth (48). In addition to FadA, hyphal proliferation is also partly regulated by the Gβγ subunit, because deletion of the *sfaD* gene encoding the Gβ subunit retards growth (39). In contrast, hyphal proliferation in *P. marneffei* at 25°C showed no significant differences between the different GasA mutants. Although the growth rate differences may be very subtle, a more likely explanation is that GasA functions predominantly in activating a signaling pathway that prevents asexual development and has only a minor or no role in the regulation of growth. A similar situation has been noted for *M. grisea* (15). These findings imply the presence of another signal transduction pathway with a more pronounced role in regulating growth. The presence of two different signal transduction pathways regulating mycelial proliferation and asexual development and involving the Gα subunits GNA1 and GNA3 has been reported for *N. crassa*. During vegetative growth, Δ*gna1* strains exhibit slower apical extension of hyphae and slower growth. Asexual development is not affected dramatically, but Δ*gna1* mutants form more conidia per aerial hypha than does the wild type (46), whereas Δ*gna3* mutants produce short aerial hyphae and conidiate prematurely, yielding a dense conidiation pattern, and, unlike the wild type, conidiate in submerged culture (26).

It has previously been shown that the conidiation and dimorphic switching pathways share transcriptional regulatory components (5, 18, 41). In *P. marneffei*, the *abaA* gene is required for both conidiation and correct yeast cell morphogenesis (5). It was therefore important to investigate the role of the signaling component GasA at 37°C. Our inability to distinguish the different *gasA* mutants from the wild type at the colonial and microscopic levels at 37°C suggests that GasA plays no role in dimorphic switching or yeast-like growth. The significance of the two *gasA* transcripts detected at 37°C was therefore not investigated further. At 37°C, a *brlA* transcript could not be detected in the dominant interfering *gasA*^{G203R} mutant, which expresses *brlA* inappropriately at 25°C. This result suggests that an as yet unknown factor must specifically repress *brlA* transcription at 37°C. Alternatively, an unknown factor required for *brlA* expression may be present at 25°C but missing at 37°C. These suggestions lead to the hypothesis that at 37°C, *abaA* may be activated independently of GasA and BrlA. This is in contrast to the situation at 25°C, where *abaA* expression is mainly dependent on BrlA (Fig. 6).

A number of fungal G proteins closely related to GasA, such as *N. crassa* GNA1 and *M. grisea* MagB, are suggested to signal through a cAMP-PKA cascade, and FadA signaling in *A. nidulans* has recently been shown to be mediated through PkaA, the catalytic subunit of PKA (34, 43, 46). In *P. marneffei*, elevation of the intracellular cAMP level through the addition of dbcAMP and theophylline to the growth medium revealed an inhibition of conidiation in the wild type, the dominant interfering *gasA*^{G203R} mutant, and the Δ*gasA* strain. This result suggests that GasA signals via the production of cAMP and the activation of PKA to inhibit the expression of *brlA* and, subsequently, asexual development (Fig. 6) and implies that GasA may function as a $G\alpha_s$ subunit that stimulates adenylyl cyclase despite its sequence similarity to mammalian $G\alpha_i$ subunits. Therefore, GasA is another example of the group of fungal Gα proteins with similarity to those of the $G\alpha_i$ class but which more likely function as activators of adenylyl cyclase, as do FadA of *A. nidulans* and GNA1 of *N. crassa* (25, 43), while CPG1 of *C. parasitica* seems to work as a bona fide $G\alpha_i$ protein (9). As the discrepancy between the sequences and functions of several fungal $G\alpha_i$ subunits becomes more apparent, the subdivision of the fungal $G\alpha_i$ class into two subclasses may be warranted.

We show in this study that the Gα subunit GasA of *P. marneffei* is a key regulator of asexual development. We pro-

pose that GasA signaling involves a cAMP-PKA pathway. Of particular significance is the fact that GasA does not play a role in dimorphic switching or yeast-like growth. We are currently characterizing two additional *P. marneffei* Gα subunits to give further insight into the complex regulation of mycelial proliferation and asexual development in *P. marneffei* at 25°C and to explain the possible role of these Gα subunits in dimorphic switching and yeast-like growth at 37°C.

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