The Heterotrimeric G-Protein Subunits GNG-1 and GNB-1 Form a Gβγ Dimer Required for Normal Female Fertility, Asexual Development, and Ga Protein Levels in *Neurospora crassa*

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We have identified a gene encoding a heterotrimeric G protein γ subunit, *gnγ-1*, from the filamentous fungus *Neurospora crassa*. *gnγ-1* possesses a gene structure similar to that of mammalian Gγ genes, consisting of three exons and two introns, with introns present in both the open reading frame and 5' untranslated region. The GNG-1 amino acid sequence displays high identity to predicted Gγ subunits from other filamentous fungi, including *Giberella zeae*, *Cryptococcus parasitica*, *Trichoderma harzianum*, and *Magnaporthe grisea*. Deletion of *gnγ-1* leads to developmental defects similar to those previously characterized for Δ*gnb-1* (Gβ) mutants. Δ*gnγ-1*, Δ*gnb-1*, and Δ*gnγ-1 Δgnb-1* strains conidiate inappropriately in submerged cultures and are female sterile, producing aberrant female reproductive structures. Similar to previous results obtained with Δ*gnb-1* mutants, loss of *gnγ-1* negatively influences levels of Ga proteins (GNA-1, GNA-2, and GNA-3) in plasma membrane fractions isolated from various tissues of *N. crassa* and leads to a significant reduction in the amount of intracellular cyclic AMP. In addition, we show that GNB-1 is essential for maintenance of normal steady-state levels of GNG-1, suggesting a functional interaction between GNB-1 and GNG-1. Direct evidence for a physical association between GNB-1 and GNG-1 in vivo was provided by coimmunoprecipitation.

G-protein-linked pathways evolved to allow responses to extracellular agonists (hormones, neurotransmitters, odors, chemoattractants, light, and nutrients) in eukaryotic cells, ranging from simpler systems, including yeasts, filamentous fungi, and slime molds, to more complex organisms, such as mammals. The G protein βγ dimer performs numerous roles during the signal transduction process (for reviews, see references 14 and 32), including membrane targeting of the α subunit (23), recognition of receptors (46), activation of downstream effectors (14), and modulation of different proteins affecting signal intensity or duration (47). Multiple isoforms, including 6 Gβ and 12 Gγ subunits, have been identified in mammals (14, 32, 50). In mammals, a major challenge for in vivo identification of Gβγ dimers and establishment of their roles in particular signaling pathways arises from the variety of possible combinations between β and γ subtypes.

In contrast to the situation with mammals, only one Gβ subunit is present in all sequenced fungal genomes (http://www.yeastgenome.org; http://www.genedb.org/genedb/pombe/index.jsp; http://www.broad.mit.edu/annotation/fungi) (27). For the budding yeast *Saccharomyces cerevisiae*, previous studies have indicated that the Ste4p Gβ functions as a positive regulator of the pheromone response in haploid cells by activation of the downstream mitogen-activated protein kinase cascade, leading to cell cycle arrest, shmoo formation, cell fusion, and karyogamy (for reviews, see references 22 and 42). Gpa1p, the Go protein that interacts with Ste4p, functions as a negative regulator of the pathway. In the fission yeast *Schizosaccharomyces pombe*, the Gβ subunit Git5 is required for glucose sensing and mating through activation of cyclic AMP (cAMP) signaling (45). In the basidiomycete human pathogenic fungus *Cryptococcus neoformans*, deletion of the Gβ subunit gene *GFB-1* results in sterility and defective monokaryotic fruiting (72). Mutation of the Gβ gene *sfaD* from the filamentous fungus *Aspergillus nidulans* leads to hyperactive conidiation (aseptate sporulation) and reduced vegetative growth (56). In the chestnut blight pathogen *Cryptonectria parasitica*, disruption of the *cgpb-1* Gβ subunit gene negatively affects virulence, conidiation, pigmentation, and hyphal branching, while stimulating growth on vegetative solid medium (40). In *Magnaporthe grisea*, the causative agent of rice BLAST disease, mutants disrupted in the Gβ subunit *MGβ1* exhibit reduced growth and conidiation, defective appressorium formation, and reduced intracellular cAMP levels (51). Loss of *gnb-1* in the filamentous fungus *Neurospora crassa* leads to inappropriate conidiation in submerged culture, altered mass accumulation on solid medium, production of aberrant fertilized female reproductive structures, reduced intracellular cAMP levels, and low levels of all three Gα subunits (80).

Gγ subunits belong to a large family of small proteins consisting of 68 to 75 amino acids with different primary structures in various species (6, 20, 28). All Gγ proteins contain the CaaX box motif at the carboxy terminus that is subject to posttranslational modification, including isoprenylation and subsequent carboxyl methylation (28, 82). This posttranslational modification of Gγ subunits determines the subcellular localization of the Gβγ complex, in that it targets the heterodimer to the plasma membrane (36, 48, 58). The carboxy-terminal modification of Gγ is also necessary for effective interaction of Gβγ with other proteins, including Go, downstream effectors, and receptors (12).

Only a single Gγ subunit gene has been identified in the yeasts *S. cerevisiae* (*STE18*) and *S. pombe* (*git11*) (45, 76). In *S.
during homology searches (BLAST) (1) of the N. crassa conidia were used to inoculate all cultures. Plasmids were maintained in development of female reproductive structures. Sorbose-containing medium was used growth, while synthetic crossing medium (SCM) (74) was used to induce develop-
113-1 FH1
5-5-12

Materials and methods
Strain manipulations and media. Neurospora strains used in this study are listed in Table 1. Vogel’s minimal medium (VM) (70) was used for vegetative growth, while synthetic crossing medium (SCM) (74) was used to induce development of female reproductive structures. Sorbose-containing medium was used to facilitate colony formation on plates (16). If required, hygromycin B (Calbiochem) was added to media at a concentration of 200 µg/ml. Seven-day-old conidia were used to inoculate all cultures. Plasmids were maintained in Esch. ene coli strain DH5α (33).

Isolation and sequencing of the gng-1 gene. A Gy gene was initially identified during homology searches (BLAST) (1) of the N. crassa cDNA database at the University of Oklahoma (http://www.genome.ou.edu) using the protein sequence of S. cerevisiae Ste18p. Two cDNA clones, b7a10ne and a8h02ne, encoding hypothetical proteins similar to Gy subunits, were identified. The 1.2-kb insert of a8h02ne was used to screen a BARGEM-7a genomic library (53). Two positive plaques were obtained and converted to double-stranded plasmids (53), and they were subsequently subjected to Southern analysis using the insert from a8h02ne as a probe. Both cDNA clone a8h02ne and one of the genomic clones (designated #31; insert size, 4.5 kb) were sequenced (Core Sequencing Facility, Department of Microbiology and Molecular Genetics, University of Texas–Hous-

TABLE 1. N. crassa strains

<table>
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<tr>
<th>Strain</th>
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<th>Source or reference</th>
</tr>
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<td>Strain expressing FLAG-tagged GNG-1</td>
<td>This study</td>
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a FGSC, Fungal Genetics Stock Center, Kansas City, Mo.

b FH1, a forced heterokaryon between the Δgng-1 his-3 (h+J) strain and the a−1 FGSC #4564 helper strain, was crossed to Δgng-1 strain 5-5-3. Progeny carry genes from the h+J and 5-5-3 strains backgrounds only, as the a−1 nucleus is not passaged during a cross.
detected were analyzed with Western blotting and Coomassie Blue staining.
proteins were subsequently transferred to PVDF membranes (Millipore Corp.). Western analysis was performed as described above, using anti-FLAG M2 monoclonal (1:1,000; Sigma), anti-GNG-1 (1:3,000), and anti-GNB-1 (1:5,000) as primary antibodies.

**Phenotypic analysis.** To determine apical extension rates, 1 μl of a conidial suspension was inoculated in the center of VM plates and the plates were incubated at 30°C in the dark. The colony diameter was measured at 2-h intervals. To analyze phenotypes in submerged cultures, liquid VM was inoculated with conidia at a final concentration 10^6 cells/ml and incubated with shaking at 200 rpm for 16 h at 30°C. Cultures were then viewed and photographed using a BX41 fluorescent microscope and a C-4040 digital camera (Olympus, Lake Success, N.Y.). Unfertilized (6-day-old protoperithecia) and fertilized (3-day-old perithecia) female tissues were grown on SCM plates in light and were observed using an SZX9 stereomicroscope with an ACH 1 objective lens outfitted with an SZX9 stereomicroscope with an ACH 1 objective lens (Olympus).

**Measurement of intracellular steady-state cAMP levels.** For measuring in vivo cAMP levels, 16-h submerged cultures and tissues grown on VM plates for 3 days at 30°C in the dark and SCM plates incubated at 25°C in constant light were ground in liquid nitrogen and extracted as previously described (38). cAMP levels were quantified using a protein binding assay following the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, N.J.). The protein concentration was determined using the bichinonic acid method (Pierce) as described elsewhere (38).

Nucleotide sequence accession number. The GenBank accession number for the gng-1 cDNA clone a8h02ne is AY823297.

**RESULTS**

**gng-1 isolation, gene structure analysis, and mRNA expression profile.** Two cDNA clones (b7a10ne and a8h02ne) similar to the S. cerevisiae G protein subunit Ste18p were identified using BLAST (1) in the Neurospora database at the University of Oklahoma (http://www.genome.ou.edu). The gng-1 ORF is 279 bp, and the predicted GNG-1 protein consists of 93 amino acid residues with a molecular mass of 10 kDa (Fig. 1). GNG-1 shows relatively high identity to G proteins from other filamentous fungi: 90% to G. zeae, 92% to C. parasitica, 86% to Trichoderma harzianum, 86% to M. grisea, 65% to A. nidulans, and 55% to Ustilago maydis. Interestingly, N. crassa GNG-1 shares only 40% identity with S. cerevisiae Ste18p (76) and even less identity with S. pombe Git11 (9%) (45), indicating evolutionary divergence between filamentous fungi and yeasts. In addition, as a group, fungal G proteins display very little identity (less than 20%) to mammalian G proteins (data not shown). Predicted G proteins from U. maydis (UM 06109.1) and M. grisea (MG10193.4) were identified in genome databases at http://www.broad.mit.edu/annotation/fungi. However, the positions of introns and exons in the two genes were predicted incorrectly by the automatic gene caller. Therefore, both genes were annotated manually, and the resulting protein sequences were used in the alignment (Fig. 1).

In order to isolate a genomic clone, the 1.2-kb insert of cDNA clone a8h02ne was used to screen a BARGEM-7α genomic library (53). The screen resulted in isolation of two genomic clones designated #31 (4.5-kb insert) and #2231 (6.5-kb insert). The entire nucleotide sequence of the a8h02ne cDNA and partial nucleotide sequence of genomic clone #31.
were used to determine the gene structure of gng-1 (Fig. 2A). The gng-1 gene contains one 96-bp intron in the ORF, from +162 to +257. Another 315-bp intron is present in the 5′-untranslated region (UTR) of the mRNA (−510 to −196). All of the exon-intron boundaries conform to the GT-AG rule for intron splice sites.

The sequence of the 5′ region upstream of the gng-1 ORF was obtained (http://www.broad.mit.edu/annotation/fungi/neurospora) and analyzed for potential transcriptional regulatory motifs (Fig. 2A). No identifiable pyrimidine-rich regions (31) or TATA box consensus sequences (75) were present. Nevertheless, two putative transcriptional regulatory motifs were observed: one CTTTG at −320 (4) and one CAAAT box at −453 (31).

In order to elucidate the expression of gng-1 throughout development, Northern analysis was used to examine gng-1 transcript levels in conidia, 8- and 16-h submerged cultures, and VM and SCM plates. gnb-1 message levels were also measured during the experiment. A 1.2-kb gng-1 transcript was detected in all tissues (Fig. 2B and data not shown). This size is similar to the insert sizes (1,198 bp) of the two independent cDNA clones (b7a10ne and a8lt02ne). The results show that gng-1 is differentially expressed during the life cycle of N. crassa and that the highest expression levels of gng-1 are in 8-h submerged cultures and protoperithecial tissue from SCM plates (Fig. 2B). The lowest levels of gng-1 were detected in conidia and in tissues grown on VM plates. Comparison of gnb-1 and gng-1 message levels shows that these two genes share a similar expression pattern (Fig. 2B) (80). A possible exception is on SCM plates, where gng-1 may have higher relative expression levels than gnb-1. Observation of similar expression profiles has also been reported for the single Gβ and Gγ in Dictyostelium discoideum (83).

Deletion of gng-1 by targeted gene replacement and isolation of a Δgng-1 gng-1 recomplemented strain. A Δgng-1 mutant was isolated after electroporation of a wild-type strain with a construct in which the gng-1 ORF was replaced by the hygromycin B cassette (Fig. 2A) (66). Genomic DNA from transformants was digested with NcoI and subjected to Southern analysis using the 1.8-kb DNA fragment (SalI-EcoRV) from pSVK3 as a probe (Fig. 2C). Under these conditions, the wild-type strain produces a 5.7-kb hybridizing fragment, while a 2.8-kb fragment is obtained (http://www.broad.mit.edu/annotation/fungi/neurospora). No identifiable pyrimidine-rich regions were observed: one CTTTG at −320 (4) and one CAAAT box at −453 (31).

The Δgng-1 mutation was complemented in trans using the 6.5-kb gng-1 genomic fragment in the his-3 targeting vector pRAUW123 (2). Transformants were screened for conferral of histidine prototrophy. Homokaryons were obtained by using microconidial isolation (21). Both gng-1 mRNA and GNG-1 protein were detected at appreciable levels in Δgng-1 gng-1 recomplemented strains (Fig. 2D and E).

Δgng-1 strains are female sterile and male fertile. In N. crassa, sexual development is induced by nitrogen starvation, with formation of female reproductive structures (protoperithecia) containing specialized hyphae, termed trichogynes (55). Trichogynes exhibit chemotropic growth towards male gametes (conidia or other vegetative cells) of opposite mating type (9), followed by fusion and recruitment of a male nucleus to the base of the protoperitheicum. The nuclei from the male and female parents recognize one another and migrate to croziers (ascogenous hyphae), where they undergo mitosis. Subsequent fusion of male and female nuclei is followed by two meiotic divisions and one episode of postmeiotic mitosis. Each resulting ascus contains eight homokaryotic, haploid ascospores. About 200 to 400 ascii are enclosed in each mature fruiting body (perithecium).

Previous studies have shown that Δgnb-1 mutants are female sterile but are fertile as males during sexual crosses (80). Δgnb-1 mutants are able to form protoperithecia but fail to develop fruiting bodies after fertilization (80) (Fig. 3A). Δgng-1 strains and Δgng-1 Δgnb-1 double mutants exhibit a phenotypic pattern identical to that of Δgnb-1 strains (Fig. 3A). Although they produce reproductive structures, development of normal perithecia after fertilization is blocked (Fig. 3A), and no ascospores are produced (data not shown). In contrast, Δgng-1 gng-1Δgng-1−rescued strains are phenotypically identical to the wild type (Fig. 3A).

Our laboratory has demonstrated that Δgnb-1 mutants are deficient in both trichogyne attraction and perithecial development (44, 80). In order to determine whether a similar defect is present in Δgng-1 strains or Δgng-1 Δgnb-1 double mutants, microconidia of opposite mating type were applied at a distance from wild-type, Δgng-1, Δgnb-1, or Δgng-1 Δgnb-1 double mutant protoperithecia. Growth of trichogyne tips towards male cells was then followed microscopically (8, 44). In a previous study (44), Δgska-1 and Δgnb-1 mutants did not display directional migration but instead grew in random directions and failed to undergo fusion with male cells, even when in direct contact. Similarly, trichogynes of Δgng-1 and Δgnb-1 Δgng-1 Δgnb-1 Δgng-1 strains did not respond to microconidia and exhibited random orientation on the agar surface during this analysis (Fig. 3B). Δgng-1 gng-1 Δgng-1−complemented strains resembled the wild type, with normal trichogyne migration and fusion with microconidia (Fig. 3B). These data support the hypothesis that GNA-1 and Gβγ (GNB-1/GNG-1) are essential for trichogyne chemotropism during the pheromone response and for subsequent fusion with male gametes. The observations from previous work suggested that GNA-1 is coupled to PRE-1 (the matA pheromone receptor), because Δpre-1 strains exhibit the same defects in trichogyne chemotaxis as Δgna-1 mutants (44).

Δgng-1 mutants conidiate inappropriately in submerged culture. During vegetative growth, N. crassa produces tubular filaments (hyphae) characterized by tip-based polarized growth. We analyzed the rate at which strains extended vegetative hyphae on VM medium. Apical extension rates of Δgnb-1 and Δgng-1 single and double mutants are similar to those of the wild type and Δgna-3 mutants (41, 80, and data not
FIG. 2. Structure of the N. crassa gng-1 genomic region and construction of Δgng-1- and Δgng-1 gng-1⁺-rescued strains. (A) gng-1 genomic clone and gene replacement vector. The grey area indicates the gng-1 ORF, and the hatched region corresponds to the gene conferring hygromycin resistance, hph, under control of the A. nidulans trpC promoter. The dashed lines illustrate the region replaced by hph that is between the second SpeI and first KpnI sites. The open triangles indicate intron positions (-511 to -197; +162 to +258). The arrows show the direction of transcription of gng-1 and hph. Abbreviations for restriction sites: N, NcoI; EV, EcoRV; Sp, SpeI; S, SalI; K, KpnI; B, BamHI; C, ClaI; St, StuI; E, EcoRI; X, XbaI; Sm, SmaI. KpnI 2, SpeI3, and the unique XbaI and SmaI are artifacts of cloning. pSVK3 was the probe used for Southern analysis (see the legend to panel B). pSVK7 was used as the gene replacement construct, while the portion of gng-1 in pSVK17 was present in the his-3-targeted rescue construct. (B) Expression of gng-1 and gnb-1 during the N. crassa life cycle. Samples from wild-type strain 74A tissues (20 μg of total RNA) were subjected to Northern analysis using as probes a 1,074-bp PCR product amplified from pBR2 for detection of the gnb-1 transcript and a 279-bp PCR product amplified from pSVK1 to detect the gng-1 ORF. The tissues used in the experiment were as indicated.
shown) but differ from those of Δgna-1 strains that display reduced apical extension rates (37).

Asexual spore formation (conidiation) is induced in wild-type strains of N. crassa cultured on solid medium. In contrast, submerged cultures form vegetative nonconidiating hyphae unless starved for carbon or nitrogen or exposed to stress conditions, such as high temperature (54, 67). Our laboratory previously showed that Δgna-1, Δgna-3, and Δgnb-1 strains conidiate inappropriately in submerged culture; in the case of Δgna-1 strains, submerged conidiation is cell density dependent (39, 41, 80).

The conidiation patterns of Δgng-1, Δgng-1, and Δgnb-1 Δgng-1 mutants cultured on solid medium are similar (80 and data not shown), with the mutants exhibiting shorter aerial hyphae and increased conidiation relative to the wild type. Like Δgna-1, Δgna-3, and Δgnb-1 strains, Δgng-1 single and Δgnb-1 Δgng-1 double mutants also form conidia in 16-h submerged cultures (Fig. 4). Rescued Δgng-1 gng-1-1 strains are phenotypically identical to the wild type (Fig. 4).

Δgng-1 and Δgnb-1 mutants have decreased levels of intracellular cAMP. Study of fungal Gs subunits has revealed functions for these proteins in regulation of cAMP levels. In N. crassa, GNA-1 is required for GTP-dependent adenylyl cyclase activity, while GNA-3 regulates the levels of the adenyllyl cyclase protein (CR-1) (38, 41). Levels of cAMP are greatly reduced in both submerged and plate cultures of Δgna-3 mutants, and many defects of Δgna-3 strains can be reversed by supplementation with cAMP (41). On the other hand, Δgna-1 mutants have normal intracellular cAMP levels during submerged growth but low levels in cultures grown on solid media. The normal concentration of cAMP in submerged cultures may result from a compensatory mechanism involving reduced cAMP-phosphodiesterase activity (38). Δgna-2 mutants have normal cAMP amounts in submerged cultures and on VM plates but smaller amounts on SCM solid medium (38). Δgna-1 Δgna-2 strains have normal cAMP levels in submerged cultures but greatly reduced concentrations on VM and SCM plates (38). Similar to Δgna-1 and Δgna-2 strains, Δgnb-1 mutants have normal levels of cAMP in submerged cultures but low cAMP levels on VM (Table 3) (80) and SCM plates (Table 3). Furthermore, like Δgna-1 and Δgna-2 mutants, Δgnb-1 strains have normal levels of CR-1 protein but reduced GTP-dependent adenylyl cyclase activity (80).

The results from previous studies indicating effects on cAMP levels due to loss of heterotrimeric G proteins in N. crassa prompted measurement of cAMP levels in Δgng-1 strains. As expected, Δgng-1 strains have concentrations of cAMP very similar to those of Δgnb-1 mutants (Table 3). Wild-type amounts of cAMP are produced in submerged cultures, while reduced levels are obtained when Δgng-1 mutants are cultured on VM (55% of wild type) or SCM (21% of wild type) plates. Δgng-1 strains have reduced Gβ and Gα protein levels. Gβ and Gγ subunits form a tight complex and are not known to dissociate from one another in vivo. Coexpression of the Gβ and Gγ subunit and the presence of an intact CaaX domain in the Gγ protein are required for plasma membrane targeting (58, 60). Mutation of Gγ genes has been shown to suppress the level of Gβ protein(s) in various organisms (34, 62, 71). To determine whether a similar mechanism exists in N. crassa, the plasma membrane fraction of Δgng-1 and Δgnb-1 mutants was subjected to Western analysis using GNG-1- and GNB-1-specific antisera (Fig. 2E). The results demonstrate that the amount of GNB-1 was reduced ~60% in Δgng-1 mutants (Fig. 2E) and that GNG-1 is almost completely absent from the plasma membrane of Δgnb-1 mutants (Fig. 2E). We were not able to detect GNG-1 in nonmembrane fractions of wild-type or mutant strains (data not shown), presumably due to low concentrations of GNG-1 in the cytosol. Interestingly, the levels of GNB-1 protein in cytosolic fractions from the Δgng-1 mutant and wild-type are comparable (data not shown), demonstrating that the major reduction in GNB-1 levels occurs in plasma membrane fractions of the Δgng-1 strain. The effect of the mutations appears to be largely posttranscriptional, as either normal (gng-1 in Δgnb-1) or 50% reduced (gnb-1 in Δgng-1) levels of the corresponding mRNAs are present in those cases where the partner protein is absent (Fig. 2D). In addition, the reduced amount of gnb-1 in Δgng-1 mutants is similar to that of rescued Δgng-1 gng-1-1 strains that have normal levels of GNB-1 (Fig. 2E) and are phenotypically comparable to the wild type.

Tethering of the Gβ protein by isoprenylated Gγ also facilitates interactions between Gβ and its other partner protein, Gα, at the plasma membrane. Deletion of the Gγ subunit can not only affect the levels of Gβ but also affect the levels of Gα proteins. For example, it has been shown in mice that Gγ2 is required for the stability of a G-protein heterotrimer (εα3βγ2), in that loss of Gγ2 results in an 82% reduction in Goαα protein levels in Gng7-/- mice (61). Deletion of the mouse Gγ3 gene, which results in a phenotype distinct from that of Gng7-/- mice, leads to reduced levels of Gβ2 and Goαα proteins. And, as mentioned above, deletion of the Gβ gene gnb-1 suppresses the level of Gα subunits in N. crassa (80).

Because GNG-1 is the only Gγ subunit in N. crassa and, by extension, is the only Gγ subunit capable of interacting with GNB-1, it was reasonable to test whether loss of gng-1 would affect expression of the three Gα proteins. Western analysis was used to measure levels of Gα proteins in wild-type, Δgnb-1, Δgng-1, and Δgnb-1 Δgng-1 strains in three different tissues:
16-h submerged cultures and VM and SCM plate cultures (Fig. 5A, B, and C). The amounts of GNA-1, GNA-2, and GNA-3 were significantly diminished in all mutants analyzed, and the magnitude of the reduction was almost identical. There were significant differences observed in the levels of single Gα proteins in 16-h submerged cultures. GNA-1 and GNA-2 levels were greatly reduced in all mutants, while changes in GNA-3 were much more subtle (~30 to 50%). The amount of all Gα proteins was dramatically lowered in VM and SCM plate cultures. To determine whether the effects on Gα protein levels were pre- or posttranscriptional, we examined levels of mRNA for the \textit{gna-1}, \textit{gna-2}, and \textit{gna-3} genes in 16-h submerged cultures of \textit{gng-1} and \textit{gnb-1} mutants (Fig. 5D). Similar to previous results from our laboratory (80), Gα message amounts were either normal (\textit{gna-1} and \textit{gna-3}) or reduced only ~50% (\textit{gna-2}), consistent with mainly posttranscriptional regulation of Gα subunit levels in both \textit{gng-1} and \textit{gnb-1} mutants.

\textbf{GNB-1 associates with GNG-1.} We confirmed by coimmunoprecipitation that the GNB-1 and GNG-1 proteins physically interact in \textit{N. crassa}. The FLAG epitope sequence was engineered at the amino terminus of the GNG-1 ORF, and the fragment was cloned into the \textit{his-3} targeting vector, pMF272 (see Materials and Methods). The resulting plasmid was electroporated into \textit{gng-1} \textit{his-3} recipient strain #113, and \textit{his-3} transformants were selected on minimal medium. Homologous recombination at the \textit{his-3} locus was verified by Southern analysis (see Materials and Methods); strains with such events were purified, and one of the strains (#5A) was used for coimmunoprecipitation studies. Phenotypic analysis of strain 5A showed that the FLAG-GNG-1 construct complemented some, but not all, of the \textit{gng-1} defects (data not shown). Although strain 5A conidiates abundantly during incubation on VM plates, conidiation is partially suppressed in 16-h submerged cultures; hyphal tips of strain 5A are swollen, but mature conidiophores similar to those of \textit{gng-1} or \textit{gnb-1} mutants were not observed. Strain 5A is also female fertile, producing perithecia and ascospores after fertilization.

Plasma membrane fractions were extracted from wild-type

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\centering
\includegraphics[width=\textwidth]{figures.png}
\caption{Phenotypes in submerged culture. Cultures grown for 16 h at 30°C under submerged conditions with shaking were photographed at ×400 magnification. Arrows indicate conidiophores formed in \textit{gng-1} and \textit{gnb-1} cultures.}
\end{figure}
We first analyzed the amount of tagged and untagged GNG-1 proteins present in the input membrane extracts by using Western analysis (Fig. 6A). Untagged GNG-1 and FLAG-GNG-1 were detected using two different antibodies: the GNG-1-specific peptide antiserum described above and anti-FLAG antiserum. The GNG-1-specific antiserum was used to determine levels of FLAG-GNG-1 or GNG-1 protein associated with the plasma membrane.

TABLE 3. Intracellular cAMP levels

<table>
<thead>
<tr>
<th>Strain</th>
<th>cAMP (pmol/mg protein) (%) of wild type on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Submerged culture</td>
</tr>
<tr>
<td>74A (wild type)</td>
<td>4.49 ± 0.72 (100)</td>
</tr>
<tr>
<td>5-5-12 (Δgng-1)</td>
<td>4.21 ± 0.27 (94)</td>
</tr>
</tbody>
</table>

Values are the means ± the standard errors of the means, calculated using data from two independent experiments, comprising four total replicates.

### FIG. 5. Analysis of Go protein and transcript levels.

(A) Go protein levels in 16-h submerged cultures. Samples containing 30 μg of protein from plasma membrane fractions were subjected to Western analysis using specific antisera (see Materials and Methods). The asterisk indicates a nonspecific band. (B) Go protein levels in VM plate cultures. Protein samples were as indicated in panel A. (C) Go protein levels in SCM plate cultures. Protein samples were as indicated in panel A. (D) Analysis of gna-1, gna-2, and gna-3 transcript levels. Total RNA was extracted from 16-h submerged cultures, and 20 μg was subjected to Northern analysis using a 5.6-kb EcoRI-ClaI genomic fragment from pPNO5, a 967-bp gna-2 PCR product amplified from plasmid 13M2A5-2, or a 1,068-bp gna-3 PCR product amplified from pAK1 as probes. The amounts of the two major rRNA species are indicated as a loading control.
membrane (Fig. 6A, top panel). Addition of the FLAG epitope results in a protein that migrates at a larger apparent molecular weight and that can be distinguished from the untagged GNG-1 protein by using the GNG-1-specific antiserum during Western analysis (see the shift in Fig. 6A, top panel). In contrast, the FLAG antibody is specific for the tagged FLAG-GNG-1 protein present in the corresponding transformants (Fig. 6A, middle panel). The level of FLAG-GNG-1 protein in strain 5A was significantly lower than the corresponding level of untagged GNG-1 in the wild type (Fig. 6A, top panel).
result may be explained by the difference in promoters, in that expression of the FLAG-GNG-1 construct is driven by the cgg-1 promoter (26). The lower level of FLAG-GNG-1 versus native GNG-1 presumably leads to the observed reduction in GNB-1 amount in the FLAG-GNG-1 strain relative to that of the wild type (Fig. 6A, bottom panel) and may explain why only partial phenotypic complementation of the Δgng-1 mutation was observed by using the FLAG-GNG-1 construct (data not shown).

For immunoprecipitation experiments, extracts were incubated with anti-FLAG-agarose beads (see Materials and Methods), and precipitated proteins were then subjected to Western blot analysis (Fig. 6B). We were able to immunoprecipitate FLAG-GNG-1 in strain 5A by using anti-FLAG agarose beads (Fig. 6B, top and middle panels). Importantly, GNB-1 was also present in the immunoprecipitate (Fig. 6B, bottom panel). The reaction is specific for FLAG-tagged GNG-1, as no GNB-1 can be detected in precipitated material from wild-type or Δgng-1 his-3 recipient strains, although the former contains appreciable amounts of GNB-1 protein (Fig. 6A, bottom panel).

**DISCUSSION**

BLAST searches of the expressed sequence tag databases and the complete N. crassa genome sequence produce evidence for only one Gy protein, GNG-1. Although we cannot rule out the possibility of another Gy with a very different sequence, previous studies of mammals and plants have shown that Gy proteins from the same species usually share a relatively high level of similarity (28, 49). The predicted GNG-1 protein possesses a typical Gy secondary protein structure (2.5 helices) (63, 83) and the conserved CaaX box motif at the carboxy terminus. As shown in other species, the CaaX motif is subjected to isoprenylation (farnesylation or geranylgeranylation) at the cysteine residue, followed by proteolytic removal of the last three amino acids and methylation of the carboxy terminus (28). If the last amino acid residue (X) of the CaaX box is M, S, Q, or A, the cysteine is a substrate for farnesylation, whereas leucine (X = L) results in geranylgeranylation (59). Amino acids at the X position of the CaaX box in characterized fungal Gy proteins are M (GNG-1 and Ste18p), S (Git11), or Q (Gg1 from *Lentinula edodes*), indicating that the CaaX motif is likely to be farnesylated.

Like Ste18p from *S. cerevisiae* and Git11 from *S. pombe*, GNG-1 contains two cysteine residues near its carboxy terminus (Fig. 1). In Ste18p, one cysteine, at position 107, is contained in the farnesyl-directing CaaX box (CTLM) (24), while the other cysteine (106) is a potential site for palmitoylation (35). In *S. cerevisiae*, substitution of serine for cysteine at position 106 or 107 resulted in failure of Gβγ to bind to the plasma membrane (35). The Cys 107 substitution also resulted in reduced steady-state levels of Ste18p, suggesting that Cys 107 farnesylation is required for Ste18p stability (35). Furthermore, previous genetic studies (30, 78) have demonstrated that yeast mutants with substitutions at either cysteine residue are unresponsive to pheromone. Further experimentation is needed to determine the importance of these two conserved cysteine residues to GNG-1 function in *N. crassa*.

The intron-exon boundaries and mRNA splicing patterns for several mammalian Gy-subunit genes have already been characterized (19, 20, 25, 28, 52). In all cases, the 5′-untranslated region of the mRNA contains one intron. A second intron is located in the ORF, and its position relative to the amino acid sequence is conserved between the Gy subunits (20). The *S. cerevisiae STE18* ORF does not contain an intron (http://www.yeastgenome.org). In contrast, both *S. pombe* git11 (http://www.genedb.org/genedb/pombe/index.jsp) and *N. crassa* gng-1 have introns in their ORFs. However, there are no reports of introns in the 5′ UTRs of STE18 and git11. In this study, we have identified two introns in *N. crassa* gng-1 at positions that correspond to those found in mammalian Gy-subunit genes. We previously reported a similar phenomenon with respect to conserved intron positions in mammalian and *N. crassa* Gy genes (68). The remarkable conservation of intron positions between mammalian and *N. crassa* Gy and Ga genes suggests that these sequences play a regulatory role in mRNA synthesis or stability. Future studies will investigate these possibilities.

The Δgng-1 mutant displays phenotypes identical to those observed in Δgnb-1 strains (44, 80), and the Δgng-1 Δgnb-1 double mutant is indistinguishable from either single mutant. Our results also demonstrate that loss of gng-1 or gnb-1 results in a significant reduction in GNB-1 or GNG-1 protein levels, respectively, from plasma membrane fractions (Fig. 2E), suggesting interdependence between GNB-1 and GNG-1 for their stability in vivo. This is similar to the situation of *S. cerevisiae*, in which Ste18p is barely detectable in ste4 mutants while Ste4p is reduced only 50% in ste18 cells (34). Taken together, our data support the hypothesis that GNB-1 and GNG-1 regulate identical events in *N. crassa* and form an active Gβγ complex in vivo. The finding that GNB-1 is coprecipitated with GNG-1 using an antibody directed against an epitope on GNG-1 provides strong evidence for a direct, physical association between these two proteins in vivo.

Like Δgnb-1 mutants (80), Δgng-1 strains have lower levels of Ga proteins than the wild type. This is in contrast to results reported for *S. cerevisiae*, where Gpa1p is present at normal levels and is localized to the plasma membrane in the absence of Gβγ (64). The major effect caused by loss of the Gβγ dimer in *N. crassa* appears posttranscriptional, because normal or appreciable levels of gna-1, gna-2, and gna-3 transcripts are produced in Δgng-1 and Δgnb-1 strains. In contrast, deletion of a single Ga does not greatly influence GNB-1 levels (38, 41, 43); a significant reduction in GNB-1 amount is only observed in a mutant lacking both GNA-1 and GNA-3 or all three Ga proteins (43). This finding suggests that the absence of multiple Ga proteins can influence the amount of Gβγ dimer anchored to the plasma membrane of *N. crassa*.

Many of the defects shared by Δgnb-1 and Δgng-1 strains can be explained by reduced amounts of Ga proteins. The female sterility of these mutants is similar to that of Δgna-1 and Δgna-1 Δgna-2 mutants (37, 44). These strains are defective in trichogyne attraction toward the male cell and form small aberrant perithecia with no ascospores after fertilization (37, 80). In contrast to GNA-1 and GNA-2, GNA-3 levels in submerged cultures were not greatly reduced (30 to 50%), suggesting that the Gβγ subunit is not crucial for GNA-3 stability in vegetative hyphal tissue. However, GNA-3 levels are significantly reduced in VM and SCM plate cultures. Based just on protein amount, it is not easy to predict the phenotypic outcome of lower GNA-3 levels in the various tissues. It is possible...
that GNA-3 is coupled to different receptors, and thus its turnover might be regulated differently in various cell types. On the other hand, GNB-1 may act as a direct regulator of downstream effectors, while GNA-3 is only required to regulate GNB-1 function. Such a scenario has been described for S. cerevisiae, where Gpa1p negatively regulates Ste4p function during pheromone signal transduction (18, 65, 81).

It was demonstrated previously that Δgmb-1 strains have low levels of intracellular cAMP when cultured on solid medium but normal amounts of cAMP in submerged culture (80). We have obtained similar results with Δgng-1 mutants (Table 3). The Δgmb-1 mutant conidiates abundantly on solid medium and in submerged cultures, and phenotypically it resembles Δgna-1, Δgna-2, and Δgna-3 mutants. It was hypothesized that the smaller amount of GNA-1 and GNA-2 in Δgmb-1 mutants is responsible for the reduction in cAMP levels (80). This hypothesis is supported by results from previous studies with both Δgna-1 deletion and gna-1 constitutively activated alleles (38, 39, 79). The observation of normal cAMP levels in submerged cultures of Δgmb-1 and Δgng-1 strains is similar to results determined for Δgna-1 and Δgna-2 mutants (38). In contrast, submerged liquid cultures of Δgna-3 mutants produce low levels of intracellular cAMP, presumably due to reduced amounts of adenylyl cyclase protein (41). Tissue-specific effects on cAMP metabolism due to loss of a Gγ-subunit gene have also been observed in mice, where the Gγ protein regulates adenylyl cyclase activity in specific regions of the brain (61).

Some phenotypes observed in Δgmb-1 mutants cannot be explained by low levels of Gα proteins. For example, Δgmb-1 mutants have essentially normal apical extension rates on various media (80), while a mutant lacking all three Gα proteins exhibits severely restricted growth (43). A possible explanation is that although Gα protein amounts are reduced in Δgmb-1 (and Δgng-1) mutants, free Gα proteins, untethered by GNB-1, can regulate downstream effectors. A similar model for G-protein functional interactions has been suggested for S. pombe, where Gpa2 remains partially active during cAMP signaling in git5(Gβ) mutants (45).

In this study, we provide evidence that GNG-1 is the sole Gγ subunit in N. crassa and that this protein forms a physical association with the only Gβ protein, GNB-1. Levels of GNG-1 and GNB-1 are decreased in the absence of the other subunit, consistent with decreased protein stability. The GNB-1/GNG-1 Gβγ heterodimer acts as a unit during signaling, with loss of either protein leading to similar defects, including a severe reduction in Gα protein levels. Future studies will focus on elucidation of the mechanism whereby loss of GNG-1 leads to smaller amounts of GNB-1 and the three Gα proteins and on understanding the contribution of individual G protein subunits to regulation of downstream effectors in N. crassa.

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REFERENCES


