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Shared and Independent Roles for a $G\alpha_i$ Protein and Adenylyl Cyclase in Regulating Development and Stress Responses in *Neurospora crassa*

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Growth and development are regulated using cyclic AMP (cAMP)-dependent and -independent pathways in *Neurospora crassa*. The *cr-1* adenylyl cyclase mutant lacks detectable cAMP and exhibits numerous defects, including colonial growth habit, short aerial hyphae, premature conidiation on plates, inappropriate conidiation in submerged culture, and increased thermotolerance. Evidence suggests that the heterotrimeric Gα protein GNA-1 is a direct positive regulator of adenylyl cyclase. Δ*gna-1* strains are female-sterile, and Δ*gna-1* strains have reduced apical extension rates on normal and hyperosmotic medium, greater resistance to oxidative and heat stress, and stunted aerial hyphae compared to the wild-type strain. In this study, a Δ*gna-1 cr-1* double mutant was analyzed to differentiate cAMP-dependent and -independent signaling pathways regulated by GNA-1. Δ*gna-1 cr-1* mutants have severely restricted colonial growth and do not produce aerial hyphae on plates or in standing liquid cultures. Addition of cAMP to plates or standing liquid cultures rescues *cr-1*, but not Δ*gna-1 cr-1*, defects, which is consistent with previous results demonstrating that Δ*gna-1* mutants do not respond to exogenous cAMP. The females of all strains carrying the Δ*gna-1* mutation are sterile; however, unlike *cr-1* and Δ*gna-1* strains, the Δ*gna-1 cr-1* mutant does not produce protoperithecia. The Δ*gna-1* and *cr-1* mutations were synergistic with respect to inappropriate conidiation during growth in submerged culture. Thermotolerance followed the order wild type < Δ*gna-1* < *cr-1* = Δ*gna-1 cr-1*, consistent with a cAMP-dependent process. Taken together, the results suggest that in general, GNA-1 and CR-1 regulate *N. crassa* growth and development using parallel pathways, while thermotolerance is largely dependent on cAMP.

Development in fungal systems frequently occurs in response to specific environmental cues and stressors. In the presence of abundant nutrients, the filamentous fungus *Neurospora crassa* extends hyphae that elongate and fuse to form the multicellular mycelium (for a review, see reference 51). Desiccation or nutrient deprivation causes the mycelium to differentiate aerial hyphae that give rise to conidiophores and multinucleate asexual spores, macroconidia (referred to here as conidia). Elaboration of conidiophores and production of conidia require an air-water interface; however, submerged cultures can be induced to undergo conidiation by carbon or nitrogen starvation or exposure to high temperatures (7, 18, 43, 55). Nitrogen limitation initiates the sexual cycle by stimulating production of female reproductive structures, or protoperithecia (reviewed in reference 44). Fertilization by a conidium or hypha of the opposite mating type results in formation of the fertilized structure (perithecium), within which sexual spores (ascospores) develop. Mature ascospores are subsequently ejected from the perithecium in the direction of blue light.

Heterotrimeric GTP-binding proteins, consisting of α, β, and γ subunits, transduce various environmental signals to stimulate morphogenesis and cellular responses in fungi (for reviews, see references 5, 19, and 34). Ligands, such as nutrients and pheromones, are recognized by seven transmembrane helical receptors coupled to G proteins. Ligand binding results in a conformational change within the receptor, leading to the exchange of GDP for GTP on the Gα subunit and subsequent dissociation of the GTP-Gα and Gβγ moiety. Depending on the system, both the freed GTP-Gα and Gβγ moiety may regulate downstream effectors, such as adenylyl cyclase and mitogen-activated protein kinase (MAPK) cascades.

The *N. crassa cr-1* mutant lacks adenylyl cyclase activity and cyclic AMP (cAMP) (47, 53, 54), and studies support *cr-1* as the structural gene for the adenylyl cyclase enzyme (28, 29). When the *cr-1* mutant is cultured on a solid medium, it grows colonially, lacks appreciable aerial hyphae, and prematurely produces macroconidia on the surface of the mycelium, yielding a "crisp" appearance. Previous work from our laboratory suggests that the Gα subunit GNA-1 positively stimulates CR-1 activity and participates in pathways regulating female fertility, apical extension, osmotic sensitivity, conidiation, and resistance to heat and oxidative stress (23, 24, 59).

Incongruous development has been observed in signal transduction mutants from *N. crassa* and the related filamentous fungus *Aspergillus nidulans*. Deletion of the *N. crassa* Gα subunit *gna-3* gene or the putative glucose sensor *rco-3* gene causes inappropriate differentiation of conidiophores in submerged cultures and transcription of the conidiation-specific gene *con-10* (27, 38). The *cr-1* mutant also conidiates in submerged culture; however, the submerged conidiation phenotype is not as dramatic as that observed in Δ*gna-3* and *rco-3* strains (27, 38, 41). These results suggest that development in

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N. crassa utilizes both cAMP-dependent and -independent pathways. In *A. nidulans*, overexpression of the RGS (named for regulator of G-protein signaling) protein gene *flbA*, expression of the dominant negative Gα allele $fadA^{G203R}$, or deletion of the Gβ gene *sfaD* leads to submerged culture conidiation (32, 33, 46, 60). Deletion of the cAMP-dependent protein kinase A catalytic subunit gene *pkaA* does not cause inappropriate conidiation in submerged cultures, suggesting that the conidiation defects of FlbA, FadA, and SfaD are cAMP independent (50).

Results from studies of *N. crassa* and *Saccharomyces cerevisiae* support an inverse correlation between cAMP levels and resistance to high temperatures. In *N. crassa*, *cr-1* and *gna-1* mutants possess increased thermotolerance (8, 59). Conversely, strains with GTPase-deficient alleles of *gna-1* are more sensitive to lethal temperatures than the wild type (59). Low cAMP levels induced by loss-of-function mutations in *ras2* or by deletion of the Gα subunit *GPA2* lead to increased heat stress resistance in *S. cerevisiae* (6, 22). In contrast, the elevated cAMP levels of strains carrying the constitutively activated $ras2^{Val19}$ mutation are correlated with hypersensitivity to lethal temperatures (11, 26).

In this study, we investigate cAMP-dependent and -independent pathways regulated by adenylyl cyclase and the stimulatory Gα protein GNA-1 in *N. crassa*. Wild-type, Δ*gna-1*, *cr-1*, and Δ*gna-1 cr-1* strains are analyzed for phenotypes and expression of G-protein subunits and adenylyl cyclase. Thermotolerance, submerged culture conidiation, and transcript levels of a conidiation-regulated gene are also investigated. Our results suggest that thermotolerance is largely dependent on cAMP and that GNA-1 and CR-1 act synergistically to regulate development in *N. crassa*.

MATERIALS AND METHODS

Strains, media, and culture conditions. *N. crassa* strain 74-OR23-1A (mating type *A*) (Fungal Genetics Stock Center [FGSC]) (University of Kansas Medical Center, Kansas City, Kans.) was used as the wild type in all experiments except those involving fertilization of mating type *A* strains; in such cases, wild-type strain Sta73a (mating type *a*) was used as the male. A Δ*gna-1 cr-1* mating type *A* double mutant (strain 1.2) was constructed through a sexual cross between the parental *cr-1* mating type *A* strain (FGSC 4008; allele B123) and Δ*gna-1*::hph^+ mating type *a* strain (3B10a) (24). Ascospores were plated on medium containing sorbose (9) containing 200 μg of hygromycin B per ml. Southern blot analysis was performed (23) to verify that hygromycin-resistant progeny contained the Δ*gna-1*::hph^+ gene replacement (data not shown). Since the *cr-1* gene and the mating type gene are linked, hygromycin B-resistant progeny were tested for mating type to determine the *cr-1* allele present (9, 41). *Escherichia coli* strain DH5α was used to maintain plasmids (20).

N. crassa strains were cultured on Vogel's minimal medium containing 1.5% sucrose (VM) (56) to facilitate production of vegetative hyphae in liquid cultures or production of vegetative hyphae, aerial hyphae, and conidia on plates. Synthetic crossing medium (SCM) (57) was used to induce production of female reproductive structures during the sexual cycle. Conidia were used to inoculate shaken or standing liquid cultures and agar plates. Media were supplemented with peptone (2% wt/vol) or cAMP (2.0 mM) where indicated.

Overexpression of *N. crassa* adenylyl cyclase (CR-1) and antiserum generation. Cosmid pSV50-4:7C (4:7C) containing the structural gene for *N. crassa* adenylyl cyclase (*nac*/*cr-1*) has previously been identified (28). This cosmid was used to generate a construct to allow overexpression of a 56-kDa protein corresponding to the 5′ end of the *cr-1* open reading frame (ORF). PCR mutagenesis was used to create restriction sites for cloning into the pET22 vector (Novagen, Madison, Wis.) (allows protein overexpression in *E. coli*) and to remove the first two introns from the 5′ end of the ORF in the 4:7C genomic clone. Templates for PCR were generated by cloning (i) a 9.14-kb *Nde*I fragment from 4:7C into the *Nde*I site of pGEM5Zf (pDIV6), (ii) the *Pst*I-*Bam*HI fragment from pDIV6 into pBluescript II KS^+ digested with the same enzymes (pDIV6.5), and (iii) a 4-kb *Eco*RI fragment from 4:7C that contains the 5′ end of the *cr-1* ORF and ~1-kb untranslated sequence into pBluescript II KS^+ to yield pDIV8.

The first *cr-1* intron was deleted through creation of a silent mutation at the intron splice junction that also encodes an *Nhe*I restriction site. Two PCR products flanking the intron were generated using pDIV8 as a template and ligated together to create a fragment extending from the ATG translational initiation codon to the end of the second exon of *cr-1*. The primers used for the two PCRs are (i) NAC1 (creates an *Nde*I site at the translational start site of *nac*; plus strand) (G GTG CAT ATG ACG AGA AAT GAC), (ii) NAC2 (encodes an *Nhe*I site at the 3′ end of exon 1; minus strand) (TCA GCT AGC AAA TTG GGT GCC GTC), (iii) NAC3 (creates an *Nhe*I site at the 5′ end of exon 2; plus strand) (ATC GCT AGC AGG TCA TCG AGA G), and (iv) NAC4 (recognizes an *Eco*RI site in exon 2; minus strand) (GG GAA TTC AAT GAC AAC TGC). The NAC1/NAC2 and NAC3/NAC4 PCRs were performed using *Pfu* polymerase and 625 ng (each) of the two indicated primers according to the manufacturer's recommendations (Stratagene, La Jolla, Calif.). The NAC1/NAC2 and NAC3/NAC4 PCR products were then digested with the appropriate enzymes and then ligated into pSP72 (Promega, Madison, Wis.) digested with *Nde*I and *Eco*RI to create pDIV13.

The second intron in *cr-1* was removed using inverse PCR with pDIV6.5 as a template. The primers were NAC5 (recognizes the 5′ end of intron 2 junction; minus strand) (GTC GGC TTC TTG GTA CAG AAA CGG) and NAC6 (anneals at 3′ end of intron 2 junction; plus strand) (GAC ATC GCT CGC TAT GGC GAA GCA CCC). The NAC5/NAC6 reactions were performed using *Pfu* polymerase and 125 ng (each) of the two indicated primers according to the manufacturer's recommendations (Stratagene), except for the presence of 5% dimethyl sulfoxide. The NAC5/NAC6 3.7-kb linear PCR product containing vector and insert was ligated and transformed into *E. coli* DH5α cells. Approximately 56% (27 of 48) of the resulting clones contained a 700-bp *Pst*I-*Bam*HI fragment, indicating deletion of the second intron. Sequence analysis revealed that all clones contained additional small deletions at the intron splice site. Clone pDIV12 contained a n^{-3} mutation that resulted in an in-frame deletion of one of two successive aspartate residues (at position 441 or 442 in the amino acid-coding sequence). The absence of one amino acid is not expected to dramatically affect the ability of the protein to function as an effective antigen in rabbits; therefore, this clone was used to make the final overexpression construct (see below).

The 700-bp *Pst*I-*Bam*HI fragment from pDIV12 and the 673-bp *Nde*I-*Pst*I fragment from pDIV13 were ligated into pET22b (Qiagen, Inc., Valencia, Calif.) digested with *Nde*I and *Bam*HI to create the final overexpression construct, pDIV18. *E. coli* strain HMS174(p*lysS*) (52) was transformed with pDIV18, and the strain was cultured to facilitate overexpression of the CR-1 protein fragment as recommended by the manufacturer (Qiagen). The 56-kDa protein was subsequently purified by virtue of its carboxy-terminal six-His epitope tag using Ni-nitrilotriacetic acid agarose according to the manufacturer's recommendations (Qiagen). The fractions enriched for the CR-1 protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide), and the CR-1 protein band was excised and used to raise a specific antiserum in rabbits (Cocalico Biologicals, Reamstown, Pa.).

Western and Northern blot analyses. The particulate fractions used for the adenylyl cyclase assays described above were subjected to Western blot analysis using GNA-1 (23), GNA-2 (3), GNB-1 (24), or CR-1 antisera at final dilutions of 1:2,500, 1:5,000, 1:5,000, and 1:10,000, respectively. The proteins were detected by using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, N.J.) with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad, Hercules, Calif.) as the secondary antibody.

For isolation of total RNA, 8-day-old conidia were inoculated into 50-ml portions of liquid VM and cultured in constant darkness for 16 h at 30°C with shaking at 200 rpm. Mycelia were collected by vacuum filtration and ground to a fine powder in liquid nitrogen, and total RNA was isolated as previously described (48). Northern blot analysis was performed as described previously (4), using a 200-bp *Bam*HI-*Eco*RI fragment of pBW100 containing the *con-10* cDNA (45) or the *cox-5* gene insert from pSRCOX-5 (48) as probes.

Adenylyl cyclase assays. Eight-day-old conidia were inoculated at a final concentration of 2.5×10^6 cells/ml in 500 ml of VM and cultured at 30°C for 16 h in darkness with shaking at 200 rpm. Mycelia were collected by filtration and ground to a fine powder in liquid nitrogen. Samples were suspended in 10-ml portions of extraction buffer [25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.9), 1 mM $MgCl_2$, 0.25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride] and stirred gently at 4°C for 20 min. Extracts were centrifuged at 1,000 × *g* for 15 min at 4°C. The resulting supernatant was centrifuged at 180,000 × *g* at 4°C. The pellet (particulate fraction) was resuspended in extraction buffer, and

protein concentration was determined using the Bradford microassay kit (Bio-Rad) with bovine serum albumin as a standard. Samples were assayed for Mn^{2+}-ATP- and Mg^{2+} -ATP-dependent adenylyl cyclase activity as described previously (24).

Phenotypic analysis and thermotolerance measurements. Colony growth and morphology were assessed by inoculating VM plates with conidia followed by incubation at 30°C in the dark for approximately 36 h. Standing liquid cultures were inoculated and grown as previously described (27) in the presence or absence of 2.0 mM cAMP. For analysis of conidiation of submerged cultures, liquid VM cultures were inoculated with conidia at a density of 1×10^6 or 3×10^6 cells/ml, incubated for 16 h with shaking at 200 rpm in the dark, and then photographed. For analysis of female fertility, strains were cultured on SCM plates for 10 days in constant light at room temperature to induce production of protoperithecia. Perithecia were photographed as described previously (23) 6 days after fertilization with conidia of the opposite mating type. Resistance of 3-h-old germlings to a 52°C lethal heat treatment with and without prior exposure to 45°C (induced versus uninduced thermotolerance, respectively) was measured as previously described (59). Percent survival was obtained by dividing the number of colonies from plates containing heated germlings (induced or uninduced) by the number of colonies on plates from germlings held at 30°C throughout the experiment (controls).

RESULTS

Δ*gna-1 cr-1* strains contain wild-type levels of GNA-2 and GNB-1 but lack full-length CR-1 protein and adenylyl cyclase activity. A regulatory component is required for adenylyl cyclase to utilize Mg^{2+}-ATP as a substrate for both basal and GTP-stimulated activities; in contrast, Mn^{2+}-ATP adenylyl cyclase activity is independent of auxiliary proteins and thus serves as a measurement of the amount of active enzyme (15). *N. crassa cr-1* strains do not contain Mg^{2+}- or Mn^{2+}-ATP adenylyl cyclase activity or intracellular cAMP (47). We have previously shown that while Mn^{2+}-ATP adenylyl cyclase activity is only slightly reduced in Δ*gna-1* mutants, basal and GTP-stimulated Mg^{2+}-ATP activities are severely affected in these strains (24). These results, in combination with the observation that an anti-GNA-1 antibody inhibits Mg^{2+}-ATP-dependent adenylyl cyclase activity in preparations of the wild-type strain, suggested that GNA-1 is the regulatory component in *N. crassa* adenylyl cyclase assays (24).

Certain Δ*gna-1* defects, such as female sterility, are not shared by *cr-1* strains, suggesting that the affected functions are regulated using cAMP-independent pathways. Furthermore, exogenous cAMP does not rescue Δ*gna-1* phenotypes (23), despite the apparent requirement for GNA-1 in regulation of adenylyl cyclase activity. To differentiate between cAMP-dependent and -independent functions of *gna-1*, a Δ*gna-1 cr-1* mutant was constructed through a sexual cross. An observation of identical defects in *cr-1* and Δ*gna-1 cr-1* strains would be evidence for cAMP-dependent functions for *gna-1*. In contrast, an observation of different phenotypes for these two strain backgrounds would offer support for cAMP independence.

The Δ*gna-1* mutation (strain 3b10*a* [mating type *a*]) is marked by an *E. coli hph* gene insertion, and the *cr-1* mutation (FGSC 4008 [mating type *A*]) is followed by linkage to the mating type (41). Therefore, homokaryotic hygromycin-resistant (hyg^R) ascospore progeny should carry the *gna-1* gene replacement (data not shown) and should not contain the GNA-1 protein (Fig. 1A). Hyg^R progeny that were phenotypically identical to the Δ*gna-1* parent were tested for mating type, and all were mating type *a*, consistent with the presence of the *cr-1*$^+$ allele. Of 10 hyg^R phenotypically crisp progeny, 2 were mating type *a* and 8 were mating type *A*. Southern blot analysis demonstrated that all 10 strains carried the Δ*gna-1*::*hph*$^+$ deletion mutation (data not shown). Thus, the mating type *a* hyg^R crisp progeny represented strains that were *cr-1* mating type recombinants.

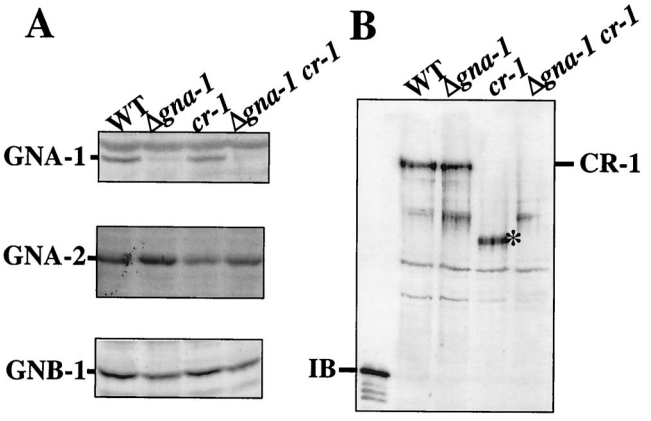

FIG. 1. Levels of Gα, Gβ, and adenylyl cyclase proteins. (A) Levels of GNA-1, GNA-2, and GNB-1. Samples containing 30 μg of total membrane protein from strains with the indicated genotypes were subjected to Western blot analysis using specific antisera. WT, wild type. (B) Levels of CR-1. Samples containing 30 μg of total membrane protein were analyzed by Western blot analysis using a CR-1 antiserum. The position of full-length CR-1 in the wild-type (WT) and Δ*gna-1* mutant preparations is indicated to the right of the blot. The migration position of the amino-terminal 56-kDa CR-1 protein fragment (IB) used as an antigen is indicated. The asterisk indicates the 150-kDa species found in the *cr-1* strain preparation.

Western blot analysis showed that *cr-1* and wild-type strains contain wild-type levels of GNA-1, GNA-2, and GNB-1, as previously observed (Fig. 1A) (27). The Δ*gna-1 cr-1* mutants lack GNA-1 but contain wild-type levels of the Gα and Gβ subunits GNA-2 and GNB-1, respectively (Fig. 1A). A 56-kDa protein corresponding to the extreme amino terminus of CR-1 was overexpressed in *E. coli* and used to generate a polyclonal antibody in rabbits. Western blot analysis with the preimmune serum showed no hybridizing proteins (data not shown). Analysis with the immune serum indicated reaction with a 220-kDa species present at similar levels in Δ*gna-1* and wild-type strains (Fig. 1B). The 220-kDa protein was not observed in *cr-1* preparations (Fig. 1B), but a prominent ~150-kDa species was present. Since the CR-1 antibody is directed against the amino terminus, this 150-kDa protein may be the result of a nonsense mutation at the carboxy terminus, producing a truncated protein. Mutation of *gna-1* in the *cr-1* background leads to loss of the ~150-kDa species, implying that GNA-1 influences the level of the mutant protein; however, we do not know the mechanism underlying this effect. The observation that levels of the 220-kDa protein are similar in wild-type and Δ*gna-1* strains is consistent with the modest effect of the Δ*gna-1* mutation on Mn^{2+}-ATP activity noted in our earlier study (24).

As expected, no Mn^{2+}-ATP or Mg^{2+}-ATP-dependent adenylyl cyclase activity could be detected in *cr-1* or Δ*gna-1 cr-1* strains (data not shown). Δ*gna-1* mutant preparations contain substantial levels of Mn^{2+}-ATP-dependent activity, consistent with previous results (87% of wild-type activity [24]). Similar to what has been noted previously (24), Δ*gna-1* mutants have basal and GTP-stimulated Mg^{2+}-ATP-dependent activities that are only 18 and 9.5% of wild-type activity, respectively.

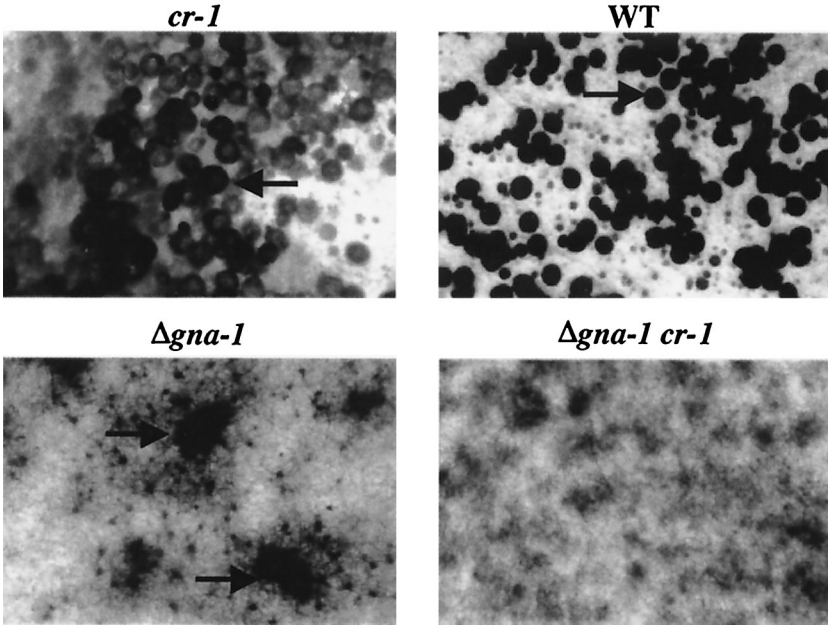

FIG. 2. Female fertility. Strains with the indicated genotypes were cultured on SCM for 7 days prior to fertilization using wild-type (WT) strain 74-OR23-1A or Sta73a conidia. Perithecia were photographed 6 days after fertilization. Perithecia (in *cr-1* and wild-type strains) and aberrant perithecia (in a Δ*gna-1* strain) are indicated by arrows.

***cr-1* and *gna-1* contribute to female fertility.** Previous work from our laboratory demonstrated that Δ*gna-1* mutants are male-fertile but female-sterile. Δ*gna-1* strains have aberrant protoperithecia that do not develop into normal perithecia after fertilization and that only rarely produce ascospores (Fig. 2) (23). In contrast, *cr-1* mutants are able to function as males or females during a sexual cross but exhibit delayed perithecial and ascospore development compared to the wild type (Fig. 2) (41). These observations supported the hypothesis that cAMP is not essential for female fertility in *N. crassa*.

Functions for GNA-1 and cAMP in regulating fertility were further examined by analyzing the sexual cycle in strains lacking both *gna-1* and *cr-1*. Similar to Δ*gna-1* mutants, the males of Δ*gna-1 cr-1* strains are fertile but the females are sterile (data not shown and Fig. 2). However, unlike Δ*gna-1* strains, Δ*gna-1 cr-1* double mutants do not form observable protoperithecia or perithecia (data not shown and Fig. 2). Therefore, the *cr-1* mutation blocks protoperithecial formation in the Δ*gna-1* background, suggesting that cAMP may play a role in protoperithecial development. This contrasts with the known effects of *cr-1* on perithecial development and may reflect a requirement for cAMP during protoperithecial formation that is exacerbated by mutation of *gna-1* and which impedes later perithecial development.

Loss of *gna-1* blocks the response of *cr-1* mutants to exogenous cAMP. As mentioned above, *cr-1* mutants have a greatly reduced apical extension rate and produce much shorter aerial hyphae compared to those of the wild type. The apical extension rate of Δ*gna-1* strains is approximately 50% of the wild-type rate, and Δ*gna-1* mutants produce shorter aerial hyphae (23). Supplementation of *cr-1* plate and standing liquid cultures with cAMP restores the wild-type morphology and growth rate to *cr-1* mutants but has no effect on Δ*gna-1* mutants (23, 27; unpublished observations in reference 53).

The inability to rescue the apical extension rate and aerial hypha height defects of Δ*gna-1* strains by adding cAMP could stem from the fact that these processes are controlled using distinct cAMP-dependent (*cr-1*) and cAMP-independent (*gna-1*) mechanisms in *N. crassa*. Alternatively, our previous observations could reflect a more general requirement for *gna-1* in the response to exogenous cAMP. To distinguish between these possibilities, we explored the effect of cAMP supplementation on the Δ*gna-1 cr-1* double mutant. Loss of *gna-1* in a *cr-1* background causes a greater restriction of apical growth than that observed in a *cr-1* background alone (57% decrease) (Fig. 3 and data not shown). Similar to previous observations with Δ*gna-1* mutants, exogenous cAMP has no effect on plate cultures of Δ*gna-1 cr-1* strains (data not shown). In standing liquid cultures, *cr-1* strains produce short aerial hyphae, while hyphae are completely absent in Δ*gna-1 cr-1* mutants (Fig. 3B). Addition of cAMP restored wild-type aerial hypha height to *cr-1* mutants but had no effect on Δ*gna-1 cr-1* (Fig. 3B). Thus, not only are Δ*gna-1* strains refractory to exogenous cAMP, but the presence of the Δ*gna-1* mutation also blocks the normally robust response of *cr-1* mutants. A similar effect is observed upon loss of *gna-1* in the Δ*gna-3* genetic background (A.M. Kays and K. A. Borkovich, unpublished observations). These observations are consistent with a more general requirement for *gna-1* in the response of *N. crassa* to exogenous cAMP.

Relationship between *cr-1* and *gna-1* in regulating thermotolerance. The heat shock response is a biological phenomenon that occurs in all cells as a mechanism to survive the stress of nonphysiological temperatures. In response to heat stress, cells quickly synthesize heat shock proteins (Hsps) that act to stabilize endogenous proteins, prevent denaturation, and protect cells from subsequent lethal heat exposure. The mechanism of Hsp induction is referred to as induced thermotolerance (for

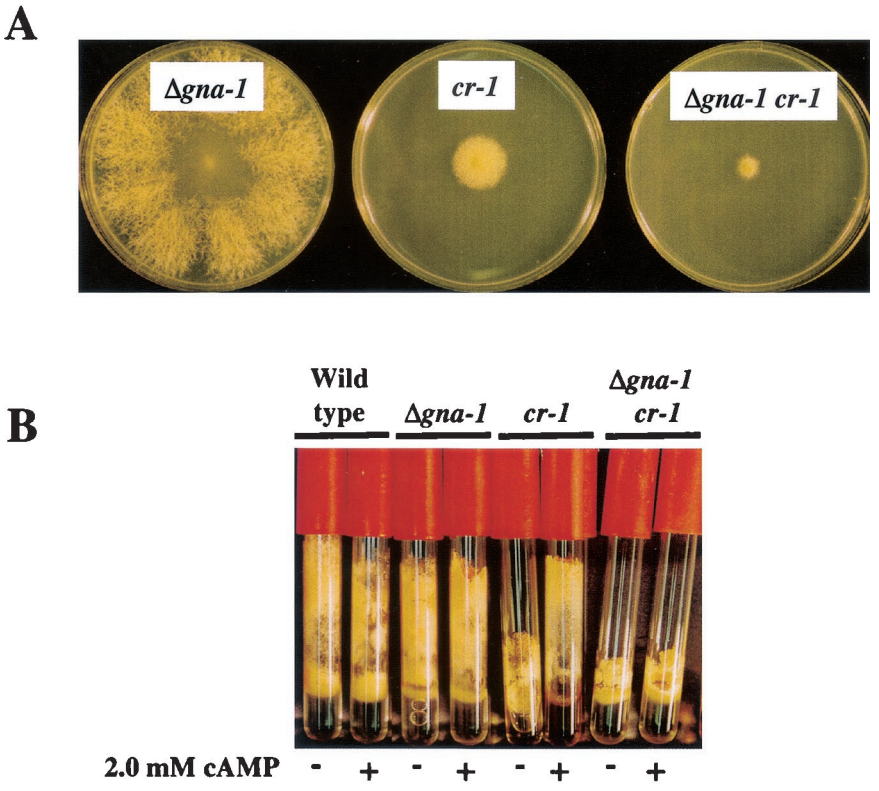

FIG. 3. Vegetative phenotypes in the presence of an air-water interface. (A) Growth of colonies on solid medium. Strains with the indicated genotypes were grown on VM plates at room temperature under constant light and photographed when the Δ*gna-1* colony had reached the edge of the plate (approximately 36 h). Wild-type colonies are approximately two times larger than Δ*gna-1* colonies under these conditions (data not shown). (B) Standing liquid cultures. Strains were inoculated into 2-ml portions of liquid VM and incubated in a stationary position first for 3 days at 30°C in the dark and then for 4 days at room temperature in constant light. The presence (+) or absence (−) of 2.0 mM cAMP is indicated below the test tubes.

reviews, see references 39 and 49). A sudden increase in temperatures is lethal when cells do not have the opportunity to adapt and is referred to as a lethal or uninduced heat shock. Exposure to temperatures over 50°C is lethal to *N. crassa*; however, previous exposure of cells to temperatures of 40 to 47°C allows the induction of Hsps (25, 42).

Studies have shown that mutation of *cr-1* or *gna-1* results in heightened thermotolerance in *N. crassa* (8, 59). Analysis of thermotolerance in Δ*gna-1*, *cr-1*, and Δ*gna-1 cr-1* strains was performed to determine the relationship between Δ*gna-1* and *cr-1* in regulating this phenomenon. If GNA-1 exerts its effect through control of cAMP levels and cAMP alone controls thermotolerance, then the response to high temperatures in the *cr-1* and Δ*gna-1 cr-1* strains should be the same. Alternatively, if GNA-1 also controls a cAMP-independent pathway that negatively regulates the heat shock response, loss of *gna-1* in the *cr-1* background should cause increased thermotolerance.

In agreement with previous work from our laboratory, Δ*gna-1* mutants possess approximately sixfold-greater uninduced thermotolerance than that of the wild type and 4.5-fold-greater induced thermotolerance than that of the wild type (Fig. 4). In contrast to a previous report (8), we found that thermotolerance in *cr-1* strains is inducible; strains containing the *cr-1* mutation have a greater than twofold-higher survival if preexposed to a nonlethal heat treatment (Fig. 4). *cr-1* and Δ*gna-1 cr-1* strains were extremely thermotolerant relative to wild-type and Δ*gna-1* strains, and thermotolerance is inducible in both *cr-1* and Δ*gna-1 cr-1* mutants. Importantly, the thermotolerance of *cr-1* and Δ*gna-1 cr-1* strains is similar within experimental error (Fig. 4), implying that GNA-1 regulates

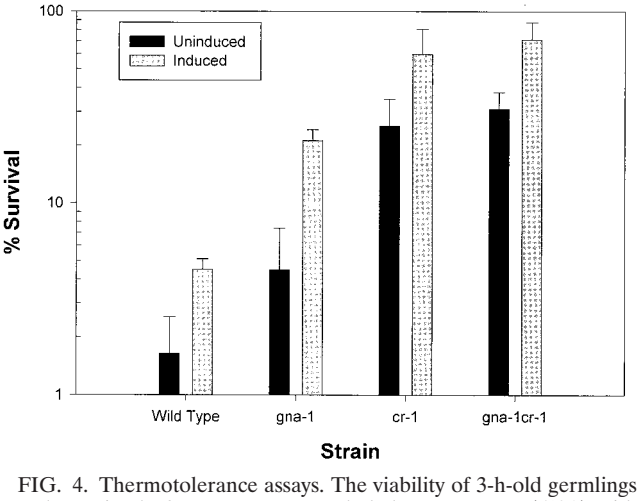

FIG. 4. Thermotolerance assays. The viability of 3-h-old germlings was determined after exposure to a lethal temperature (52°C) with (induced thermotolerance) and without (uninduced thermotolerance) prior exposure to a sublethal heat shock temperature (45°C). The error bars show standard errors of the means.

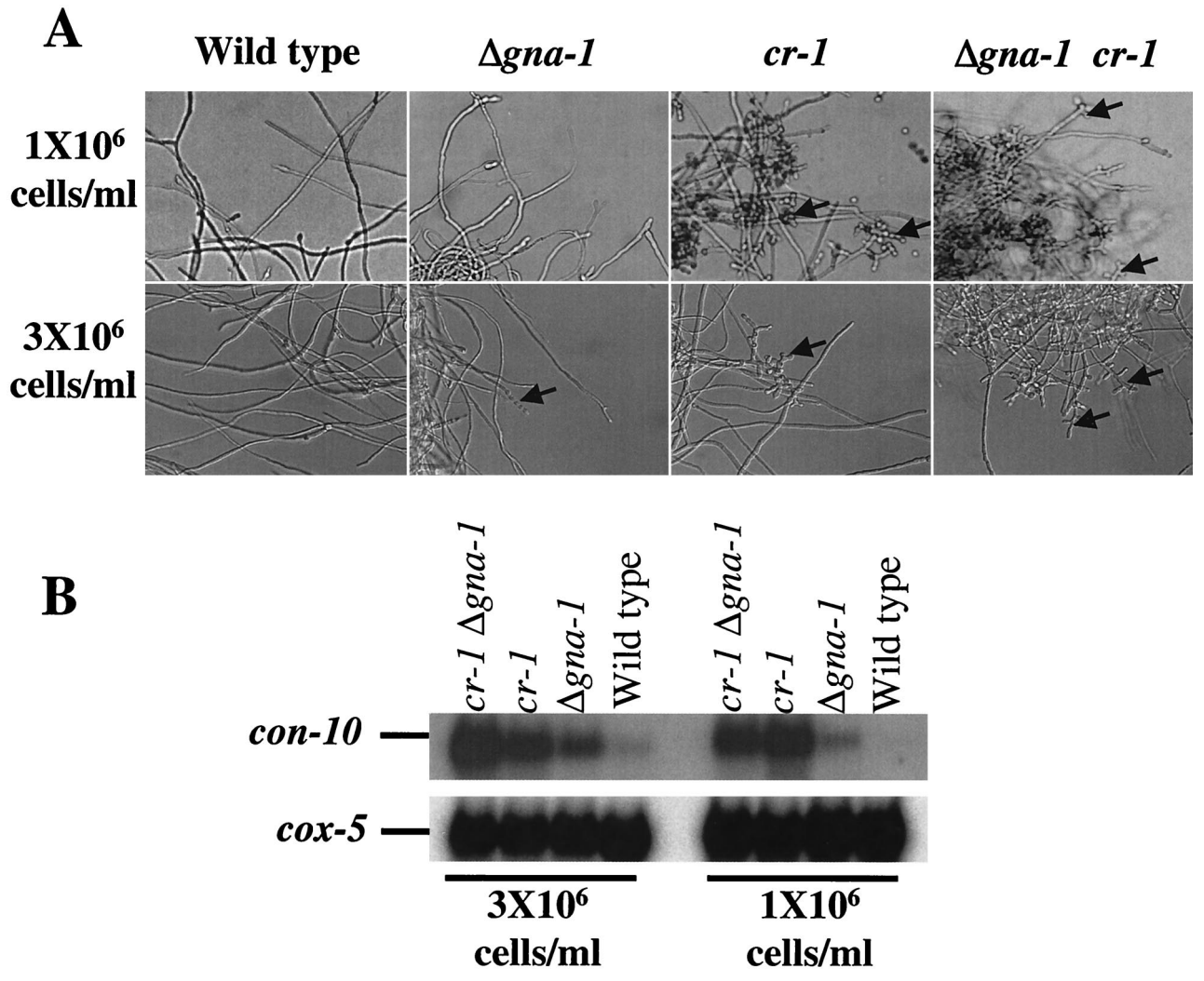

FIG. 5. Conidiophore development and conidiation-specific gene expression in submerged cultures. (A) Conidiophore development. Conidia were inoculated into liquid VM at either 1×10^6 or 3×10^6 cells/ml, incubated with shaking in the dark at 30°C for 16 h, and then photographed. Arrows indicate conidiophores. (B) Correlation between conidiophore development and conidiation-specific gene expression. Total RNA (20 μg) isolated from strains with the indicated genotypes was analyzed by Northern blot analysis using a ^{32}P-labeled *con-10* cDNA as a conidiation-specific probe; the *cox-5* gene was used as a probe to control for RNA loading and transfer.

thermotolerance through modulation of cAMP levels. The data are consistent with previous reports that the observed increase and decrease in thermotolerance of strains carrying either null or constitutively activated *gna-1* alleles result from their lower or higher levels of cAMP, respectively (59).

Δ*gna-1* mutants conidiate in submerged cultures at higher cell densities. Macroconidiation in *N. crassa* requires an air-water interface; thus, conidiation typically occurs on solid surfaces. However, wild-type *N. crassa* strains can be induced to conidiate in submerged cultures when starved for carbon or nitrogen. Conidiation of submerged cultures has also been observed for *cr-1*, *rco-1,* and *gna-3* mutants (27, 38, 41). On the basis of these observations, we postulated that the Δ*gna-1 cr-1* mutant would be derepressed for conidiation in submerged cultures.

The wild-type control strain grew to confluence and did not conidiate in submerged culture under any of the conditions tested (Fig. 5A). The Δ*gna-1* mutant conidiated in submerged culture only when cells were inoculated at a high cell density (3×10^6 cell/ml [Fig. 5A]). A threefold decrease (10^6 cell/ml) in cell density resulted in small, tight mycelial aggregates, with no observable conidiophores (Fig. 5A). *cr-1* and Δ*gna-1 cr-1* strains produced large numbers of conidiophores at both cell densities, with more conidiophores visible in the Δ*gna-1 cr-1* mutant (Fig. 5A). Therefore, submerged culture conidiation in the Δ*gna-1* mutant is cell density dependent, and the Δ*gna-1* mutation intensifies the conidiation defect of *cr-1* strains.

It has been previously demonstrated that *cr-1* mutants express the conidiation-specific gene *con-10* in submerged culture (27). To determine if the conidiation observed in Δ*gna-1* and Δ*gna-1 cr-1* strains correlated with expression of *con-10*, RNA was isolated and analyzed for the presence of the *con-10* transcript using Northern blot analysis. Although no conidiophores were observed, *con-10* was weakly transcribed at the

higher cell density for the wild-type strain, suggesting that conidiation may just be initiating. For the Δ*gna-1* strain, *con-10* was expressed at both cell densities, with greater transcription occurring at the higher inoculation density (Fig. 5B). *cr-1* and Δ*gna-1 cr-1* strains transcribe the *con-10* message at both cell densities (Fig. 5B), with the highest level produced by the Δ*gna-1 cr-1* mutant at the high cell density. The relative amounts of *con-10* in *cr-1* and Δ*gna-1 cr-1* cultures inoculated at the lower concentration were more variable; however, the amounts of transcript produced by Δ*gna-1 cr-1* strains were generally the same as or greater than that made by *cr-1* mutants (data not shown and Fig. 5B).

These results indicate that loss of *cr-1* and *gna-1* is synergistic with respect to derepression of *con-10* expression, particularly in cultures inoculated at a high cell density, as the Δ*gna-1 cr-1* strain contains more *con-10* mRNA than the *cr-1* strain. Furthermore, because adenylyl cyclase activity is absent from both *cr-1* and Δ*gna-1 cr-1* strains, the observation that the Δ*gna-1 cr-1* strain contains the greatest amount of *con-10* transcript indicates that *con-10* gene expression is also regulated by a pathway involving GNA-1, independent of cAMP.

DISCUSSION

Previous work has demonstrated that GNA-1 is necessary for normal stress responses and female fertility and that GNA-3 is a global negative regulator of conidiation (23, 27, 59). Biochemical studies indicate dual regulation of adenylyl cyclase by GNA-1 and GNA-3 in *N. crassa*: GNA-1 positively modulates adenylyl cyclase activity, and GNA-3 maintains adenylyl cyclase enzyme levels (24, 27). Here we have further defined the role of GNA-1 in cAMP-dependent and -independent pathways by constructing and analyzing a Δ*gna-1 cr-1* double mutant. In contrast to the *cr-1* strain, Δ*gna-1 cr-1* grows even more slowly and is refractory to exogenous cAMP. Deletion of *gna-1* in the *cr-1* background leads to female sterility. Thermotolerance experiments show that Δ*gna-1 cr-1* and *cr-1* strains are similarly resistant to high temperatures. Conidiation in Δ*gna-1* submerged cultures is dependent on cell density, and the Δ*gna-1 cr-1* strain conidiates more abundantly than the *cr-1* strain.

The inability of Δ*gna-1 cr-1* strains to respond to exogenous cAMP was consistent with our earlier observation that Δ*gna-1* mutants are insensitive to cAMP supplementation (23). As mentioned above, the same phenomenon is seen in Δ*gna-1* Δ*gna-3* strains (Kays and Borkovich, unpublished). Previous work has shown that both wild-type and Δ*gna-1* strains secrete similar amounts of cAMP into the medium during growth in submerged cultures (24). This indicates that cAMP may function as an extracellular signal in *N. crassa* and that the mechanism that allows movement of cAMP in and out of the cell is intact in Δ*gna-1* mutants. Therefore, GNA-1 may function in a pathway that senses extracellular cAMP levels to inhibit conidiation of submerged cultures or other developmental processes. Alternatively, GNA-1 may be required for the expression or stability of a component in the cAMP sensing or signaling pathway in *N. crassa*.

A requirement for a Gα protein in the response to exogenous cAMP has not been previously reported in *N. crassa* or other related fungi. In fact, there are several instances of fungal adenylyl cyclase and Gα mutants that can respond to exogenous cAMP. As mentioned above, the wild-type morphology is restored to standing liquid cultures of *cr-1* and Δ*gna-3* strains by cAMP supplementation (27, 53). Treatment with cAMP suppresses the developmental defects of the *Magnaporthe grisea* Δ*mac1* and *Ustilago maydis* Δ*uac1* adenylyl cyclase mutants (1, 16). Phenotypic defects resulting from mutation of the *gna-3* homologue in the saprophyte *Podospora anserina* and the pathogens *U. maydis*, *Ustilago hordei*, and *Cryptococcus neoformans* are all rescued by exogenous cAMP (2, 31, 35, 37). Addition of cAMP to a mutant of the *gna-1* homologue, *cpg-1*, in the plant pathogen *Cryphonectria parasitica* suppresses its virulence defect (13).

Sexual development in *N. crassa* is initiated by formation of protoperithecia in response to nitrogen limitation. Deletion of *gna-1* causes an early stage defect in the sexual cycle, resulting in the formation of aberrant protoperithecia (23, 59). In contrast, although *cr-1* strains lack intracellular cAMP, they are still able to complete the sexual cycle and are only delayed in postfertilization development, including perithecial and ascospore production (41). Female sterility in the *cr-1* strain background is observed only upon mutation of *gna-1*, supporting a cAMP-independent role for GNA-1 in regulating female fertility. Furthermore, cAMP supplementation has no effect on the sexual cycle defects of either Δ*gna-1* or Δ*gna-3* mutants (27; F. D. Ivey and K. A. Borkovich, unpublished observations). In contrast to *N. crassa*, the sexual cycle of *M. grisea* and *U. maydis* is dependent on cAMP, and the Δ*mac1* and Δ*uac1* adenylyl cyclase mutants mate only when exogenous cAMP is supplied (1, 16).

Thermotolerance in *N. crassa* and *S. cerevisiae* is inversely correlated with cAMP levels (12). Strains with mutations in genes encoding either adenylyl cyclase or its positive regulators are more resistant to heat stress (6, 8, 22, 59), and heat shock transcription factors are active during periods of low protein kinase A activity in *S. cerevisiae* (17). The thermotolerance of Δ*gna-1 cr-1* and *cr-1* strains is identical, consistent with this process being largely dependent on cAMP in *N. crassa*. However, the observation that thermotolerance can be induced in strains carrying the *cr-1* mutation suggests that there may also be a cAMP-independent mechanism for the induction of heat shock stress regulators. Studies in *S. cerevisiae* support cAMP-independent pathways in regulating the heat shock response. Disturbing the integrity of the cell wall by chemical agents or by mutation of genes important for cell wall synthesis leads to phosphorylation and activation of the MAPK Slt2p and changes in cell wall composition (10). Such disruptions to the cell wall also result in an increased resistance to high temperatures, supporting the observation that additional kinases may also function during the heat shock response (10, 14).

Conidiation of submerged cultures occurs in Δ*gna-1* mutants in a cell density-dependent manner. Conidiation of submerged cultures is much more extensive in *cr-1* cultures than in Δ*gna-1* mutant cultures. Deletion of *gna-1* and *cr-1* is synergistic, with respect to expression of the conidiation-specific gene *con-10* at high cell densities. The observations that Δ*gna-1* strains produce conidiophores only at higher cell densities and that Δ*gna-1 cr-1* strains exhibit more conidiation than *cr-1* mutants suggest that GNA-1 functions independently of cAMP as a negative regulator of conidiation of submerged cultures. The

GNA-1 homologue FadA in *A. nidulans* also serves as a negative regulator of conidiation of submerged cultures (21, 60). cAMP independence is supported by the observation that *A. nidulans pkaA* catalytic subunit mutants do not conidiate in submerged culture (50).

The results from comparison of Δ*gna-1*, *cr-1*, and Δ*gna-1 cr-1* strains support the hypothesis that hyphal development and conidiation involve cAMP-dependent and MAPK pathways. A gene encoding a MAPK kinase kinase (MAPKKK), *nrc-1*, has been identified in *N. crassa* (30). NRC-1 is most similar to the MAPKKK proteins Ste11p and Byr-2 from *S. cerevisiae* and *Schizosaccharomyces pombe*, respectively (30). Mutation of *nrc-1* results in formation of short aerial hyphae, constitutive conidiation on solid medium and in submerged culture, thin basal hyphae, semicolonial growth morphology, defective conidial separation, and female sterility (30); some of these phenotypes are also found in *cr-1* and Δ*gna-1* strains. The presence of shared defects for *nrc-1* and *gna-1* mutants suggests that GNA-1 may act upstream of NRC-1 to suppress submerged conidiation. Another possibility is that a conidiation-specific, cAMP-dependent pathway influences the NRC-1 MAPK cascade. Cross talk between cAMP and MAPK pathways during regulation of fungal development has been observed in *M. grisea*; the MAPK PMK-1, the GNA-1 homologue MAGB, and cAMP all regulate the invasive growth habit of this organism (36, 40).

The results presented here provide further evidence that GNA-1 controls multiple signal transduction pathways in *N. crassa*. The previous demonstration of differential complementation of Δ*gna-1* phenotypes by mammalian Gα protein genes (58), as well as the results from functional analysis of *N. crassa gna-3* and *P. anserina modD* mutants, showed that these three Gα subunits function in more than one pathway (27, 37). GNA-1 regulates cAMP-dependent functions, such as thermotolerance, by positively modulating adenylyl cyclase activity. Female fertility and submerged conidiation are regulated by GNA-1 through cAMP-independent pathways. Of the three *N. crassa* Gα subunits, GNA-1 has the greatest role in resistance to cellular stresses, including hyperosmotic conditions and elevated temperatures. Identification of signaling components that act upstream and downstream of GNA-1 will elucidate the molecular mechanisms that enable filamentous fungi to sense and respond to environmental stresses and will further reveal the extent of conservation between signaling pathways in yeast and filamentous fungal species.

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