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The GanB Gα-Protein Negatively Regulates Asexual Sporulation and Plays a Positive Role in Conidial Germination in *Aspergillus nidulans*

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ABSTRACT

We isolated the *ganB* gene encoding the Gα-protein homolog from *Aspergillus nidulans*. To investigate the cellular function of GanB, various mutant strains were isolated. Deletion of constitutively inactive *ganB* mutants showed conidiation and derepressed *brlA* expression in a submerged culture. Constitutive activation of GanB caused a reduction in hyphal growth and a severe defect in asexual sporulation. We therefore propose that GanB may negatively regulate asexual sporulation through the BrlA pathway. In addition, deletion or constitutive inactivation of GanB reduced germination rate while constitutive activation led to precocious germination. Furthermore, conidia of a constitutively active mutant could germinate even without carbon source. Taken together, these results indicated that GanB plays a positive role during germination, possibly through carbon source sensing, and negatively regulates asexual conidiation in *A. nidulans*.

GUANINE nucleotide-binding proteins (G proteins) are key components in various signal transduction pathways and show great complexity in structure and function. Heterotrimeric G proteins consist of three subunits, α, β, and γ. The α-subunit has intrinsic GTPase activity and associates with βγ-subunits with bound GDP in the inactive state (Gilman 1987; Simon *et al.* 1991). When ligands interact with their seven cognate transmembrane receptors, the GDP bound to the α-subunit is replaced with GTP, resulting in dissociation of the αGTP-subunit from the βγ-complex. The α-subunit with GTP bound and the free βγ-complex may interact with many types of downstream effectors such as enzymes (adenylate cyclases, phosphodiesterases, phospholipases, etc.) and ion channels, which in turn initiate signals via second messengers such as cyclic AMP, diacylglycerol, inositol triphosphate, and/or critical ions in the signal transduction pathway (Neer 1995; Bohm *et al.* 1997; Neves *et al.* 2002). Thus signals emanating from the cell surface are delivered to diverse downstream effectors by switching G-protein functions on and off. The integrated G-protein signals are responsible for orchestrating a coherent specific biological response.

In fungi, G proteins are known to regulate various morphogenetic events and pathogenicity, including dimorphic switching, appressorium formation, asexual or sexual development, and vegetative growth (Bölker 1998). Fungal Gα-proteins are classified into three major groups according to their amino acid sequences (Bölker 1998). Groups I and III are related to the mammalian Gαi- and Gαs-subfamilies, respectively. Group II fungal Gα-proteins have no corresponding mammalian counterpart. Group III fungal Gα-proteins play crucial roles in growth and development, including sporulation and germination, and pathogenesis in diverse fungi (Liu and Dean 1997; Kays *et al.* 2000; Liebmann *et al.* 2003; Zuber *et al.* 2003). The cAMP/PKA system is also involved downstream of group III Gα-protein signaling in some processes such as cell proliferation, development, stress response, mating, and virulence (Kübler *et al.* 1997; Kruger *et al.* 1998; Alspaugh *et al.* 2002; Ivey *et al.* 2002; Liebmann *et al.* 2003).

A dominant activating mutation in the *fadA* gene, encoding a Gα-subunit of the filamentous fungus *Aspergillus nidulans* in which the intrinsic GTPase activity has been eliminated, thereby fixing FadA in its active GTP-bound state, showed a proliferative growth phenotype and no conidiation (Yu *et al.* 1996). This means that the active Gα-subunit of a heterotrimeric G protein of *A. nidulans* stimulates hyphal growth but suppresses development of conidia. Developmental activation necessary for asexual reproduction requires at least partial inactivation of FadA and this is executed by the product of another gene, *flbA* (Yu *et al.* 1996). The FlbA protein has an RGS domain (for *r*egulator of *G* protein *s*ignaling) that negatively regulates G-protein-mediated signaling pathways (Dietzel and Kurjan 1987; Dohlman *et al.* 1995; Dohlman and Thorner 1997). Deletion

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mutations of *flbA* showed a phenotype similar to that of dominant activating mutations in *fadA* that caused failure of asexual sporulation accompanied by uncontrolled growth (Lee and Adams 1994; Yu *et al.* 1996, 1999). Furthermore, both loss-of-function and dominant interfering mutations in *fadA* result in growth reduction and suppression of the developmental defects of *flbA* deletion mutant strains. On the other hand, deletion of the Gβ-subunit encoded by *sfaD*, which acts not only as a regulator of the Gα-subunit but also as a signal transducer by itself, causes various phenotypes such as reduction of mycelial growth, hyperactive sporulation in submerged culture, and lack of cleistothecium formation (Rosén *et al.* 1999).

The asexual spore conidium is critical for dispersal and constitutes a safe genomic storage device that can survive in adverse environments for filamentous fungi; thus spore germination is an essential developmental stage in the life cycle (d'Enfert 1997; Osherov and May 2001). Whereas genetic and biochemical analysis of conidial development has been extensively studied, the signaling system for induction of conidial germination is not well understood (Osherov and May 2001). In *A. nidulans*, some components transmitting signals for germination have been characterized. It was reported that RasA, a small GTP-binding protein, is involved in conidial germination in *A. nidulans* (Som and Kolaparthi 1994). Overexpression of a constitutively active form of RasA produced multinucleate swollen conidia that failed to form germ tubes, while overexpression of an inactivated form of RasA resulted in delayed germination. Also overexpression of an activated form of RasA induces germination without carbon source, indicating that RasA plays a role in carbon source sensing during conidial germination (Osherov and May 2000). Recently, the cAMP/PkaA pathway has been shown to be involved in conidial germination (Fillinger *et al.* 2002). Deletion of *cyaA*, the adenylate cyclase gene, resulted in severe delays in germ tube formation, and inactivation of *pkaA* resulted in slower germ-tube-formation kinetics even though the degree of delay was less severe than that in the *cyaA* deletion mutant (Fillinger *et al.* 2002). More recently in *Penicillium marneffei*, GasC, a homolog of the group III fungal Gα-protein, was shown to control conidial germination but was not involved in carbon source sensing. Deletion of GasC results in delay of conidial germination, and dominant activation of GasC causes accelerated germination (Zuber *et al.* 2003).

The *veA* gene has been known to have dual functions in regulating developmental processes as a positive regulator of sexual and a negative one of asexual development (Kim *et al.* 2002). The *veA1* mutant that was first isolated and characterized by Käfer (1965) reduced sexual development and increased conidium formation (Champe *et al.* 1981). While the overexpression of *veA* reduced the formation of conidia, deletion of *veA* caused no cleistothecium formation even under conditions where sexual development preferentially occurs (Kim *et al.* 2002). These results suggest that VeA may have a key role in regulating both developmental processes.

To elucidate the signal transduction pathway regulating development of *A. nidulans*, we have isolated two genes, *ganA* and *ganB*, encoding Gα-protein homologs. Therefore there are at least three Gα proteins in *A. nidulans*. In this study, loss-of-function, overexpression, and constitutively active and constitutively inactive mutants of *ganB* were constructed and characterized. Phenotypes of these mutants suggested that GanB negatively regulates asexual development and plays a critical role in early events, including carbon source sensing during germination in *A. nidulans*. This is the first report that a Gα-protein is involved in carbon source sensing for germination in fungi.

MATERIALS AND METHODS

Fungal strains, growth condition, and genetic manipulations: *A. nidulans* RMS011 (Stringer *et al.* 1991) from the Fungal Genetics Stock Center, Kansas City, and VER7 (Han *et al.* 2001) were used as host strains for integration of manipulated genes. All *A. nidulans* strains used in this study (Table 1) were cultured on complex medium (CM) and minimal medium (MM). MM containing appropriate supplements was prepared as described (Pontecorvo *et al.* 1953; Käfer 1977) and CM was prepared by adding 1.5 g of casamino acids, 1.5 g of yeast extract, and 10 ml of vitamin solution per liter to MM. If necessary, 0.1% yeast extract or 0.1% casamino acids was added to MM. Transformation of *Escherichia coli*, plasmid DNA preparation, colony hybridization, Southern and Northern analysis, and other general molecular biological methods were as described in Sambrook and Russell (2001). Transformation and genetic manipulations of *A. nidulans* were also as described elsewhere (Pontecorvo *et al.* 1953; Yelton *et al.* 1984; Timberlake 1990).

Isolation of the *ganB* gene: The *ganB* gene was isolated using a *fadA* fragment (Yu *et al.* 1996) as a heterologous probe. Southern analysis and colony hybridization showed that the chromosome VIII-specific genomic library (Brody *et al.* 1991) contained the *ganB* gene. Two cosmid clones containing the *ganB* gene, W02C05 and W04H02, were isolated from this library. The cDNA of *ganB*, isolated by colony hybridization from an *A. nidulans* cDNA library in pBluescriptSK, were obtained from Jae Hyuk Yu (University of Wisconsin, Madison). Genomic DNAs and cDNAs of *ganB* were digested with various restriction enzymes, subcloned into pUC18 or pBluescriptSK, and sequenced.

Deletion of the *ganB* gene: To isolate a *ganB* knockout mutant, a 1.4-kb fragment of the GanB open reading frame (ORF; Figure 1A) was removed by inverse PCR. Two primers were designed to amplify in an outward direction from the *ganB* ORF. Primer gbup1 was directed downstream of *ganB* and contained a *Sma*I site. Primer gblp2 was directed upstream of *ganB* and contained a *Cla*I site. The PCR product using these two primers contained the vector region plus insert, minus the 1.4-kb *ganB* ORF fragment. An ~3.0-kb *Cla*I/*Sma*I fragment containing *argB* was ligated to the above PCR product that had been cut with *Cla*I/*Sma*I. The resulting plasmid, pdgBAR, was used as template in a PCR reaction amplifying from the outside the deleted *ganB* gene toward *argB* with another two PCR primers

TABLE 1

***A. nidulans* strains used in this study**

Strains	Genotype	Source
FGSC4	Wild type, *veA*$^{+}$	FGSC
VER7	*paba1 yA2;* Δ*argB::trpC; trpC801 veA*$^{+}$	Han *et al.* (2001)
RMS011	*paba1 yA2;* Δ*argB::trpC; trpC801 veA1*	Stringer *et al.* (1991)
RJY918.9	*paba1 yA2;* Δ*argB::trpC;* Δ*fadA::argB; trpC801 veA1*	Yu *et al.* (1996)
V7p16	*paba1 yA2;* Δ*argB::trpC; trpC801 veA*$^{+}$	This study
RMp16	*paba1 yA2;* Δ*argB::trpC; trpC801 veA1*	This study
V7dgB08	*paba1 yA2;* Δ*argB::trpC;* Δ*ganB::argB; trpC801 veA*$^{+}$	This study
RMdgB03	*paba1 yA2;* Δ*argB::trpC;* Δ*ganB::argB; trpC801 veA1*	This study
V7gBCI1521	*paba1 ya2;* Δ*argB::trpC; ganBG207R::argB; trpC801 veA*$^{+}$	This study
RMgBCI1633	*paba1 ya2;* Δ*argB::trpC; ganBG207R::argB; trpC801 veA1*	This study
V7gBQL705	*paba1 yA2;* Δ*argB::trpC; ganBQ208L::argB; trpC801 veA*$^{+}$	This study
RMgBQL801	*paba1 yA2;* Δ*argB::trpC; ganAQ208L::argB; trpC801 veA1*	This study
V7ogB105	*paba1 yA2;* Δ*argB::trpC; niiA(p)::ganB::argB; trpC801 veA*$^{+}$	This study
RMogB204	*paba1 yA2;* Δ*argB::trpC; niiA(p)::ganB::argB; trpC801 veA1*	This study

FGSC, Fungal Genetics Stock Center (Kansas City).

(gBpr7 and gBpr8). The PCR product with these two primers contained *argB* flanked on both sides by the remains of the *ganB* gene and was used to transform *A. nidulans* to obtain a knockout mutant. Knockout mutants of *ganB* were screened by PCR and Southern hybridization. We also confirmed by Northern analysis that the *ganB* gene was not expressed in these mutants.

Isolation of *ganB* overexpression mutants: The *niiA* promoter (Punt *et al.* 1995) was isolated by PCR amplification with the primers nAup1 and nAlp3 and was then cloned into the *Aat*II-*Xba*I sites of pGEM7ZF (Stratagene, La Jolla, CA). Plasmid pnAPGAR was constructed by inserting the *argB* gene into *Pst*I-*Bam*HI sites in the resulting plasmid. For *ganB* overexpression, the whole *ganB* ORF was isolated by digestion with *Bam*HI and *Xba*I (Figure 1A) and cloned into *Bam*HI-*Xba*I sites in pnAPGAR. The resulting plasmid (pOGB6) containing *niiA(p)-ganB* was introduced by homologous recombination into *A. nidulans* VER7 and RMS011. Mutants were screened by PCR and Southern blotting, and overexpression was confirmed by Northern analysis. Expression of *ganB* in these clones was induced by addition of 0.3% sodium nitrate and repressed by adding 0.2% ammonium tartrate as a nitrogen source (Johnstone *et al.* 1990).

Construction of *ganB* constitutively active and inactive mutants: To construct the constitutively active mutant of *ganB*, Gln208 was switched to Leu using site-directed PCR mutagenesis as described elsewhere (Ho *et al.* 1989; Kadowaki *et al.* 1989). For GanB^{Q208L}, we designed primer pair gBQ208Lup11 and gBQ208Llp12, containing sequences in which the CAG (Q) codon was changed into the CTG (L) codon and introducing an *Fsp*I site. PCR reaction I was performed with gBmbup7 and gBQ208Llp12, and PCR reaction II with gBQ208Lup11 and gBmblp8. A third PCR reaction was performed using PCR products I and II as a template to give a final product containing the Q208L mutation. After cloning this fragment into a vector containing the wild-type *argB* gene, the mutation site was confirmed by nucleotide sequencing the whole constitutively active mutant gene before transformation of *A. nidulans*. Screening and confirmation of mutant clones were performed by PCR, nucleotide sequencing, and Southern analysis after digestion of genomic DNA by *Fsp*I.

The procedure for constructing the constitutively inactive mutant was similar to that used for constitutively active mutants. GanB Gly207 was substituted with Arg. We introduced a *Nae*I (GCCGGC) site in primers gbG207Rup13 and gbG207Rlp14, which were used for switching codon GGC (G) to CGG (R). Again, mutant alleles were screened and confirmed by PCR, Southern hybridization, and nucleotide sequencing. The nucleotide sequences of all primers used in this study are listed in Table 2.

Growth rate and conidiation: We investigated growth rate by calculating radial colony extension and cell density of mycelia. After 2 μl of conidial suspension (1×10^6conidia/ml) of wild-type and *ganB* mutant strains were point inoculated onto supplemented minimal medium plates, the suspension was incubated for 4 days, then mycelial blocks 0.5 cm in diameter were isolated from the colony margin, and 30 separated mycelial blocks were inoculated into 50 ml of liquid minimal medium in the 250-ml flask and incubated for 4 days, shaking at 80 rpm. Mycelial cultures were harvested, freeze dried, and weighed.

For radial extension measurements, conidial suspensions were point inoculated into the centers of minimal medium plates and incubated for 5 days before measuring colony diameter. Radial extension rate was determined as extension length per hour (in millimeters per hour) and the values were expressed as percentages of the wild type.

To count the number of conidia, conidia (1×10^6) of wild type and *ganB* mutant strains were spread on minimal medium plates. After 5-day incubation, 0.8-cm agar blocks were vortexed in 1 ml of 0.08% Triton X-100 solution for 2 min and the resulting conidial suspension was counted with a hemacytometer. The number of conidiophores per square centimeter of medium plates was counted with a low-power microscope. The values for growth, conidia, and conidiophores are shown in Tables 3 and 4 as percentages of the wild type.

Germination of conidia and ascospore: Conidia of wild-type and mutant strains were inoculated into 20 ml of minimal medium in petri dishes containing autoclaved cover slips and incubated at 37°. We removed coverslips every 2 hr and observed swelling and germ tube emergence under the microscope. We also observed conidial germination of mutants in carbon-source-free medium.

To investigate germination of ascospores in *ganB* mutant alleles, wild-type and *ganB* mutants were incubated under sexual-development-inducing conditions for 10 days, when cleistothecia from each strain were isolated and suspended in 0.08% Triton X-100 solution. Cleistothecia and asci were bro-

TABLE 2

Primers used in this study

Primers	Sequence
GPPPR	CGG GTA CCT NGG NGC YGG NGA RTC NGG NAA RTC
GPGPR	GGG TAC CTC YTC RAA NAG RTC RAT YTT RTT NAG RAA
GPG′PR	CGG GAT CCA YTT YTT NCG YTC NGA NCG YTG NCC
gBup1	TCC CCC GGG ACG ACA ACT GAG TCG CCT TAG C
gBlp2	CCA TCG ATT TCT AGA TAC TGC CTT CGA CAG C
gBpr7	GAA GGC TCG TCG AGT CCT GAC
gBpr8	CCA CTG ATC GAG CGA CTA TGG
nAup1	CCG ACG TCC GAT CCA CTT CAG GGC TCC AT
nAup2	TAC TGC AGC CCG ACG TCA AC
nAlp3	GCT CTA GAT GAG CAG GGG GTC TGA GAA AA
gBmbup7	CGG ACG CTG TCG AAG GCA GT
gBmblp8	CGC AGG TGT GTG GTT TCG TTC
gBR182Lup9	CGC GCG TTA ACC AAG ACG AC
gBR182Llp10	TGG TTA ACG CGC GCA GCA C
gBQ208Lup11	GGC CTG CGC AGC GAG AG
gBQ208Llp12	GCG CAG GCC GCC AAC AT
gBG207Rup13	GTT GGC CGG CAG CGC AG
gBG207Rlp14	CGC TGC CGG CCA ACA TCA

ken with a mounted needle and vortexed. Ascospores released were inoculated into minimal medium with or without glucose and incubated as specified in the text.

RESULTS

The *ganB* gene encodes a Gα-protein homolog in *A. nidulans*: The *ganB* gene (GenBank accession no. AF198116) has five introns in its ORF and one in the 5′-untranslated region (Figure 1A). The *ganB* gene located in chromosome VIII contains an open reading frame consisting of 356 amino acids that are perfectly matched to those of AN1016.2 in the *A. nidulans* genome database at the Whitehead Institute Center for Genome Research (WICGR; http://www-genome.wi.mit.edu/annotation/fungi/aspergillus; Figure 1B). GanB has conserved domains that are considered to be directly involved in interaction with the guanine nucleotide found in all Gα-proteins (Simon *et al.* 1991; Skiba *et al.* 1996; Bohm *et al.* 1997), as well as the myristoylation site in the N terminus (Buss *et al.* 1987). The amino acid sequence of GanB shows 75–90% identity to the amino acids of GpaB (Liebmann *et al.* 2003) and GasC (Zuber *et al.* 2003) of the human pathogenic fungi *Aspergillus fumigatus* and *P. marneffei*, respectively; to the amino acids of Cpg-2 (Choi *et al.* 1995) and MagA (Liu and Dean 1997) of the phytopathogenic fungi, *Cryphonectria parasitica* and *Magnaporthe grisea*, respectively; and to those of GNA-3 (Kays *et al.* 2000) of *N. crassa*. When fungal Gα-proteins are classified according to amino acid sequence, these Gα-proteins belong to the group closely related to the mammalian $G\alpha_s$-subfamily that is involved in activation of adenylate cyclase (Bölker 1998; Zuber *et al.* 2002).

Northern blot analysis showed that the *ganB* transcript accumulated at higher levels during developmental stages than during vegetative growth, with greater levels during sexual development than during asexual development (Figure 1C).

Isolation of deletion, overexpression, constitutively active, and constitutively inactive mutants of the *ganB* gene: To address the function of GanB *in vivo*, deletion, overexpression, constitutively active, and constitutively inactive mutants of *ganB* were isolated. To obtain deletion mutants, we constructed a plasmid in which the GanB ORF was deleted and *argB* substituted as a selectable marker (see materials and methods). Because the presence of functional VeA affects the development pathway in *A. nidulans*, we isolated mutants from both strains of veA^+ (VER7) and *veA1* (RMS011). Eight *ganB* deletion mutants from VER7 and six from RMS011 were isolated and the deletion of *ganB* was checked by genomic Southern analysis. The fact that the transcripts of the *ganB* gene were not detected by Northern analysis of these mutants and sequencing the flanking region of inserted *argB* showed that the deletion was successful (data not shown).

We also isolated overexpression mutants of *ganB* using the *niiA* promoter that is induced in the presence of nitrate but repressed by ammonium (Johnstone *et al.* 1990; see materials and methods). Seven *ganB* overexpression mutants were isolated from VER7 and three from RMS011. Transcript levels for *ganB* were much higher in these mutants than in the parental strains VER7 and RMS011 (data not shown).

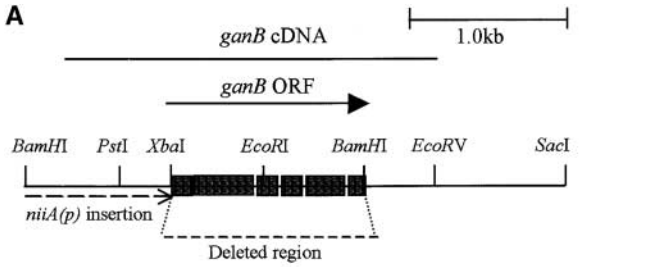
Gln208 in the switch II region and Arg182 in the switch I region of the GTPase domain of Gα protein (Figure 1) play important roles in the GTPase activity that hydrolyzes GTP bound to Gα protein to GDP (Simon

et al. 1991; Skiba *et al.* 1996; Bohm *et al.* 1997). Mutations at either of these sites largely abolish the hydrolytic activity of intrinsic GTPase and thus stabilize the active, GTP-bound state (Simon *et al.* 1991; Coleman *et al.* 1994). Many constitutively active mutants with substitutions of these amino acid residues have been isolated in a variety of fungal Gα proteins (Wieser *et al.* 1997; Yang and Borkovich 1999; Yu *et al.* 1999; Zuber *et al.* 2003). The constitutively active mutants (*ganB*^{Q208L} and *ganB*^{R182L}), in which the glutamine or arginine residue was substituted by leucine, were isolated by site-directed mutagenesis using PCR amplification (see materials and methods). Ten *ganB*^{Q208L} constitutively active mutants from VER7 and 5 from RMS011 were isolated. In addition, 12 *ganB*^{R182L} mutants were isolated from VER7. Since *ganB*^{R182L} and *ganB*^{Q208L} mutants showed the same phenotypes, we here present the results from only the *ganB*^{Q208L} mutant as phenotypes of *ganB* constitutive activation.

In addition, we isolated constitutively inactive mutants by substituting arginine for Gly207. This glycine residue in the conserved DXXGQ motif of the switch II domain (Figure 1) has been proposed to act as a hinge involved in the conformational change that accompanies guanine nucleotide exchange (Kurjan 1992; Bohm *et al.* 1997). The Gly207Arg mutation prevents conformational changes to the GTP-bound Gα-subunit, resulting in inhibition of the release of Gβγ; the G protein thus stays in a constitutively inactive state (Kurjan 1992; Yu *et al.* 1996; Zuber *et al.* 2003). The difference between deletion and inactivating mutants of the Gα-protein is that the former releases the Gβγ-subunit but the latter does not. Because of this, signal transduction by Gβγ as well as by Gα is blocked in the inactivated mutant. We isolated six *ganB*^{G207R} inactivated mutants through site-directed mutagenesis of VER7 and RMS011 (see materials and methods).

Constitutive activation of GanB causes reduction in mycelial growth: To determine the effect of GanB on growth, we compared the growth rates of wild-type and *ganB* mutants by measuring cell mass or radial colony expansion. While cell mass and radial extension rates for deletion, constitutively inactive, and overexpression mutants of *ganB* were similar to those of the wild type, constitutive activation of GanB caused decreases in cell mass and radial extension to 69 and 74% of wild type, respectively (Figure 2 and Table 3). No differences in the effect of *ganB* mutations on growth were observed between *veA*$^{+}$ and *veA1* strains.

Absence or constitutive inactivation of GanB induces hyperactive sporulation and derepression of *brlA* in submerged culture: To investigate the function of GanB in asexual development, we incubated *ganB* mutant strains in liquid minimal medium containing 0.1% yeast extract to observe conidiophore development in submerged culture where asexual development is normally sup-



B

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MGSCFSSESA GDVEQKKRSQ AIDRKLEEDS RRLRRECKIL LLGSGESGKS
TIVKQMKIIH QNGYTVEELA LYRLTVYKNL LECAKALIGA YHQFNLEPTS
QKVRDNIEFL ANYNIDPDPN IPLDPAVGDA ITYIWNDPCT STALERQNEF
                                 L
YLMDSAPYFF EEAKRITSPD YIPNVNDVLR ARTKTTGIYE TRFTMGQLSI
      RL                           Switch I
HMFDVGGQRS ERKKWIHCFE NVTSIIFCVA LSEYDQVLLE EGNQNRMMES
        Switch II                      Switch III
LVLFDSVVNS RWFMRTSIIL FLNKVDLFRQ KLPRSPLSNY FPDYSGGNDV
NRAAKYLLWR FNQVNRAHLN LYPHLTQATD TTNIRLVFAA VKETILQNAL
KDSGIL
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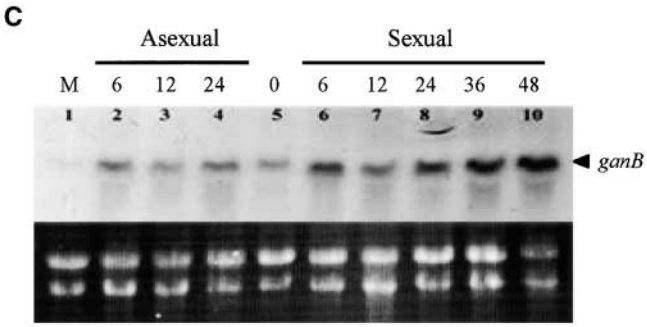


Figure 1.—The gene structure and transcription profile of *ganB* and the deduced amino acid sequence of GanB. (A) The gene structure and partial restriction map of *ganB*. The arrow indicates the direction of *ganB* transcription. Shaded boxes represent approximate exons of *ganB* ORF and interrupted regions indicate introns. Dashed line and dashed arrow indicate deleted region in the Δ*ganB* mutant and the *niiA* promoter insertion in *niiA(p)::ganB* mutant, respectively. (B) The deduced amino acid sequence of GanB. Three regions within the GTPase domain, termed switch I, II, and III, are underlined. The amino acids R182 and Q208 (in the constitutively active mutations) and G207 (in the constitutively inactive mutation) that were substituted by site-directed mutagenesis are in boldface type and the myristoylation site is underlined. (C) The expression of the *ganB* gene during the *A. nidulans* life cycle. The mycelia of the wild-type strain cultured in CM broth for 14 hr were transferred to a MM plate (for inducing conidiation) containing 0.1% casamino acid (for inducing sexual development) and incubated for the time indicated above lanes. For sexual development induction, plates were tightly sealed with parafilm for 20 hr. The total RNA was isolated from mycelia of vegetative growth (lane 1, M), asexual development inducing (lanes 2–4) and sexual development inducing (lanes 5–10), and hybridized with a *ganB*-specific probe.

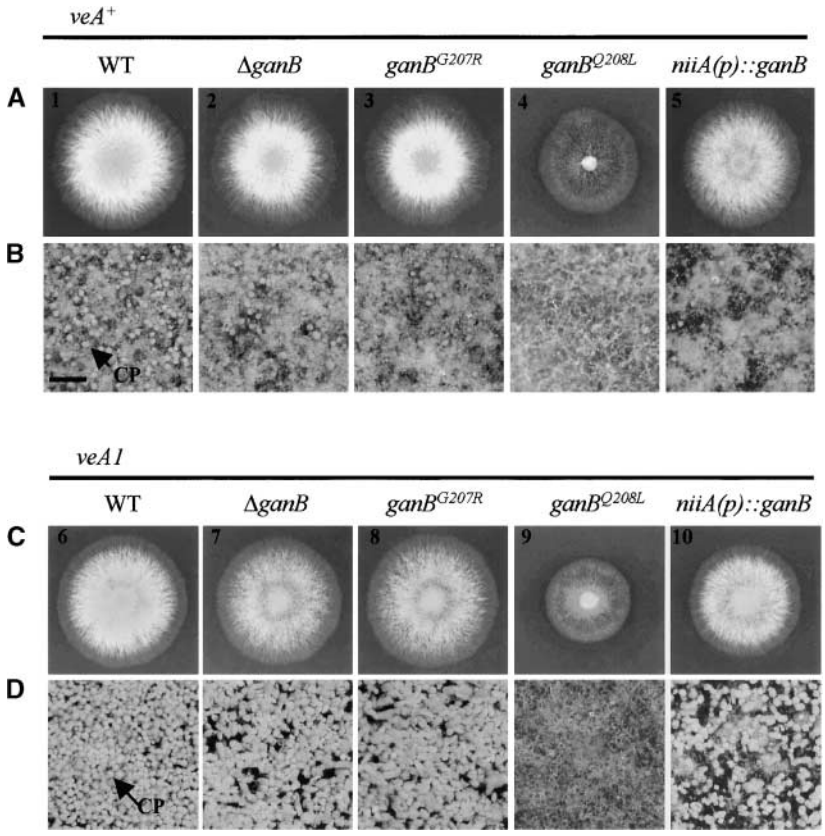


FIGURE 2.—Phenotypes of *ganB* mutant strains. (A and C) Conidia of wild types (1 and 6, V7p16 and RMp16), *ganB* deletion mutants (2 and 7, V7dgB08 and RMdgB03), *ganB* constitutively inactive mutants (3 and 8, V7gBCI1521 and RMgBCI1633), *ganB* constitutively active mutants (4 and 9, V7gBQL705 and RMgBQL801), and *ganB* overexpression mutants (5 and 10, V7ogB105 and RMogB204) were point inoculated in the centers of minimal medium plates and incubated for 3 days at 37°. Note that the radial extension of the *ganB* constitutively active mutant is reduced. (B and D) The strains are the same as above and were grown on minimal medium plates for 3 days after spreading to observe asexual development. CP indicates conidiophore. Note that conidia are not observed in the picture of constitutively active mutants. Bar in B, 200 μm.

pressed in the wild type even after >24 hr incubation. It was reported that the addition of yeast extract or casamino acid to minimal medium increased growth and the number of conidiophores (ROSÉN *et al.* 1999). The deletion and constitutively inactive mutants of *ganB* formed complete asexual conidiophores by 18 hr, whereas no development was seen in wild type, overexpression, or constitutively active mutants (Figure 3A). However, this submerged conidiation was observed only in a *veA1* background, not in *veA*$^+$ strains.

To determine whether the hyperactive sporulation phenotype observed in deletion and constitutively inactive GanB mutants was due to *brlA* expression, total RNA isolated from 18-hr submerged cultures was probed with a *brlA*-specific probe. The *brlA* transcript level was increased in deletion and constitutively inactive mutant strains, whereas no transcript was detected in the wild type (Figure 3B). The fact that the transcript of *brlA* was not detected in the submerged culture of any mutants in the *veA*$^+$ background (data not shown) might explain

TABLE 3

Radial extension of colony and mycelial density in *ganB* mutants

Relevant genotype	Radial extension (%)[a]	Mycelial density (%)[b]
WT, *veA*$^+$	100.0[c]	100.0[c]
Δ*ganB*, *veA*$^+$	94.4 ± 1.2	110.2 ± 6.5
ganB^{G207R}, *veA*$^+$	95.0 ± 2.8	106.7 ± 17.3
ganB^{Q208L}, *veA*$^+$	73.7 ± 4.0	69.0 ± 11.1
niiA(p)::ganB, *veA*$^+$	90.3 ± 2.0	94.5 ± 6.1
WT, *veA1*	100.0[c]	100.0[c]
Δ*ganB*, *veA1*	98.4 ± 1.5	96.5 ± 5.5
ganB^{G207R}, *veA1*	99.7 ± 1.5	97.4 ± 4.4
ganB^{Q208L}, *veA1*	71.0 ± 1.7	70.8 ± 12.7
niiA(p)::ganB, *veA1*	94.0 ± 1.1	84.0 ± 13.4

WT, wild type. ±, standard errors of the means.
[a] Three different plates from each strain were tested and the experiment was repeated six times.
[b] The values are the mean of three independent experiments.
[c] No difference was found between the 100% values of *veA*$^+$ and *veA1* strains.

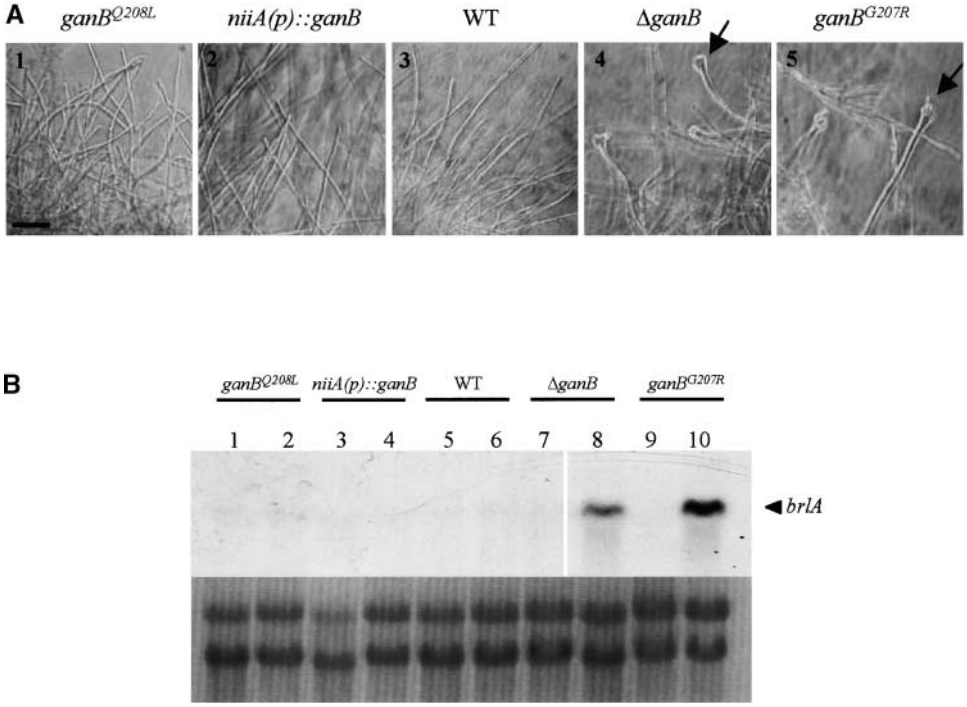


FIGURE 3.—Deletion and constitutively inactive mutants of the *ganB* gene show hyperactive sporulation and derepress *brlA* expression in submerged culture. (A) Conidia of designated strains in a *veA1* background were inoculated into 50 ml liquid minimal medium containing 0.1% yeast extract and incubated at 37°, shaking at 200 rpm. The photographs were taken of samples harvested at 18 hr. Arrows indicate complete conidiophores. (B) Total RNA was isolated from $ganB^{Q208L}$ (lanes 1 and 2), *niiA(p)::ganB* (lanes 3 and 4), wild type (lanes 5 and 6), Δ*ganB* (lanes 7 and 8), and $ganB^{G207R}$ (lanes 9 and 10) grown for 12 hr (lanes 1, 3, 5, 7, and 9) and 18 hr (lane 2, 4, 6, 8, and 10) under the same conditions as above. The RNA blots were probed with a *brlA*-specific probe. Arrowhead in B indicates *brlA* transcript. Bar in A, 50 μm.

why no submerged conidiation was observed in deletion or constitutively active mutants with veA^+ background. These results suggest that activation of GanB may repress conidiophore formation in liquid culture and downregulate *brlA* transcript levels.

Activation or overexpression of GanB reduces conidiation on solid medium: In surface cultures, formation of both conidiophores and conidia by constitutively active *ganB* mutants was dramatically decreased to <2.2% of the wild type in veA^+ strains, with a slightly less marked reduction in conidophore formation in the *veA1* background (Figure 2 and Table 4). Overexpression mutants also showed a reduction in conidiation but to a lesser extent than constitutively active mutants. Deletion or constitutive inactivation of GanB reduced conidiation to a certain extent on solid medium (Figure 2 and Table 4). This contrasts with the hyperactive sporulation phenotype of these mutants in submerged culture. Furthermore, conidiophore formation and the conidiation rate in the mutants having the veA^+ allele is more aggravated, compared with the mutants having *veA1* mutation. Especially, the relative conidiophore formation rate (6.7 ± 3.7%) of the *ganB* overexpression mutant to a wild type in the veA^+ background is much lower than that (35.3 ± 1.5%) in the *veA1* background (Table 4), suggesting that VeA may act synergistically with the GanB subunit to regulate asexual sporulation negatively. This is consistent with the suggestion of KIM *et al.* (2002) that *veA* may act as a negative regulator of asexual development.

TABLE 4

Conidiation of *ganB* mutants

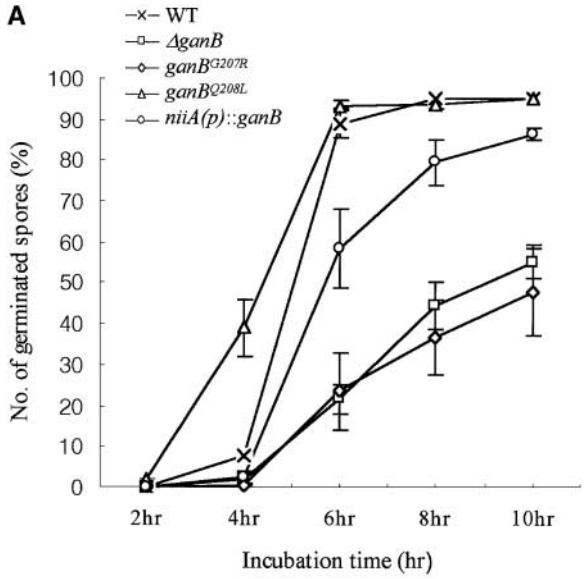
Strains	Conidia (%)[a]	Conidiophores (%)[a]
WT, veA^+	100.0[b]	100.0[b]
Δ*ganB*, veA^+	48.4 ± 37.8	37.8 ± 25.3
$ganB^{G207R}$, veA^+	36.4 ± 21.1	29.2 ± 16.7
$ganB^{Q208L}$, veA^+	2.5 ± 1.4	0.2 ± 0.2
niiA(p)::ganB, veA^+	5.8 ± 0.9	6.7 ± 3.7
WT, *veA1*	100.0[b]	100.0[b]
Δ*ganB*, *veA1*	94.4 ± 21.3	56.8 ± 4.0
$ganB^{G207R}$, *veA1*	105.1 ± 32.7	64.3 ± 12.5
$ganB^{Q208L}$, *veA1*	1.1 ± 0.9	2.2 ± 1.8
niiA(p)::ganB, *veA1*	54.8 ± 13.8	35.3 ± 1.5

±, standard errors of the means.

[a] Three different plates from each strain were tested and the experiment was repeated three times.

[b] In the two wild-type strains, the actual number of conidia and conidiophore of the veA^+ strain were, respectively, one-twelfth and one-fifth of those in the *veA1* background.

GanB plays a crucial role in spore germination: When conidia of deletion or constitutively inactive mutants of *ganB* were cultured in liquid medium, the number of mycelial balls was reduced relative to the conidium inoculum size (data not shown). This suggested that GanB might affect germination or conidial viability. To examine germination in various mutant strains, conidial swelling and germ tube emergence were observed under the microscope. Whereas conidial swelling and germ tube formation in deletion or constitutively inactive *ganB* mutants were delayed, those of constitutively active mutants were facilitated (Figure 4A). Wild-type conidia started to swell 2 hr after inoculation and only 8% of them had formed germ tubes by 4 hr. Meanwhile, ~40% of conidia of constitutively active *ganB* mutants had germ tubes by 4 hr. By 6 hr, >90% of conidia of both wild



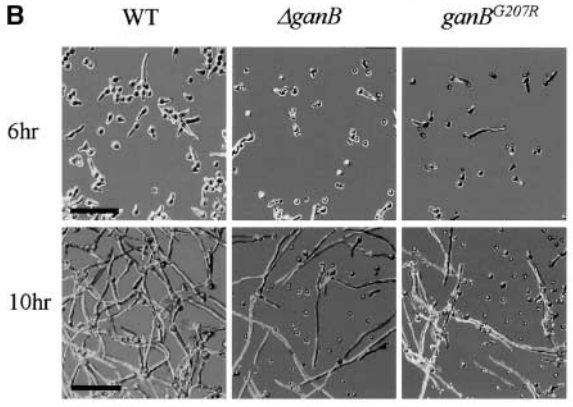


FIGURE 4.—Conidial germination rate was delayed in *ganB* deletion and constitutively inactive mutants but was accelerated in a constitutively activate mutant. (A) Kinetics of germ tube outgrowth. Conidia of designated strains were inoculated into minimal medium and incubated at 37°. The number of conidia per microscope field showing germ tube was counted and presented as the percentage of total conidia in this field. The values are the mean of three independent experiments and ± represents standard error of the mean. (B) Deletion and constitutively inactive mutants of *ganB* showed asynchronous and delayed germination. Photographs were taken after 6 and 10 hr incubation at 37°. Bars in B, 50 μm.

type and the constitutively active mutant had formed the germ tubes. In contrast, very few conidia of deletion or constitutively inactive mutants had germ tubes by 4 hr, and only half of the conidia had produced germ tubes even after 10 hr. Deletion and constitutively inactive mutants also showed a highly asynchronous germination pattern (Figure 4B). These results imply that activation of GanB seems to play an important but dispensable role in early signaling for germination.

We further examined the effects of *ganB* mutations on ascospore germination. Ascospores were inoculated into minimal medium containing glucose. As with conidia, the constitutive activation of GanB led to precocious ascospore germination while *ganB* deletion and constitutively inactive mutants germinated very poorly (data not shown). These results indicate that activated GanB is a positive signal transducer for initiation of germination in both kinds of spores.

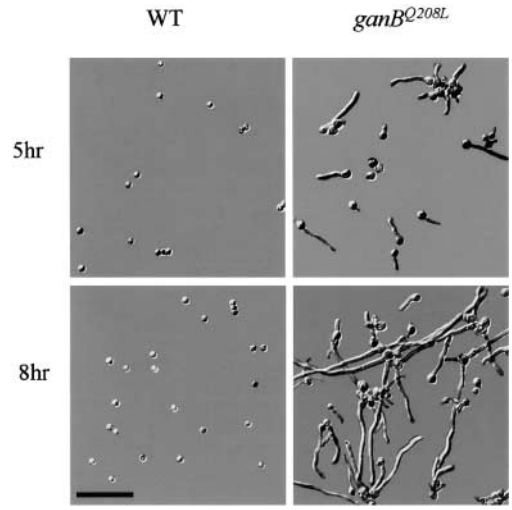


FIGURE 5.—Constitutive activation of GanB led to germination without carbon source. Conidia of wild type (WT) and the *ganB* constitutively active mutant (*ganB*^{Q208L}) were inoculated into minimal medium without carbon source and incubated at 37°. Photographs were taken after 5 and 8 hr incubation. Bar, 40 μm.

Constitutive activation of GanB allows germination without carbon source: When the constitutively active mutant form of RasA is overexpressed in *A. nidulans*, germinating conidia form multinucleate swollen spheres but fail to produce germ tubes (SOM and KOLAPARTHI 1994), and this occurs even in the absence of a carbon source (OSHEROV and MAY 2000). To examine whether *ganB* constitutively active mutants could germinate without carbon source, conidia of mutant strains were inoculated into carbon-free minimal medium. After 5 hr incubation most conidia of the constitutively active mutant formed germ tubes (Figure 5). In contrast, very few swollen cells or germ tubes were observed in wild type even after 24 hr.

In minimal medium without carbon source, ascospore germination showed a slightly different pattern from that of conidia. The germination rate of wild-type ascospores was comparatively higher (~20–30%) than that of conidia under conditions without a carbon source. Whereas the germination rate of constitutively active mutant ascospores increased to ~50% when compared to that of wild type, deletion or constitutively inactive mutants showed a germination defect as observed in conidia (data not shown).

DISCUSSION

Constitutive activation of GanB reduces hyphal growth: G proteins, for example, FadA Gα-subunit and SfaD

Gβ-subunit of *A. nidulans*, are involved in regulating vegetative growth in fungi. To address whether GanB signaling regulates vegetative growth, we examined growth rate in *ganB* mutants constructed in this study. Of these, only constitutively active mutants showed any reduction in radial extension rate and cell mass when compared to other mutants and the wild type (Table 3 and Figure 2). The fluffy phenotype and hyphal autolysis observed in the constitutively active mutant of FadA (Yu *et al.* 1996, 1999) was not seen in the equivalent mutant of GanB. The slight effect of *ganB* mutation on hyphal growth suggests that GanB may not be a key element regulating vegetative growth. Instead, GanB might act as a signal transducer in developmental processes that compete with hyphal growth. This hypothesis is supported by the phenotype of deletion or constitutively inactive mutants whose growth rate is not increased compared to the wild type.

Active GanB negatively regulates asexual sporulation: When cultured on solid media, the number of conidiophores and conidia in *ganB* mutants was reduced (Table 4, Figure 2). The constitutively active mutant especially showed a severe decrease in conidiophore formation compared with other mutants, indicating that active GanB may be a negative regulator of conidiation. To confirm this hypothesis, we cultured *ganB* mutants in liquid medium where development, including conidiation, is normally suppressed. It was reported that starvation for carbon and/or nitrogen could induce submerged conidiation in *A. nidulans* (Skromne *et al.* 1995). Inappropriate submerged conidiation also occurs in mutants of genes involved in asexual development. For example, submerged conidiation was observed in a deletion mutant of *sfaD* (Rosén *et al.* 1999), a dominant inactivating mutant of *fadA* (Yu *et al.* 1996), and in an overexpression mutant of *brlA* (Adams *et al.* 1988). Deletion and constitutively inactive mutants of *ganB* also showed inappropriate submerged conidiation (Figure 3A). Since it seems likely that there are a Gβ-subunit (AN0081.2), a Gγ-subunit (AN2742.2), and three Gα-subunit homologs (AN0651.2, AN3090.2, and AN1016.2) in the *A. nidulans* genome database of WICGR (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus), there may be three forms of heterotrimeric G proteins in this fungus. Whereas the constitutively inactive form of GanB is predicted to interfere with signaling by both the Gβγ-subunit and GanB itself, the deletion mutation of *ganB* is expected to interfere only with GanB signaling and not with Gβγ-signaling. We therefore suggest that the submerged conidiation observed in deletion and constitutively inactive *ganB* mutants is likely due to the lack of active GanB. This hypothesis is contrary to that proposed by the report of Rosén *et al.* (1999) in which the absence of the SfaD Gβ-subunit leads to submerged conidiation in liquid medium.

Recently, it has been reported that deletion of Gβ causes a reduction in Gα-protein levels in several fungi, possibly by post-transcriptional regulation. In *N. crassa*, deletion of the Gβ-gene, *gnb-1*, leads to downregulation of GNA-1, GNA-2, and GNA-3 Gα-proteins under a variety of medium conditions (Yang *et al.* 2002). Also, in *C. parasitica*, the level of the Gα-protein CPG-1 is greatly reduced in a deletion mutant of *cpgb-1* that encodes Gβ-protein (Parsley *et al.* 2003). These findings may give us an answer to the question of why a deletion mutant of *sfaD* causes submerged conidiation in liquid culture: the absence of Gβγ-signaling, caused by deletion of *sfaD*, may lead to a reduced GanB protein level, as in the situation described above for *N. crassa* and *C. parasitica*, and result in failure of active GanB to repress conidiation. However, we cannot rule out the possibility that the lack of GanB may reduce the Gβ-protein level, resulting in submerged conidiation. Endpoint dilution immunoblot analysis showed that the protein level of the CPGB-1 Gβ-subunit was slightly reduced in the absence of the CPG-1 Gα-subunit in *C. parasitica* and dramatically reduced in a double deficiency of CPG-1 and CPG-2 Gα-proteins (Parsley *et al.* 2003). These conditions lower the level of the Gα-subunit, resulting in submerged conidiation as in the *sfaD* deletion. We need more experiments to explain whether GanB alone, SfaD alone, or GanB and SfaD together negatively regulate asexual development.

Absence or inactivation of GanB derepresses the expression of *brlA* in a liquid culture: The *brlA* gene is not transcribed under conditions where conidiation is not active (Adams *et al.* 1988) and if submerged conidiation observed in deletion or constitutively inactive *ganB* mutant strains is mediated through the BrlA pathway, *brlA* should be transcribed in liquid cultures of these mutants. As expected, *brlA* transcripts were detected only in deletion and constitutively inactive *ganB* mutants (Figure 3B). These results indicate that negative regulation of asexual sporulation by active GanB may be mediated through repression of *brlA* expression at the transcriptional level.

The *veA* gene encodes a putative transcription activator that may have dual functions in regulating developmental processes, acting as a positive regulator of sexual development and a negative regulator of asexual development (Kim *et al.* 2002). Submerged conidiation of *ganB* deletion or constitutively inactive mutants was not observed in strains carrying the *veA*$^{+}$ allele but only in a *veA1* genetic background, indicating that the *veA* gene is involved in negatively regulating submerged conidiation. Submerged conidiation has also been observed in a *sfaD* deletion mutant in *veA1* but not in *veA*$^{+}$ backgrounds (Rosén *et al.* 1999). This suggests that the suppression of asexual development in submerged culture requires active GanB and VeA proteins.

GanB plays critical roles in the early events of spore germination: In filamentous fungi, early signaling events in germination, including carbon source sensing, trehalose breakdown, swelling (isotropic growth), and germ tube emergence, are not well characterized because of the difficulty of dissecting germination at a molecular level (d'Enfert 1997; Osherov and May 2001). In

several filamentous fungi, germination may be controlled by multiple pathways, resulting in complication of genetic analysis. In addition to true early germination signaling events, essential metabolic and housekeeping activities occur at the beginning of the germination, making it difficult to differentiate between true signaling events and these activities (Osherov and May 2001). Nevertheless, in *A. nidulans*, some components of a conidial germination signal transduction system have been isolated and characterized. A RasA has been shown to have a function in carbon source sensing during conidial germination (Som and Kolaparthi 1994; Osherov and May 2000). Recently, it was reported that cAMP/PkaA signaling plays an important role in conidial germination and it has also been shown that cAMP signaling and RasA act independently on conidial germination (Fillinger *et al.* 2002).

Our results indicate that active GanB plays a critical role during early events of germination of both conidia and ascospores in *A. nidulans*. GasC, the GanB homolog of *P. marneffei*, plays a role in conidial germination (Zuber *et al.* 2003). Unlike GanB, GasC does not seem to have a function of carbon sensing since conidia of the dominant activating mutant do not germinate in the absence of carbon source, suggesting that in this fungus there are separate mechanisms for carbon source sensing and for other early events of germination such as swelling and nuclear decondensation. Furthermore, the majority of ungerminated conidia of deletion or constitutively inactive *ganB* mutants cultured in minimal medium remained unswollen (data not shown), suggesting that GanB signaling is also involved in conidial swelling. Because the constitutively active *ganB* mutant showed successful swelling and germ tube emergence, and deletion or constitutively inactive mutants left many conidia unswollen, establishment of carbon source sensing is perhaps the earliest step in germination. Trehalose breakdown is defective in the *ganB* deletion mutant, suggesting that GanB signaling may play a role in the activation of adenylate cyclase (Lafon *et al.* 2003; C. d'Enfert, personal communication). Taken together, these results lead to the conclusion that GanB signaling mediates early germination events in *A. nidulans*. Similar results were obtained with ascospores: *ganB* deletion and constitutively inactive mutants showed defects in swelling and germ tube formation (data not shown). Thus GanB signaling is involved in regulating germination of ascospores as well as of conidia.

Hypothetical model for the function of GanB: The above results lead us to propose that GanB may function in signaling pathways of asexual development and germination (Figure 6). GanB negatively regulates asexual development by repressing *brlA* expression at least in submerged culture. In addition to GanB, the VeA protein is necessary to repress submerged conidiation possibly by repressing the *brlA* expression. GanB also positively controls early events, including carbon source sensing during germination. One of the candidates for GanB downstream targets in the signaling pathway regulating germination might be cAMP/PkaA, which is reported to have a function in conidial germination (Fillinger *et al.* 2002).

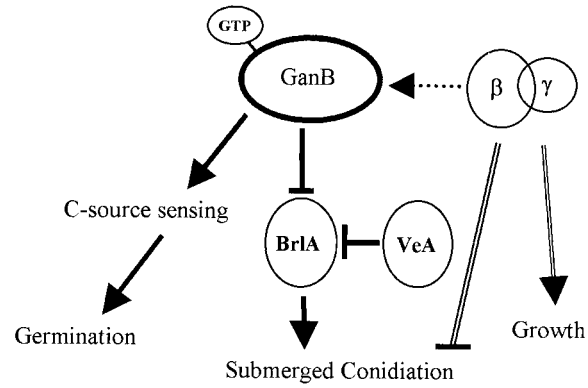


Figure 6.—Proposed model of the contribution of GanB in the signaling pathways of germination and conidiation. GanB negatively regulates asexual development by repressing *brlA* expression and also positively controls early events, including carbon source sensing during germination. The Gβ-protein SfaD is known to be a positive regulator of growth and a negative one of submerged conidiation (Rosén *et al.* 1999), which are depicted by double lines ending with an arrow and a bar, respectively. On the other hand, SfaD may function in maintaining the protein level of Gα (Yang *et al.* 2002; Parsley *et al.* 2003; also see discussion), which is represented by the dashed arrow.

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