The $G\alpha_i$ Homologue *gna-1* Controls Multiple Differentiation Pathways in *Neurospora crassa*

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Submitted February 15, 1996; Accepted May 22, 1996
Monitoring Editor: David Botstein

Heterotrimeric G proteins are components of principal signaling pathways in eukaryotes. In higher organisms, α subunits of G proteins have been divided into four families, G_i , G_s , G_q , and G_{12} . We previously identified a $G\alpha_i$ homologue *gna-1* in the filamentous fungus *Neurospora crassa*. Now we report that deletion of *gna-1* leads to multiple phenotypes during the vegetative and sexual cycles in *N. crassa*. On solid medium, $\Delta gna-1$ strains have a slower rate of hyphal apical extension than wild type, a rate that is more pronounced under hyperosmotic conditions or in the presence of a cellophane overlay. $\Delta gna-1$ mutants accumulate less mass than wild-type strains, and their mass accumulation is not affected in the same way by exposure to light. $\Delta gna-1$ strains are defective in macroconidiation, possessing aerial hyphae that are shorter, contain abnormal swellings, and differentiate adherent macroconidia. During the sexual cycle, $\Delta gna-1$ strains are fertile as males. However, the mutants are female-sterile, producing small, aberrant female reproductive structures. After fertilization, $\Delta gna-1$ female structures do not enlarge and develop normally, and no sexual spores are produced. Thus, mutation of *gna-1* results in sex-specific loss of fertility.

INTRODUCTION

Heterotrimeric ($\alpha\beta\gamma$) guanosine 5'-triphosphate (GTP)-binding proteins (G proteins) are integral components of major sensory pathways in eukaryotic organisms (for review, Simon *et al.*, 1991; Neer, 1995; Borkovich, 1996). These proteins are coupled to seven-helix transmembrane receptors, which sense the presence of environmental stimuli such as light, hormones, odorants, and neurotransmitters (Birnbaumer *et al.*, 1990; Dohlman *et al.*, 1991). Ligand binding of stimuli to the receptor facilitates exchange of GTP for guanosine 5'-diphosphate (GDP) on the α subunit of the G protein and dissociation of the α and $\beta\gamma$ units. Depending on the pathway, either the α -GTP protein and/or the

$\beta\gamma$ dimer have been shown to activate downstream effectors (Neer, 1995).

Mammalian α proteins are grouped into four classes, $G\alpha_i$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$, based on amino acid sequence identity (Simon *et al.*, 1991). The $G\alpha_i$ family includes the $G\alpha_i$, $G\alpha_o$, $G\alpha_t$ (transducin), and $G\alpha_z$ subfamilies. The effectors regulated by $G\alpha_i$ family members are quite diverse, and include adenylylcyclase, K^+ and Ca^{2+} ion channels, and cyclic guanosine 5'-monophosphate phosphodiesterase (Neer, 1995). $G\alpha_i$ proteins are distinguished by possession of consensus sequences for myristoylation (Buss *et al.*, 1987) and, with the exception of $G\alpha_z$, ADP-ribosylation by pertussis toxin (Ui, 1990).

$G\alpha_i$ superfamily proteins have been implicated in control of cell proliferation, behavior, and development in higher organisms (Lyons *et al.*, 1990; Gupta *et al.*, 1992a; Mendel *et al.*, 1995; Segalat *et al.*, 1995). An activated $G\alpha_{12}$ mutation is found in adrenal cortex and ovarian endocrine tumors (Lyons *et al.*, 1990), and

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expression of activated $G\alpha_{i2}$ or $G\alpha_o$ proteins in cultured cells leads to a transformed phenotype (Gupta *et al.*, 1992b). In the nematode *Caenorhabditis elegans*, loss of function mutations in a gene encoding a $G\alpha_o$ protein (*goa-1*) led to hyperactive movements, premature egg laying in hermaphrodites, and impotence in males (Mendel *et al.*, 1995; Segalat *et al.*, 1995).

Previously, we reported isolation of a $G\alpha_i$ gene (*gna-1*) from the filamentous fungus *Neurospora crassa* (Turner and Borkovich, 1993). *N. crassa gna-1* was the first identified microbial $G\alpha$ subunit that could be classified in a family found in higher organisms. The predicted amino acid sequence of Gna-1 is ~55% identical to members of the mammalian $G\alpha_i$ superfamily and contains both the consensus pertussis toxin labeling (West *et al.*, 1985) and myristoylation sites (Buss *et al.*, 1987) common to this group. Biochemical evidence for a $G\alpha_i$ in *N. crassa* was provided by the *in vitro* labeling of an *N. crassa* membrane protein of the approximate size of Gna-1 using pertussis toxin (Turner and Borkovich, 1993).

The existence of a $G\alpha_i$ in the filamentous fungus *N. crassa*, but not in the yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* (Borkovich, 1996), suggests that this protein may regulate a process not present in these organisms. Possibilities include multicellularity, elaboration of multiple cell types, or multiple differentiation processes that respond to diverse environmental stimuli, such as light, nutrients, and desiccation. *N. crassa* grows by the apical extension and branching of hyphae, which then anastomose to form a network structure called a mycelium (for review, Davis and deSerres, 1970). *N. crassa* has two mating types, *A* and *a*; the anastomosis reaction requires that the two hyphal tubes be isogenic at the mating type and several other loci (termed heterokaryon compatibility genes) (Davis, 1966; Perkins, 1975). The mycelial hyphae have incomplete septa between adjacent cells, which facilitates the movement of cytoplasmic contents and even organelles through the body of the organism (Davis and deSerres, 1970).

N. crassa produces two types of vegetative spores (macro- and microconidia) during the vegetative life cycle (Davis and deSerres, 1970). The macroconidiation developmental pathway is complex, requiring many gene products (Springer and Yanofsky, 1989). The pathway initiates with nutrient deprivation and desiccation of the vegetative mycelium. Under these conditions, *N. crassa* differentiates aerial hyphae, structures which grow approximately perpendicular to the agar surface. The macroconidia-forming structures, conidiophores, are then elaborated at the tips of aerial hyphae. In contrast to macroconidia, microconidia are uninucleate spores that are differentiated from short stalks on the vegetative hyphae (Davis and deSerres, 1970). In nature, both macro- and microconidia can be

dispersed by wind currents to facilitate colonization of new nutrient sources.

Under conditions of nitrogen starvation, *N. crassa* differentiates multicellular female reproductive structures called protoperithecia (reviewed, Raju, 1992). The female gametes are located in the base of the protoperithecium, whereas vegetative cells such as hyphal fragments, or conidia, function as the male gametes. In contrast to vegetative anastomosis, mating requires that the female and male be of opposite mating type (Davis and deSerres, 1970). After fertilization, meiosis occurs and the protoperithecium darkens and enlarges to become a perithecium. Sexual spores (ascospores) develop in asci within the perithecium and are violently ejected when mature.

In this study, we present the organization of the *N. crassa gna-1* gene, including potential upstream regulatory sequences and intron positions. We describe a targeted gene replacement technique used to delete the *gna-1* gene in *N. crassa*. We demonstrate that the *gna-1* mutants are defective in multiple differentiation pathways during the vegetative and sexual life cycles, including vegetative hyphal extension, aerial hyphae formation, conidiogenesis, and protoperithelial development. These lesions may be related to the observed sensitivity of *gna-1* mutant strains to hyperosmotic media.

MATERIALS AND METHODS

Strains and Growth Conditions

The *N. crassa* strains used in this study are listed in Table 1. Media with elevated levels of NaCl or KCl were made by adding the indicated amounts to that normally found in Vogel's minimal medium (VM), 26 and 37 mM, respectively (Vogel, 1964). L-histidine (100 μ g/ml) and pyridoxine-HCl (10 μ g/ml) were used as supplements for auxotrophic strains. Conidia were propagated and harvested using standard procedures (Davis and deSerres, 1970). Plasmids were maintained in *Escherichia coli* strain DH5 α (Hanahan, 1983).

Subcloning and Sequencing of the *gna-1* Genomic Clone

The 3' portion of the *gna-1* gene was previously subcloned from a genomic cosmid (Turner and Borkovich, 1993) as a 4.5-kb *Xho*I insert in pBluescript II KS⁺ (Stratagene, La Jolla, CA). This vector has been named pGET3. Plasmid pPNO3, which contains a smaller fragment of the 3' end of *gna-1*, was obtained by isolating a 2.9-kb *Xho*I-*Cl*aI fragment from pGET3 and inserting it into pGEM-7Zf (Promega, Madison, WI) digested with these same enzymes. The 5' portion of the *gna-1* gene was isolated as a 2.5-kb fragment after digestion of the cosmid with *Eco*RI and *Xho*I. This fragment was inserted in pBluescript II KS⁺, yielding plasmid pGET5. Another plasmid, pPNO2, was generated by cloning the pGET5 *gna-1* insert into vector pGEM-7Zf. A plasmid containing the entire *gna-1* gene (pPNO5) was constructed by inserting the *Eco*RI-*Xho*I insert from pPNO2 and the *Xho*I-*Cl*aI insert from pPNO3 into vector pBluescript II KS⁺ digested with *Eco*RI and *Cl*aI.

Templates for sequencing were obtained by *Exo*III deletion to obtain overlapping fragments or by restriction fragment analysis of the *gna-1* subclones described above. Specific primers were de-

signed to fill in gaps. The sequence of both DNA strands was determined manually using Sequenase version 2.0 (United States Biochemical, Cleveland, OH). Sequences were analyzed using the GCG package for the VAX (Devereux *et al.*, 1984) and the SeqEd program for the Macintosh (Applied Biosystems, Foster City, CA). The GenBank/EMBL accession number for the *gna-1* genomic fragment is U56090.

Gene Replacement and Rescue Constructs

Gene Replacement Construct. Plasmid pPNO2 was digested with *Stu*I, and *Bam*HI linkers were inserted at this site, yielding pPNO6. Plasmid pPNO6 was digested with *Eco*RI and *Bam*HI, and the 1.7-kb fragment containing the 5' end of *gna-1* was purified. Plasmid pNC807 (Koo and Stuart, 1991) was digested with *Bam*HI and *Eco*RV, and the 2.5-kb fragment containing the *mtr* gene was isolated. The fragments from pPNO6 and pNC807 were inserted into vector pBR322 (Bolivar *et al.*, 1977a,b) digested with *Eco*RI and *Eco*RV. The resulting plasmid, pPNO8, was then digested with *Eco*RI and *Eco*RV, and the 4.2-kb *gna-1::mtr* fragment was purified. Plasmid pPNO3 was digested with *Eco*RV and *Clal*, and the 2-kb fragment containing the 3' end of *gna-1* was isolated. The fragments from pPNO8 and pPNO3 were then inserted into pBR322 digested with *Eco*RI and *Clal*. The resulting plasmid (pPNO9) was used as the gene replacement construct.

Rescue Construct. A cosmid containing the *N. crassa* *his-2* gene was obtained from W.D. Stuart (University of Hawaii, Honolulu, HI). The *his-2* gene was isolated from the cosmid as a *Hind*III fragment and subcloned into pBluescript II KS⁺ to yield pKB49. Plasmid pPNO5 was then digested with *Clal*, the site filled in with DNA Polymerase Klenow fragment, and a *Not*I linker was inserted. This vector was then treated with *Eco*RI, and the *Eco*RI-*Not*I fragment containing *gna-1* was isolated and inserted into pKB49 cut with the same restriction enzymes. The resulting plasmid (pDIV1) contains both the wild-type *his-2* and *gna-1* genes.

Transformation, Electroporation, and Strain Purification

N. crassa Stadler strain spheroplasts were transformed (Vollmer and Yanofsky, 1986) with either 5.2 μ g of gel-purified *Eco*RI-*Hind*III insert from pPNO9 or 270 ng of gel-purified *Bam*HI-*Eco*RV insert from pNC807. Transformants were selected for the presence of the *mtr* gene (Koo and Stuart, 1991) by plating on sorbose plates with regeneration agar (Case *et al.*, 1979) supplemented with L-histidine (100 μ g/ml), pyridoxine (10 μ g/ml), and L-arginine (2 mg/ml). Heterokaryotic pPNO9 transformants were checked for the presence of the gene replacement event by both PCR and Southern analysis (see below). Heterokaryotic pNC807 transformants were tested for the presence of an ectopic copy of *mtr* by Southern analysis. In both cases, positive strains were purified to homokaryons by plating on the histidine, pyridoxine, and arginine medium. Verification of homokaryosis in both pPNO9 (Δ *gna-1*) and pNC807 (control) strains was achieved by testing lethality of *p*-fluorophenylalanine, a toxic amino acid analogue transported by the *mtr* gene product (Koo and Stuart, 1991). The pPNO9 strains were analyzed for the absence of a product corresponding to *gna-1* in PCR reactions (see below), and the substitution of gene replacement fragments for wild-type fragments of *gna-1* during Southern analysis. Approximately 10% of the original transformants carried the gene replacement.

Nine-day-old *N. crassa* conidia were electroporated using an Electroporator II (Invitrogen, San Diego, CA) as described (Vann, 1995). The Δ *gna-1* mutant conidia were electroporated separately with 1 μ g supercoiled pDIV1 or pKB49, and control strains were electroporated with 1 μ g pKB49. Electroporation conditions for all strains were 1500 V, 500 Ω , and 50 μ F. Electroporants were selected for the presence of the *his-2* gene by plating on sorbose plates with regeneration agar (Case *et al.*, 1979) containing pyridoxine (10 μ g/ml).

The apical extension rates of 50 pDIV1 electroporants were measured, and 15 strains that grew at near wild-type rates were subjected to Southern analysis (described below). Such heterokaryotic pDIV1 strains, as well as control pKB49 strains, were purified to homokaryons by repeated plating on sorbose-selective medium.

Polymerase Chain Reactions and Southern Analysis

N. crassa genomic DNA from the pPNO9 transformants was isolated (Metzenberg and Stevens, 1982) and used as a template in Polymerase Chain Reactions (PCR). The 5' primer (*mtr*c1), which corresponds to the extreme carboxyl terminus of *mtr*, has the sequence 5'-CTGAACATACCTTGCC-3'. The 3' primer (*gn*1ct) corresponds to the sequence on the *gna-1* chromosome just beyond the 3' end of the *gna-1* genomic insert in pPNO9. The *gn*1ct sequence (5'-GCAG-CAGCATCATAAC-3') was obtained from plasmid pGET3 (see above). These PCR primers amplify a 2-kb fragment only if the *gna-1* gene has been replaced with *mtr* on the chromosome (Aronson *et al.*, 1994). The PCR reactions contained 1 μ l DNA, 300 ng each oligonucleotide primer, 0.3 U of Tfl polymerase (Epicenter Technologies, Madison, WI), and 200 μ M of each deoxynucleoside triphosphate in the buffer supplied by the manufacturer. Amplification conditions were 92°C denaturation for 1 min, 50°C annealing for 1 min, and 72°C extension for 3 min for a total of 30 cycles. To check for the presence of the wild-type *gna-1* gene, degenerate primers to *Ga* genes were used in PCR reactions (Turner and Borkovich, 1993).

Genomic DNA from *N. crassa* strains was digested with various restriction endonucleases and used to prepare Southern blots (Sambrook *et al.*, 1989). DNA fragments to be used as probes were gel-purified and then labeled using the Prime-a-Gene Labeling System (Promega). The *Eco*RI-*Clal* fragment from pPNO5 was used to detect *gna-1* sequences, and the *Hind*III insert from pKB49 was the probe for *his-2*.

Overexpression of Gna-1 in E. coli and Antiserum Generation

A plasmid directing overexpression of full-length Gna-1 protein in *E. coli* (pKB46) was constructed as follows. The intron in plasmid pPNO3 was deleted by site-directed mutagenesis (Kunkel *et al.*, 1987; Berger and Christie, 1994) to yield plasmid pKB44. Plasmid pKB44 was digested with *Hinc*II and *Bam*HI, and the 872-bp fragment containing the 3' end of *gna-1* was isolated. The plasmid containing the 3' truncated *gna-1* cDNA clone (8D1; Turner and Borkovich, 1993) was treated with *Hind*III and *Hinc*II, and the 560-bp fragment of *gna-1* was purified. Both fragments were inserted into pBluescript II KS⁺ digested with *Hind*III and *Bam*HI to yield pKB45.

A primer was designed that would amplify a fragment of *gna-1* with an *Nde*I site at the translational start codon. This primer, along with one that was a perfect match to the *Hind*III site in the *gna-1* coding sequence, was used in PCR reactions containing plasmid 8D1 as template, Vent Polymerase (New England BioLabs, Beverly, MA) and 300 μ M deoxynucleoside triphosphates in buffer. The fragment was gel-purified and digested with *Nde*I and *Hind*III. Plasmid pKB45 was linearized with *Hind*III, subjected to a partial digest with *Bam*HI, and a 1400-bp fragment was isolated. This fragment, along with the PCR product described above, was inserted into plasmid pET11a (Novagen, Madison, WI) digested with *Nde*I and *Bam*HI to yield pKB46.

E. coli strain HMS174 *plys*S (Studier *et al.*, 1990) containing plasmid pKB46 was cultured to allow overexpression of Gna-1 protein according to the manufacturer's recommendations (Novagen). Inclusion bodies containing insoluble Gna-1 were purified as described (Nagai and Thogersen, 1987), with the following modifications: 1) A 500-ml culture of *E. coli* cells was collected by centrifugation and lysed in 20-ml lysis buffer using sonication. Because this treatment sheared the DNA, the treatment with DNase I was omitted; 2) Forty milliliters of detergent buffer was added to

the lysed cells after sonication; and 3) Pellets were resuspended in the 20 ml of Triton-EDTA buffer using sonication at the first resuspension step. The Gna-1 protein in the final washed pellets was solubilized using Laemmli sample buffer and separated from contaminants on SDS-PAGE gels (Laemmli, 1970). The gels were Coomassie stained and destained (Weber and Osborn, 1969) and then soaked in water to remove residual acid. The region of the gels containing Gna-1 protein was excised and used as an antigen for generation of a Gna-1-specific antiserum in rabbits (Cocalico Biologicals, Reamstown, PA).

Western Blot Analysis and ADP-Ribosylation Reactions

Conidia from *N. crassa* strains were inoculated into liquid VM at a final concentration of 5×10^8 cells/ml and cultivated at 30°C for 16 h at 200 rpm. Subcellular fractions were isolated, and protein concentrations were determined as described (Turner and Borkovich, 1993). Western blot analysis was performed on samples containing 20- μ g plasma membrane protein fraction as described previously (Borkovich *et al.*, 1989). Anti-Gna-1 polyclonal rabbit antiserum at a 1:1000 dilution was used as the primary antibody. Reactive bands were visualized using the Protoblot Western Blot AP System with an anti-rabbit IgG (Fc) AP Conjugate (Promega).

ADP-ribosylation by pertussis toxin was conducted as described (Turner and Borkovich, 1993). Reactions contained 2 or 10 μ g of protein from either plasma membrane or whole-cell extract fractions, respectively (Turner and Borkovich, 1993).

Photomicroscopy

Conidia were photographed using an Axioskop microscope with DIC objectives (Zeiss, Thornwood, NY). Cultures grown on solid medium were photographed using an SZHX10 stereomicroscope with a PM20 Exposure Unit (Olympus America, Lake Success, NY).

Growth Measurements

Growth was measured as either dry weight accumulation in solid or liquid culture or apical extension rate on plates. For dry weights of liquid cultures, 3.5×10^8 conidia (8 d old) were inoculated into 25 ml of liquid VM and shaken at 200 rpm for various times at 30°C. The entire culture was then filtered onto Whatman 540 filter paper and dried at 80°C to remove cell water (Davis and deSerres, 1970). For dry weights of solid medium-grown cultures, VM plates were overlaid with sterile 0.0015-cm cellophane (Bio-Rad, Richmond, CA) and inoculated in the center with 1 μ l of a conidial suspension. Plates were incubated at 30°C in a humid atmosphere, either in constant light or darkness. At various times, the colonies were scraped from the plates onto Whatman #540 filter paper and dried as described for the liquid-grown cultures above. Apical extension rates were measured by inoculating solid agar plates (with or without a cellophane overlay) in the center with a conidial suspension. Plates were incubated at 30°C constant light or darkness, and colony diameters were measured at various times.

Crosses and Sexual Fertility Assays

Crosses between various *N. crassa* strains were conducted using standard techniques (Davis and deSerres, 1970). Strains *fl a* and *fl A* were used for testing mating types of progeny obtained from such crosses (Davis and deSerres, 1970).

The *his-2*⁺ control and Δ *gna-1* strains used in sexual fertility assays were constructed by crossing the homokaryotic *mtr*⁺ strains to strain KB-5 (see Table 1) for the following reasons: 1) The original Δ *gna-1* and control strains are in the Stadler *his-2* mutant background (see Table 1), and this strain has defects in female fertility. 2) Because *N. crassa* possesses a mechanism for inactivating duplicated DNA during the sexual cycle (Repeat-Induced Point [RIP] mutation;

Selker, 1990), the *his-2*⁺ pDIV1 and pKB49 transformed strains could mutate both the endogenous mutated and ectopic wild-type copy of *his-2* during the mating assay. This could result in a requirement for histidine at some point during development of the perithecia and skew interpretation of the results.

The *his-2*⁺ progeny obtained from the Δ *gna-1* mutant crosses were analyzed for the presence of the gene deletion using PCR and Southern analysis. Strains which carried the Δ *gna-1* pattern were used for the sexual fertility studies. In the case of the control cross *his-2*⁺ progeny, Southern analysis using *Hind*III revealed that one strain carried the Δ *mtr* pattern, two strains possessed fragments indicative of the wild-type *mtr* gene, and one strain showed evidence of RIP at the *mtr* locus. In all cases, the control strains were resistant to *p*-fluorophenylalanine. This suggests that the two strains that show no evidence of RIP using the *Hind*III digest must nevertheless carry a mutation in the *mtr* gene. It has been reported that *mtr* mutants produce ascospores that are slow to darken and mature (Perkins *et al.*, 1982). However, this effect appears to be variable, and large numbers of viable progeny are easily obtained from crosses using *mtr* mutant strains as either males or females (Table 2; our unpublished observations).

Mating assays were conducted on synthetic crossing medium slants (Westergaard and Mitchell, 1947) at room temperature in constant light. The formation of protoperithecia (female reproductive structures) was assessed by microscopic examination of the agar surface of the slants 6 or 8 d after incubation. At this point, washed conidia from a strain of opposite mating type (males) were applied to the protoperithecia. Scoring for the presence of ascospores on the slant tube walls was performed at 11–12 ds postfertilization; no further expulsion of ascospores was observed after this time.

RESULTS

Organization of the *gna-1* Gene

A 5.58-kb fragment containing the entire *gna-1* gene has been subcloned from a genomic cosmid (Turner and Borkovich, 1993). Comparison of the sequence of this fragment to that of the *gna-1* cDNA clone demonstrates the presence of three introns in the amino acid coding region (Figure 1). Two of these introns are in positions conserved in *G_i* and *G_s* α subunit gene families (Itoh *et al.*, 1988; Kaziro *et al.*, 1988; Kozasa *et al.*, 1988; Weinstein *et al.*, 1988; Raport *et al.*, 1989; Lochrie *et al.*, 1991), reinforcing the idea that *gna-1* is evolutionarily related to mammalian *G α* genes.

The *gna-1* genomic fragment contains several potential transcriptional regulatory sequences (Figure 1), including pyrimidine-rich regions (Gurr *et al.*, 1987), CCAAT boxes (Guarente *et al.*, 1984), GATA sequences (Ko and Engel, 1993; Chiang and Marzluf, 1994), and motifs recognized by SRY family HMG-box DNA binding proteins (Baxevanis and Landsman, 1995). Pyrimidine-rich regions are commonly found upstream of fungal genes, but their role in transcriptional regulation is not well-understood (Gurr *et al.*, 1987). One of the two consensus CCAAT boxes (Guarente *et al.*, 1984) in *gna-1* is located in the first intron of the gene (Figure 1). There is a precedent for transcriptional regulatory elements in introns of *N. crassa*. For example, normal mycelial and conidial expression of the *con-10* gene is dependent on a 17-bp enhancer

Figure 1. Gene structure of *gna-1* from *N. crassa*. The positions of various elements are numbered relative to the start site of translation (+1). The stippled area corresponds to the amino acid coding region of *gna-1*. The three intron positions are marked by triangles; the bolder triangles correspond to introns whose positions are conserved in various mammalian $G\alpha$ genes (see text). The position of the *mtr* gene replacement between the *StuI* and *EcoRV* sites of *gna-1* is indicated by the dotted line. Pyrimidine-rich tracts are present at -161 to -174, -188 to -201, -226 to -238, -776 to -805, and -1886 to -1909. CCAAT boxes are located at -2070 and +178. GATA sequences are found at -67, -100, and -112. Copies of the sequence bound by SRY family HMG-box proteins (CTTG or CAAAG) are found at -433, -473, -841, -1255, -1282, -1289, -1300, -1568, -1790, -1809, and -2132. References for these putative regulatory elements are given in the text.

contained in the first intron of this gene (Corrochano *et al.*, 1995).

GATA sequences are recognized by a family of zinc-finger proteins known as GATA factors (Ko and Engel, 1993; Chiang and Marzluf, 1994), which are implicated in diverse regulatory and developmental pathways in several eukaryotic species. In *N. crassa*, the GATA factor Nit-2 is a global nitrogen regulatory protein (Chiang and Marzluf, 1994).

The *N. crassa* mating type *a-1* protein (MT *a-1*) (Philly and Staben, 1994) is an example of an SRY-family High Mobility Group (HMG)-box protein. However, the Gna-1 protein is present in both *A* and *a* strains of *N. crassa* (our unpublished observations), although *A* strains do not contain the *a* gene. This result suggests that expression of *gna-1* is regulated by another yet-to-be identified HMG-box protein in *N. crassa*.

Targeted Deletion of *gna-1* in *N. crassa*

We made deletion mutations in *gna-1* by targeted integration of a construct in which the *gna-1* gene was replaced by the *mtr* gene (Stuart *et al.*, 1987; Figure 1). The *mtr* gene encodes a component of the neutral/aromatic amino acid transporter in *N. crassa* (Lester, 1966; Stadler, 1966). Both positive and negative selection schemes exist for the *mtr* gene, and these were exploited during selection and purification of strains (see MATERIALS AND METHODS). PCR was used for the initial screening to identify heterokaryotic transformants, which contained nuclei with the gene replacement (our unpublished observations).

Southern analysis using the 5.58-kb *gna-1* genomic fragment as a probe revealed that the heterokaryotic transformants possessed *KpnI* fragments corresponding to both the wild-type and disrupted *gna-1*

alleles (Figure 2A). The wild-type *gna-1* locus is contained on a 15.5-kb *KpnI* fragment. Because the *mtr* gene contains an internal *KpnI* site, replacement of *gna-1* with *mtr* introduces a second *KpnI* site at the *gna-1* locus. Therefore, Southern analysis of the disrupted *gna-1* allele produces two hybridizing fragments of 8.5- and 3.8-kb (Figure 2A). Purification of the heterokaryotic strains yielded $\Delta gna-1$ mutant homokaryons containing only the 8.5- and 3.8-kb fragments (Figure 2A). Isogenic *mtr*⁺ *gna-1*⁺ strains were made by transformation of the *mtr* gene into the same strain used for making the deletion mutants (Table 1). These strains were purified to homokaryons and used as controls for phenotypic studies (see below).

We verified that the $\Delta gna-1$ homokaryons contained no Gna-1 protein, using two methods. Previously, we demonstrated that *N. crassa* contains a pertussis toxin substrate with a molecular mass (39 kDa) similar to that predicted from the DNA sequence (40.9 kDa) (Turner and Borkovich, 1993). Possession of a consensus sequence for ADP-ribosylation by pertussis toxin is a feature of $G\alpha_i$ family proteins (Ui, 1990). ADP ribosylation experiments with pertussis toxin revealed that both whole-cell extracts and plasma membrane fractions from the $\Delta gna-1$ strains lack a labeled protein, whereas wild type possesses a substrate (Figure 2B). This result proves that the pertussis toxin substrate identified in our earlier studies is Gna-1. Furthermore, the results demonstrate that Gna-1 is the only pertussis toxin substrate, and therefore the only $G\alpha_i$ protein, in *N. crassa*.

We also tested for the presence of Gna-1 protein using Western blot analysis with a Gna-1 antiserum (Figure 2C). Wild-type strains possess a reactive pro-

Figure 2. Presence of the Gna-1 protein correlates with a wild-type *gna-1* allele. (A) Southern analysis: Genomic DNA from wild-type strains (WT), heterokaryotic primary transformants (HETERO-KARYONS), and purified homokaryotic mutants (HOMOKARYOTIC MUTANTS) was digested with *Kpn*I, transferred to a nylon membrane, and probed with the 32 P-labeled *gna-1* genomic fragment depicted in Figure 1. (B) Pertussis toxin labeling: samples contain 10 μ g of whole-cell extract or 2 μ g of purified plasma membrane fractions from wild-type (WT) or homokaryotic mutants (10-2 and 3-7), as indicated, and [32 P]NAD. The presence or absence of pertussis toxin (PTx) is indicated by + and -, respectively. The line marked Gna-1 corresponds to a 38-kDa PTx substrate that is absent in *gna-1* mutant extracts. (C) Western blot analysis of *his-2*⁺ strains: Samples contain 20 μ g of purified plasma membrane fraction from Δ *gna-1* mutants (Δ Gna-1) 3-7g (lane 1) and 10-2c (lane 2), pDIV1-rescued strains (Rescued) 3-7-20 (lane 3) and 10-2-9 (lane 4), and pKB49-transformed wild-type strains (WT) 4-1-17 (lane 5) and 9K2-2 (lane 6). Gna-1 in inclusion bodies (I.B.) purified from *E. coli* was used as a positive control. I.B. Gna-1 migrates as a larger molecular weight species than native Gna-1 in plasma membrane fractions.

tein of 38 kDa, whereas Δ *gna-1* mutants lack this protein. The molecular mass of the protein recognized by the Gna-1 antiserum is similar to that of the pertussis

toxin substrate and that predicted by the protein sequence (see above).

To further verify that the *gna-1* gene encodes the corresponding Gna-1 protein, we electroporated Δ *gna-1* strains with a *gna-1* genomic fragment using *his-2*⁺ as a marker (pDIV1). Strains containing a single ectopic copy of *gna-1* were purified to homokaryons. Approximately equal amounts of Gna-1 protein were found in rescued pDIV1 homokaryons and *his-2*⁺ controls (Figure 2C). These results indicate that *gna-1* encodes the Gna-1 protein and that the genomic clone contains sufficient transcriptional regulatory elements to direct expression of *gna-1* in *N. crassa*.

Δ gna-1 Mutants Have Slower Growth Rates than Wild-Type Strains on Solid Medium

The growth of Δ *gna-1* mutants on solid media and solid media overlaid with cellophane was compared with that of controls. The Δ *gna-1* mutants extend vegetative hyphae at approximately half the rate of wild-type strains on solid medium without a cellophane overlay (Figures 3A and 6). The slower apical extension rate of mutants can be explained by their spreading colonial growth habit; consistent with a slower growth rate, hyperbranching of the mycelium is also observed (our unpublished observations). The pDIV1-rescued strains have normal apical extension rates (Figure 3A).

Growth measurements on solid medium overlaid with cellophane were performed in either constant light or darkness (Figure 3B). Comparison of both dry mass accumulation and apical extension rates demonstrates that the mutant is inhibited relative to wild type in both light and dark (Figure 3B). Furthermore, in wild type the apical extension rate is affected minimally by light, but the dry mass is increased 2.8-fold (Figure 3B). In contrast, in the Δ *gna-1* mutant, apical extension rate and dry mass increase to the same extent in light (1.5- to 1.6-fold; Figure 3B).

The apical extension rate of wild-type strains in the dark is decreased by the presence of a cellophane overlay (75% of rate without cellophane; compare Figures 3B and 6A). The inhibitory effect on the Δ *gna-1* mutant is much greater; in this case, the apical extension rate with cellophane is only 32% of that without cellophane (compare Figures 3B and 6A). This difference may be due to a defect in water absorption, ion homeostasis, or nutrient assimilation in the Δ *gna-1* mutant, which is more pronounced in cultures grown on a cellophane overlay. That the defect is in water and/or ion homeostasis is substantiated by the observation that Δ *gna-1* colonies cultured on cellophane are dry in appearance, even when incubated in a humid atmosphere (our unpublished observations).

N. crassa conidia are able to germinate and form a mycelium in submerged cultures, but differentiation

Table 1. Strains used in this study

Strain	Genotype	Comments	Source
74-OR23-1A (74 A)	wild type, A		R. L. Weiss, UCLA
73a	wild type, a		R. L. Weiss, UCLA
Stadler	Δmtr , <i>his-2</i> , <i>pdx-1</i> , a		D. D. Stadler, U. of Washington
KB5	Δmtr , <i>pdx-1</i> , A	74 A X Stadler progeny	This study
4-1	<i>his-2</i> , <i>pdx-1</i> , pNC807 (<i>mtr</i> ⁺), a	Stadler pNC807 transformant	This study
9K	<i>his-2</i> , <i>pdx-1</i> , pNC807 (<i>mtr</i> ⁺), a	Stadler pNC807 transformant	This study
10-2	$\Delta gna-1::mtr$ ⁺ , <i>his-2</i> , <i>pdx-1</i> , a	Stadler <i>gna-1</i> gene replacement mutant	This study
3-7	$\Delta gna-1::mtr$ ⁺ , <i>his-2</i> , <i>pdx-1</i> , a	Stadler <i>gna-1</i> gene replacement mutant	This study
4-1#5	<i>pdx-1</i> , <i>mtr</i> ⁻ , a	KB5 X 4-1 progeny: has <i>mtr</i> gene, but is p-FPA* resistant (see MATERIALS AND METHODS)	This study
4-1#7	<i>pdx-1</i> , <i>mtr</i> ⁻ , A	KB5 X 4-1 progeny: has <i>mtr</i> gene, but is p-FPA* resistant (see MATERIALS AND METHODS)	This study
9KB#2	<i>pdx-1</i> , <i>mtr</i> ⁻ , a	KB5 X 9K progeny: has <i>mtr</i> gene, but is p-FPA* resistant (see MATERIALS AND METHODS)	This study
9KB#5	<i>pdx-1</i> , Δmtr , A	KB5 X 9K progeny: has Δmtr mutation	This study
3-7#3	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , A	KB5 X 3-7 progeny	This study
3-7#5	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , a	KB5 X 3-7 progeny	This study
10-2#12	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , a	KB5 X 10-2 progeny	This study
10-2#13	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , A	KB5 X 10-2 progeny	This study
3-7-20	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , pDIV1 (<i>gna-1</i> ⁺ :: <i>his-2</i> ⁺), a	3-7 pDIV1 transformant	This study
10-2-9	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , pDIV1 (<i>gna-1</i> ⁺ :: <i>his-2</i> ⁺), a	10-2 pDIV1 transformant	This study
3-7g	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , pKB49 (<i>his-2</i> ⁺), a	3-7 pKB49 transformant	This study
10-2c	$\Delta gna-1::mtr$ ⁺ , <i>pdx-1</i> , pKB49 (<i>his-2</i> ⁺), a	10-2 pKB49 transformant	This study
4-1-17	<i>pdx-1</i> , pKB49 (<i>his-2</i> ⁺), a	4-1 pKB49 transformant	This study
4-1-9	<i>pdx-1</i> , pKB49 (<i>his-2</i> ⁺), a	4-1 pKB49 transformant	This study
9K2-2	<i>pdx-1</i> , pKB49 (<i>his-2</i> ⁺), a	9K pKB49 transformant	This study

*p-FPA = *para*-fluorophenylalanine.

of aerial hyphae and conidiophores is repressed under these conditions (Davis and deSerres, 1970). Because growth in liquid culture represents an alternative developmental paradigm for *N. crassa*, we also compared dry mass accumulation in $\Delta gna-1$ and control strains under these conditions. However, there was no significant difference between the rates of mass accumulation in $\Delta gna-1$ mutant and control strain germlings in liquid culture (Figure 3C).

Mutation of *gna-1* Causes Multiple Defects in Macroconidiation

The $\Delta gna-1$ strains exhibit several defects in the macroconidiation developmental pathway. Wild-type strains differentiate longer aerial hyphae than $\Delta gna-1$ mutants, as evidenced by the shorter appearance of mutant cultures in agar slants (our unpublished observations). In addition, aerial hyphae from $\Delta gna-1$ strains is produced with the concurrence of a yellowish exudate (Figure 4A). Microscopic examination re-

veals that the exudate results from large swellings in the aerial hyphae; these anomalous structures are absent from control and pDIV1 rescued strains (Figure 4D; our unpublished observations). Two types of swellings are observed, referred to as smooth and rough. Smooth swellings are free of hyphal appendages and have been observed coalescing into larger smooth swellings during microscopic observation; light or higher temperature seem to enhance this effect (Figure 4B; our unpublished observations). In contrast, rough swellings appear to have hyphae emanating from the swelling and do not coalesce (Figure 4C).

The swellings produced in the aerial hyphae of $\Delta gna-1$ mutants approximate the appearance of protoperithecia, the female reproductive structures of *N. crassa* (Raju, 1992). However, unlike protoperithecia, which normally differentiate from vegetative hyphae under conditions of nitrogen starvation (Davis and deSerres, 1970), the swellings are located in aerial hyphae cultured on high-nitrogen medium. Attempts

Figure 3. Growth of $\Delta gna-1$ mutant and control strains at 30°C. (A) Colony growth on VM solid medium without a cellophane overlay. Wild-type (strain 4-1-9), $\Delta gna-1$ mutant (strain 3-7g), and rescued mutant (strain 3-7-20) colonies after growth for ~48 h. (B) Apical extension rates and dry weight accumulation on cellophane-covered VM solid medium. Strains were 4-1 (Wild type) and 3-7 (Mutant). Dry weight accumulation was measured after 40 h of growth. L, cultures grown in light; D, cultures grown in dark. (C) Dry weight accumulation in submerged liquid culture. Data for wild-type (WT) and $\Delta gna-1$ mutant strains are shown.

Figure 4. Colony morphology on VM solid medium at 30°C. (A) $\Delta gna-1$ colony. Both basal vegetative hyphae (lighter shade; right side of photograph) and aerial hyphae with clusters of conidiophores (darker shade of gold; left side of photograph) are visible. A smooth (thin arrow) and rough (thick arrow) swelling are indicated in the aerial hyphae. (B) Smooth swelling from a $\Delta gna-1$ strain at higher magnification. (C) $\Delta gna-1$ mutant rough swellings at higher magnification. (D) Wild-type colony. Clusters of conidiophores are visible on the left side of the photograph. (E) Wild-type strain grown on 1500 mM sorbitol. (F) Wild-type strain grown on 750 mM KCl. Large, smooth swellings are marked by the arrows in E and F. Scale bar is 4000 μm in A, D, E, and F and 1000 μm in B and C.

to fertilize both rough and smooth swellings with wild-type conidia of the opposite mating type were unsuccessful (our unpublished observations), indicating that these swellings are either not protoperithecia or are infertile protoperithecia.

In addition to the deformities noted above for aerial hyphae formation, $\Delta gna-1$ strains are defective in other aspects of macroconidiation. Production of conidia is delayed in the $\Delta gna-1$ strains (our unpublished observations) and, at the same age, mutant conidia are more adherent than wild-type conidia. With longer growing time, the mutant spores become more easily dispersed (our unpublished observations). Mutant conidia are also slightly smaller than those from wild-type strains (Figure 5, A and B). DAPI staining reveals that conidia from $\Delta gna-1$ strains have fewer nuclei compared with controls (~ 2 and 3 nuclei/conidium, respectively). This difference in nuclear number may reflect the difference in size of the mutant and wild-type spores.

$\Delta gna-1$ Mutants Are Sensitive to Hyperosmotic Media

Our observations that the apical extension rate of $\Delta gna-1$ strains is greatly inhibited by the presence of a cellophane overlay, that $\Delta gna-1$ vegetative hyphae contain abnormal swellings, and that $\Delta gna-1$ conidia are adherent suggested that these mutants possess a defect in ion homeostasis that was affecting the plasma membrane and/or cell wall. This defect could result from changes in ion channel or porter activity, osmosensing, or increased salt sensitivity of a membrane-bound enzyme in the mutants. In the case of ion homeostasis, there is a precedent for G protein regulation of ion channels and porters in mammals (Cantiello *et al.*, 1989; Voyno-Yasenetskaya *et al.*, 1994; Neer, 1995). Therefore, we tested the growth characteristics of $\Delta gna-1$ and wild-type strains on hyperosmotic media (Figures 4 and 6).

The $\Delta gna-1$ and control strains were tested for sensitivity to elevated levels of NaCl, KCl, and sorbitol (Figures 4 and 6). Increased concentrations of these agents inhibit the apical extension rate of $\Delta gna-1$ strains (Figure 6), with the highest levels resulting in colonial growth (growth rate ~ 0.5 mm/h). At a concentration of 750 mM added NaCl, KCl, or 1.5 M sorbitol, control strains grow at approximately the same rate as $\Delta gna-1$ strains cultured without any additions (Figure 6). The pDIV1 rescued strains restored salt sensitivity to the wild-type values (our unpublished observations).

Control strains cultured on 750 mM KCl and 1.5 M sorbitol produce large smooth swellings in their aerial hyphae (Figure 4, E and F). In addition, rough swellings are sometimes observed under these conditions (our unpublished observations). Both the rough and

Figure 5. Macroconidia from $\Delta gna-1$ and control strains. Nomarski microscopy of macroconidia from a control strain (A) and $\Delta gna-1$ mutant (B). The scale bar, 20 μ m, is relevant to both A and B. Arrows indicate representative macroconidia.

large smooth swellings are absent in controls grown in the presence of 750 mM NaCl; only small smooth swellings are present (our unpublished observations). Because exposure of wild-type strains to 750 mM KCl or 1.5 M sorbitol mimics the growth rate and swelling phenotype of mutants cultured without any additions, we conclude that these agents phenocopy aspects of the $\Delta gna-1$ mutation.

$\Delta gna-1$ Mutants Are Male-Fertile, but Female-Sterile

A role for G α proteins in mating has been demonstrated for the yeasts *S. cerevisiae* and *S. pombe* (for review, Kurjan, 1992; Hughes and Yamamoto, 1993; Borkovich, 1996). These organisms do not possess separate male and female gametes; to mate, two wild-type cells need only be of opposite mating type. In contrast, mating in *N. crassa* requires the presence of a male and a female gamete of opposite mating type (Davis and deSerres, 1970). Because of this distinction, we tested

Figure 6. Relative apical extension rates on solid medium without a cellophane overlay. (A) VM solid medium without additions. (B and C) The indicated concentrations of K^+ and Na^+ are in excess of those already present in VM solid medium. (D) Sorbitol was added to the indicated final concentration. Average apical extension rates were obtained using wild-type strains 4-1 and 9K and $\Delta gna-1$ mutant strains 10-2 and 3-7.

the fertility of $\Delta gna-1$ strains of both mating types as male or female parents during the sexual cycle.

$\Delta gna-1$ mutant conidia of either mating type are fertile when used as males in sexual crosses (Table 2). However, compared with wild-type controls, $\Delta gna-1$ A or a strains have thin hyphae and produce small, infertile protoperithecia when cultivated on low-nitrogen medium (Table 2; Figure 7, A and B). Differentiation of these aberrant protoperithecia is delayed relative to wild-type controls, and fewer total protoperithecia are produced (our unpublished observations; compare Figure 7, A and B).

In addition to the other sexual cycle defects, protoperithecia and vegetative hyphae from $\Delta gna-1$ strains cultivated on low-nitrogen medium are black-brown in color (compare Figure 7, A and B). Appearance of such pigmentation is correlated with tyrosinase activity and its end product melanin in *N. crassa* (Perkins *et al.*, 1982). This enzyme is normally induced in perithecia and during nitrogen, carbon, or sulfur starvation of vegetative mycelia (Horowitz *et al.*, 1961). Control strain perithecia darken and develop normally after fertilization and ascospores are produced (Table 2). In contrast, $\Delta gna-1$ mutants do not produce normal peri-

Table 2. Sexual fertility of wild type and $\Delta gna-1$ strains

Female parent ^a	MT ^b	Male parent ^c	MT ^b	Protoperithecia ^d	Ascospores ^e
$\Delta gna-1$ (10-2#13)	A	$gna-1^+$ (9KB#2)	a	+	—
$\Delta gna-1$ (3-7#3)	A	$gna-1^+$ (9KB#2)	a	+	—
$gna-1^+$ (4-1#7)	A	$gna-1^+$ (9KB#2)	a	++	+
$gna-1^+$ (9KB#5)	A	$gna-1^+$ (9KB#2)	a	++	+
$gna-1^+$ (KB5)	A	$gna-1^+$ (9KB#2)	a	++	+
$\Delta gna-1$ (10-2#12)	a	$gna-1^+$ (9KB#5)	A	+	—
$\Delta gna-1$ (3-7#5)	a	$gna-1^+$ (9KB#5)	A	+	—
$gna-1^+$ (4-1#5)	a	$gna-1^+$ (9KB#5)	A	++	+
$gna-1^+$ (9KB#2)	a	$gna-1^+$ (9KB#5)	A	++	+
$gna-1^+$ (KB5)	A	$\Delta gna-1$ (10-2)	a	++	+
$gna-1^+$ (KB5)	A	$\Delta gna-1$ (3-7)	a	++	+
$gna-1^+$ (KB5)	A	$gna-1^+$ (4-1)	a	++	+
$gna-1^+$ (KB5)	A	$gna-1^+$ (9K)	a	++	+
$gna-1^+$ (KB5)	A	$gna-1^+$ (Stadler)	a	++	+
$gna-1^+$ (74)	A	$gna-1^+$ (73)	a	++	+

^a All strains used as female parents were grown on synthetic crossing medium (Westergaard and Mitchell, 1947) slants with necessary supplements for 6–8 d before fertilization.

^b MT = mating type (see text).

^c Conidia were used as male parents.

^d Scored by microscopic analysis. ++ = many; + = few.

^e A + score indicates presence of ascospores on slant tube wall 11–12 d after fertilization.

Figure 7. Phenotypes during the sexual cycle. Wild-type (A) and $\Delta gna-1$ mutant (B) protoperithecia after 12 d on synthetic crossing medium. Representative protoperithecia are indicated by arrows. Scale bar, 1000 μm .

thecia or ascospores and are thus female-sterile (Table 2).

DISCUSSION

This study is the first report of phenotypes resulting from deletion of a G protein α subunit gene (*gna-1*) in a filamentous fungus. Because *N. crassa gna-1* is also a member of the G_i family, study of its functions would be expected to reveal information about the evolution of this family in eukaryotes. Since our initial isolation of *gna-1*, two other $G\alpha_i$ genes have been identified in filamentous fungi: *cgpa1* from *Coprinus congregatus* (Kozak *et al.*, 1996) and *cpg-1* from *Cryphonectria parasitica* (Choi *et al.*, 1995). However, $G\alpha_i$ genes have not been found in other lower eukaryotes, supporting our original hypothesis that the $G\alpha_i$ family may have first evolved in filamentous fungi (Turner and Borkovich, 1993). The predicted amino acid sequence of *N. crassa* Gna-1 is 68.9 and 98.3% identical to *C. congregatus* CGP α 1 and *C. parasitica* CPG-1, respectively. In addition, comparison of the genomic sequences of *N. crassa gna-1* and *C. parasitica cpg-1* reveals that all three introns in these genes are in identical positions (our unpublished observations). The high degree of relatedness between these three genes suggests the existence of a fungal $G\alpha_i$ family. Deletion mutations have not been made in *cgpa1* or *cpg-1*; however, cosuppression studies with *cpg-1* implicate this gene in fungal virulence (Choi *et al.*, 1995). Comparisons between saprophytic (*N. crassa*) and pathogenic (*C. parasitica*) fungi may reveal the evolutionary advantages of a $G\alpha_i$ to these two classes of organisms.

$\Delta gna-1$ strains are impaired in cell proliferation, as evidenced by lower apical extension rates on agar

medium. In mammalian cells, Mitogen-Activated Protein Kinase (MAPK) pathways are implicated in the control of cell proliferation (Gupta *et al.*, 1992a,b; Johnson *et al.*, 1994). The presence of an overactive MAPK pathway, through a constitutively active MAPK, causes transformation of rat fibroblast cells and loss of growth control (Gupta *et al.*, 1992b).

The growth rate defect of $\Delta gna-1$ strains is enhanced by hyperosmotic media or the presence of a cellophane overlay, implicating Gna-1 in osmosensing and/or ion homeostasis in *N. crassa*. To date, there is no data linking a specific heterotrimeric G protein to osmosensing in any species. However, there is precedence for involvement of a MAPK pathway during osmosensing in various organisms. In *S. cerevisiae*, the Hog1 MAPK can be regulated by the Sln1 histidine kinase and/or the Sho1 SH3-domain protein pathways during the response to changes in osmolarity (Maeda *et al.*, 1995). In mammalian cells, the Jnk1 MAPK and p38 MAPK are phosphorylated under hyperosmotic conditions, and both genes can complement the yeast *hog1* mutation (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994).

Cultivation of wild-type strains on high levels of NaCl phenocopies the slower growth rate but not the swelling phenotypes of $\Delta gna-1$ mutants grown in the absence of high NaCl. This result suggests that ion homeostasis, as well as osmosensing, may be perturbed in $\Delta gna-1$ strains. In higher organisms, several members of the $G\alpha_i$ superfamily are implicated in controlling ion transport systems (Neer, 1995) and Na^+/H^+ ion exchangers (Volpi *et al.*, 1985; Huang *et al.*, 1987). It is also of interest that mutation of a putative mechanosensory channel in *C. elegans* caused

swelling and degeneration of posterior lateral microtubule cells, presumably because of aberrant ion channel conductances (Hong and Driscoll, 1994; Huang and Chalfie, 1994).

Our results with *N. crassa gna-1* provide evidence for the genetic separation of male and female fertility by mutation of a $G\alpha_i$ gene. A role for the concertina-encoded (*cta*) $G\alpha_{12}$ family protein in female fertility has been demonstrated in *Drosophila melanogaster* (Parks and Wieschaus, 1991). As mentioned above, mutation of the $G\alpha_i$ superfamily member *goa-1* in *C. elegans* causes defects in both male and female fertility. In the yeasts *S. cerevisiae* and *S. pombe*, mating is also regulated by a heterotrimeric G protein pathway (Borkovich, 1996). In *S. cerevisiae*, the α subunit Gpa1 is a negative regulator of pheromone response/mating pathway; the Ste4–Ste18 $\beta\gamma$ dimer has a positive function (Kurjan, 1992). It has been demonstrated that a MAPK module is regulated by the G protein in this signal-transduction cascade (Levin and Errede, 1995). In *S. pombe*, the Gpa1 α protein is a positive regulator of mating and sexual sporulation, and acts upstream of two kinases that are highly homologous to two found in the *S. cerevisiae* pheromone response MAPK pathway (Borkovich, 1996).

By analogy to examples described above, participation of a MAPK pathway(s) is one explanation for many of the defects noted for $\Delta gna-1$ mutants. Of course, there are other possibilities for the effector pathway(s) regulated by Gna-1 and/or its $\beta\gamma$ pair. For example, Gna-1, like mammalian $G\alpha_i$ subfamily members (Tang and Gilman, 1992), may regulate cAMP levels in *N. crassa*. However, there is no data to support this hypothesis at present, because supplementation with 1 mM 8-Br-cAMP does not alter phenotypes of $\Delta gna-1$ mutant or wild-type strains (our unpublished observations), and $\Delta gna-1$ strains do not resemble *N. crassa* mutants known to have defects in cAMP metabolism (Scott, 1976; Rosenberg and Pall, 1979). Another possibility is suggested by the similar sensitivity of *N. crassa* $\Delta gna-1$ strains and *S. cerevisiae* phospholipase C (PLC) mutants to hyperosmotic media (Flick and Thorner, 1993). In eukaryotic cells, activation of PLC initiates a pathway leading to release of the second messenger Ca^{2+} from intracellular stores (for review, Berridge, 1993). Because Ca^{2+} is implicated in cell growth and morphogenesis in *N. crassa* (Reissig and Kinney, 1983; Dicker and Turian, 1990), Gna-1 may control these processes by modulating intracellular Ca^{2+} levels through a PLC-dependent or -independent pathway.

$\Delta gna-1$ strains exhibit multiple defects in macroconidiation, the major asexual reproduction pathway in *N. crassa*. The defects include shorter aerial hyphae, presence of swellings in aerial hyphae, and delayed conidial maturity. Furthermore, our observation that

$\Delta gna-1$ strains do not exhibit as great a mass increase when grown in light as wild type may stem from the macroconidiation defects of the mutant. Light normally enhances conidiation in *N. crassa* (Turian, 1977), and the wild-type strains produce abundant conidia when cultured in light. This presumably contributes to the greater increase in dry mass obtained in light conditions relative to dark-grown conditions for the wild-type strains.

The observation that $\Delta gna-1$ females produce melanin before fertilization suggests that the mutants have precociously entered the sexual cycle before fertilization has occurred. However, the nitrogen-sensing pathway required for the onset of the sexual cycle seems to be intact in that no fertile protoperithecia are formed by $\Delta gna-1$ strains on high-nitrogen media. Another possibility is that mutation of *gna-1* causes activation or induction of tyrosinase, resulting in sexual defects. In *N. crassa*, two regulatory mutants that do not express tyrosinase under conditions that induce control strains are female sterile (Horowitz *et al.*, 1961). Thus, both low and high tyrosinase activity may impair completion of the sexual cycle in *N. crassa*.

There are several possibilities for the stimuli sensed by the Gna-1 signaling pathway in *N. crassa*. During the vegetative cycle, nutrient, water, and oxygen availability regulate hyphal extension. In the case of macroconidiation, blue light enhances the abundance of conidia and decreases the time before conidiation (Turian, 1977). We know of no reports linking chemical agents to regulation of macroconidiation, but it has been observed that an air-water interface and/or dessication is necessary for this developmental pathway to commence in *N. crassa* (Davis and deSerres, 1970).

In the sexual cycle, the response to several stimuli could utilize a Gna-1 pathway. Protoperithecial development is induced by nitrogen starvation (Raju, 1992), and illumination with blue light increases the total number of protoperithecia formed (Degli-Innocenti *et al.*, 1983). Fertilization of the protoperithecium with opposite mating type conidia appears to involve a response to pheromones (Bistis, 1981, 1983). In addition, evidence has been presented for the production of a sexual morphogen in mated cultures (termed erogen B), which stimulates production of perithecia when applied to an unmated culture (Vigfusson and Cano, 1974). Another diffusible, possibly gaseous substance has been identified in mated cultures, which appears to exhibit the opposite activity; it inhibits perithecial formation (Howe and Prakash, 1969; Metzzenberg, 1993).

As described above, the decision to grow vegetatively or enter a particular differentiation pathway is influenced by a variety of agents in *N. crassa*. Gna-1 is central to the response to such stimuli in that *gna-1* mutants exhibit multiple defects in both the vegetative

and sexual growth cycles. Further study of the receptors and effectors coupled to Gna-1 will reveal details of the signaling paradigms used by this $G\alpha_i$ protein in *N. crassa*.

ACKNOWLEDGMENTS

We acknowledge L. Alex, B. Bowman, R. Davis, T. Koehler, R. Metzberg, L. Quarmby, M. Simon, E. Spudich, J. Spudich, T. Vida, and T. Wilkie for helpful discussions at various times during the course of this research. We thank T. Vida and M. Winkler for use of equipment and L. Alex, R. Baasiri, P. Rowley, and Q. Yang for critical reading of this manuscript. This work was funded by a grant from the National Institutes of Health, GM-48626 (to K.A.B.) and by an American Cancer Society Junior Faculty Research Award JFRA-495 (to K.A.B.).

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