The Neurospora crassa DCC-1 Protein, a Putative Histidine Kinase, Is Required for Normal Sexual and Asexual Development and Carotenogenesis

Carlos Barba-Ostría, Fernando Lledías, and Dimitris Georgellis

Received 26 August 2011/Accepted 23 October 2011

Two-component signaling pathways based on phosphoryl group transfer between histidine kinase and response regulator proteins regulate environmental responses in bacteria, archaea, plants, slime molds, and fungi. Here we characterize a mutant form of DCC-1, a putative histidine kinase encoded by the NCU00939 gene of the filamentous fungus Neurospora crassa. We show that this protein participates in the regulation of processes such as conidiation, perithecial development, and, to a certain degree, carotenogenesis. Furthermore, DCC-1 is suggested to exert its effect by promoting cyclic AMP production, thereby placing this protein within the context of a signaling pathway.

Signal transduction by phosphorylation and dephosphorylation of cellular proteins plays pivotal roles in regulating numerous cellular processes in both prokaryotes and eukaryotes. In prokaryotes, signaling by phosphoryl group transfer reactions relies on two-component signal transduction (TCS) pathways that depend on histidine and aspartyl residues as phosphoryl donor groups and acceptors (24, 26, 39, 43). The prototypical two-component system comprises two protein components, a sensor histidine kinase (HK) and its cognate response regulator (RR), that contain a transmitter domain with an invariant histidine residue and a receiver domain with an invariant aspartate residue, respectively. Signal reception by the HK is believed to propagate conformational changes in the protein that stimulate an ATP-dependent autophosphorylation at the conserved histidine residue. The phosphokinase then donates the phosphoryl group to the conserved aspartate residue of its receiver domain (5). Upon cessation of signaling, both the cognate RR and the HK undergo dephosphorylation, which results in silencing of the system (11). Many TCS pathways, however, are more elaborated and operate through a phosphorelay (7, 12, 23, 28, 40). In these systems, signal transmission involves, in addition to the two above-mentioned domains, an extra receiver domain and a histidine-containing phosphotransfer domain (HPT) that can be fused to the HK or can be present as independent proteins (12, 17, 20, 28, 40). Such TCS pathways have also been found in plants, slime molds, and fungi (8, 13, 28). It is noteworthy that most bacterial sensor kinases possess only one transmitter domain, whereas the sensor kinases present in eukaryotic microorganisms or plants are almost exclusively multimeric HKs (8, 13, 28, 33). Curiously, the architecture of filamentous fungal TCS pathways provides another notable difference. Prokaryotic TCS pathways are typically organized in protein pairs, whereas in filamentous fungi, multiple HKs appear to share a single HPT protein to transduce signals to two RRs. For instance, the Neurospora crassa genome predicts 11 hybrid HKs, one HPT protein, and two canonical RRs, in addition to the RR protein encoded by the NCU07378 gene and homologous to Saccharomyces cerevisiae Rim15p, which lacks the conserved aspartate residue of its receiver domain (5).

A number of studies have addressed the functions and phenotypes of various TCS proteins in N. crassa. For instance, it has been shown that RRG-1 is involved in the activation of a mitogen-activated protein kinase (MAPK) pathway that regulates female fertility and responses to osmotic and fungicide stresses (18). On the other hand, RRG-2 is implicated in cell responses against oxidative stress during vegetative growth (4). Moreover, the HPT-1 protein has been suggested to play a central role in N. crassa viability because attempts to delete the corresponding gene have been fruitless (4). Finally, the function of the OS-1, PHY-1, and PHY-2 HKs has also been addressed in N. crassa. OS-1 has been shown to participate in the activation of the above-mentioned MAPK pathway (1, 18, 36) and therefore has been placed upstream of RRG-1 in the signaling cascade responsive to osmotic and fungicide stresses (18). PHY-1 and PHY-2 show sequence similarity to bacterio-phytochromes and plant phytochromes (5), and a bacterially expressed N-terminal fragment of PHY-2 was shown to covalently bind either biliverdin or phycocyanobilin in vitro, with the resulting holoprotein displaying red/far-red light photochromic absorption spectra and a photocycle. However, deletions of phy-1 and phy-2 cause no phenotypic defects (10), although they produce alterations in the expression of the light-responsive conidiation gene con-10 (25).

Here we describe the results of experiments demonstrating that the protein encoded by the NCU00939 gene, a putative histidine
sensor kinase, participates in the regulation of asexual spora-
tion, perithecial development, and, to a certain degree, caroten-
genesis. We therefore propose to coin the NCU00939-encoded protein DCC-1, for development and carotenogenesis control, and hereafter this protein will be referred to as DCC-1. Moreover, we provide experimental data indicating that DCC-1 most likely exerts its effect by promoting cyclic AMP (cAMP) produc-
tion, thereby placing DCC-1 within the context of a signaling pathway that operates during conidiation and sexual develop-
m ent.

Probing for phenotypic differences in \textit{N. crassa} HK mutants.

The \textit{N. crassa} genome predicts 11 hybrid HKs, one HPT protein, and two canonical RRs (5). Although various studies have addressed the function of the RRs and HPT, only three HKs have been studied. To gain some insight into the TCS pathways and the functions of the various HK proteins, we pursued a phenotypic characterization of single HK deletion mutants. All strains were provided by the Fungal Genetics Stock Center (FGSC) (22), except the NCU04615 mutant, which was made in this study according to the previously published knockout procedure (9). All strains were confirmed by PCR (data not shown). For example, the \textit{dcc-1} mutant (FGSC catalog no. 13652) was verified by PCR using primers 5'-AGCGGCAAG TGGGAGCTCAT-3' (placed upstream of the \textit{dcc-1} gene and indicated as primer 1 in Fig. 1B and C), 5'-CCAATGCA TCTATGGCTCTATCGTAC-3' (placed within the \textit{dcc-1} gene and indicated as primer 2 in Fig. 1B), and 5'-TTGGGCG TTGGCTGAGCTAGT-3' (placed within the \textit{hph} gene and indicated as primer 3 in Fig. 1C). As shown in Fig. 1, primers 1 and 2 amplified a band of the expected size (5.4 kb) in the wild-type strain but not in the mutant strain, whereas in the case of the \textit{dcc-1} deletion mutant, which exhibited a slightly lower hyphal elongation rate (Fig. 2A). Moreover, although no defects in radial growth were observed in any of the mutants, a striking difference was noticeable in the \textit{dcc-1} deletion strain. Orange pigmentation, representative of carotenoid accumula-
tion, was observed over the entire surface of the plate inocu-
lated with the \textit{dcc-1} mutant strain, whereas in the case of the wild type, orange pigmentation was observed mainly at the center and near the edge of each plate (Fig. 2B). Because of these apparent differences, we decided to focus our efforts on the characterization of the \textit{dcc-1} mutant strain. The \textit{dcc-1} gene encodes a putative hybrid HK, which is conserved among Sor-
darionymycetes but whose function is unknown. The SMART protein domain prediction program (21, 35) revealed the pres-
ence of GAF and PAS/PAC motifs as sensing or input domains of the \textit{dcc-1} protein.
The DCC-1 HK regulates conidiation of *N. crassa*. *N. crassa* is a filamentous fungus that has a complex life cycle due to its ability to produce both asexual (vegetative phase of growth) and sexual spores (38). During vegetative growth, *N. crassa* hyphae elongate, intertwine, and fuse, forming the mycelial network. Under carbon source deprivation and when a water-air interface exists, *N. crassa* enters its asexual developmental cycle, during which aerial hyphae differentiate from mycelia, generate chains of proconidia, and terminate with the septation of proconidia and the release of free mature conidia (macroconidia). We therefore argued that if the DCC-1 HK acts as a repressor of the process of conidiation, growing the *dcc-1* mutant on glucose-rich medium and incubating the plates in complete darkness should result in augmented conidiation. This was tested by inoculating Vogel’s glucose medium (VGM) plates with $1 \times 10^5$ wild-type or mutant conidia, incubating the plates at 30°C in complete darkness, and harvesting and counting the conidia every 12 h. It was found that the mutant strain produced conidia as early as 12 h after inoculation, unlike the wild-type strain, in which conidia were detected after 48 h. Remarkably, even at 72 h after inoculation, the mutant strain produced approximately 3.5 times more conidia than the wild type (Fig. 3A).

As mentioned above, formation of aerial hyphae and subsequent conidiation require an air-water interface, and therefore conidiation does not occur in submerged cultures (37). Given the above-described phenotype of increased conidiation under growth-repressing conditions (glucose-rich medium and darkness), we asked whether the *dcc-1* mutant also produces conidia in submerged cultures. We therefore inoculated wild-type and mutant strains into VSM at $1 \times 10^6$ spores/ml and incubated the cultures at 30°C with agitation (200 rpm) in complete darkness. After 16 h of incubation, a sample of each strain was withdrawn and examined by light microscopy. As expected, no conidia were observed in submerged cultures of the wild type. In contrast, the mutant strain produced a significant amount of conidia (Fig. 3B). Also, the hyphae of the mutant strain appeared to be shorter than those of the wild-type strain (Fig. 3B). This is to be expected if the hyphae of the mutant strain were to enter the program of conidiation prematurely. This was tested by comparing the growth of the wild-type and mutant strains in standing liquid VSM cultures. As shown in Fig. 3C, the wild-type strain conidiated at the tip of the elongated aerial hyphae, whereas dense premature conidiation and no elongated aerial hyphae were observed in the mutant strain (Fig. 3C). Premature conidiation could also provide a plausible explanation of the above-described slower lineal growth of the *dcc-1* mutant (Fig. 2C). In view of the above results, it can be concluded that the *N. crassa* DCC-1 HK acts as a negative regulator of the asexual sporulation pathway, since its absence causes derepression of conidiation under non-sporulating conditions.

**The DCC-1 HK is required for proper perithecial development in *N. crassa***. Given the significant role of DCC-1 as a negative regulator of sporulation during vegetative growth, we asked whether it also participates in the regulation of sexual development. Under conditions of nitrogen limitation, *N. crassa* enters a sexual life cycle during which vegetative hyphae initiate a sexual differentiation pathway to form the female reproductive structure (protoperitheciun) (27, 30). The pro-
To test for possible effects of the DCC-1 HK on the processes of the *N. crassa* sexual cycle, the 74-ORS-6a (FGSC catalog no. 4200) wild-type (wt; A, C, and E) and *dcc*-1 mutant (B, D, and F) strains were inoculated as females onto Vogel’s N modified agar plates. After 7 days of incubation at 25°C in the presence of light, the plates were inoculated with strain 74-OR23-1VA (FGSC catalog no. 2489) as the male, and 7 days later, the plates were examined. Representative pictures of the surface of the wild type (A, C) and the *dcc*-1 mutant (B, D) are presented. The cross sections of the plates with the wild-type (E) and *Δdcc*-1 mutant (F) strains are presented. Arrows indicate the localization of the perithecia formed.

To test for possible effects of the DCC-1 HK on the processes of the *N. crassa* sexual cycle, the 74-ORS-6a (FGSC catalog no. 4200) wild-type and *dcc*-1 mutant strains were inoculated as females onto 10-cm plates under nitrogen-limiting conditions (Vogel’s N modified medium [31]) and incubated at 25°C with constant light. At day 7 postinoculation, wild-type strain 74-OR23-1VA (FGSC catalog no. 2489) was inoculated as a male and the plates were incubated at 25°C with constant light. Seven days later, it was observed that, in contrast to the wild-type strain, which formed mature pigmented perithecia on the surface of the plate, only an insignificant number of small unpigmented mutant fruiting bodies were observed on the surface of the plate (Fig. 4A to D), suggesting that deletion of *dcc*-1 leads to sterility. However, after careful inspection of the plates inoculated with the mutant strain, it was observed that an almost equal number of mature perithecia were formed, although they were submerged in the agar (Fig. 4F). This phenotype should result in the ascospores, if they were formed, remaining enclosed in the perithecia or being ejected into the agar but unable to be released into the air.

To test whether mature and viable ascospores are produced in the *dcc*-1 mutant, submerged perithecia were harvested from the agar and disrupted with a homogenizer to release the ascospores. Indeed, mature ascospores...
able to germinate after heat activation and form colonies at percentages similar to those of the wild type were obtained (data not shown). It can therefore be concluded that although the DCC-1 HK does not affect ascospore production and viability, it is required for proper localization of perithecia. The DCC-1 HK does not affect ascospore production and viability (data not shown). It can therefore be concluded that although the DCC-1 protein plays a role not in the regulation of rhythmicity (2), we asked whether DCC-1 exerts its effect, we looked for mutant strains that exhibit phenotypes similar to the ones described for the DCC-1 mutant strain. Interestingly, similar phenotypes have been described for mutants encoding proteins that are implicated in cAMP metabolism. For example, a gna-3 (Gα subunit which, together with CR-1, participates in cAMP production) mutant shows dense premature conidiation and formation of short aerial hyphae, two phenotypes that are reversed by exogenous cAMP (19). Another phenotype due to gna-3 that is not reversed by exogenous cAMP is the formation of submerged perithecia (19). Considering the similar phenotypes of the gna-3 and DCC-1 mutants, the ability of cAMP to reverse the Δdcc-1-dependent phenotypes was tested. It was found that addition of cAMP to solid VGM and standing liquid cultures resulted in a drastic reduction of premature conidiation (Fig. 6A and B) and improved aerial hypha elongation of the Δdcc-1 mutant (Fig. 6C). It should be mentioned that, as has been reported for the Δgna-3 strain (19), cAMP addition to submerged cultures does not reverse the conidiation phenotype of the Δdcc-1 mutant strain (data not shown). Furthermore, carotenoid accumulation in the mutant strain was reduced to wild-type levels (Fig. 6D) whereas conidiation was decreased approximately 2-fold when the liquid VSM containing 0.2% Tween 80 as a wetting agent was supplemented with cAMP (data not shown). It may therefore be concluded that DCC-1 participates in the regulation of asexual sporaulation and carotenogenesis most likely by affecting cAMP metabolism in a pathway that may include GNA-3 and CR-1.

**Exogenous cAMP reverses the dcc-1-dependent differentiation phenotypes.** To gain some insight into the pathway(s) through which DCC-1 exerts its effect, we looked for mutant strains that exhibit phenotypes similar to the ones described for the Δdcc-1 mutant strain. Interestingly, similar phenotypes have been described for mutants encoding proteins that are implicated in cAMP metabolism. For example, a gna-3 (Gα subunit which, together with CR-1, participates in cAMP production) mutant shows dense premature conidiation and formation of short aerial hyphae, two phenotypes that are reversed by exogenous cAMP (19). Another phenotype due to gna-3 that is not reversed by exogenous cAMP is the formation of submerged perithecia (19). Considering the similar phenotypes of the gna-3 and DCC-1 mutants, the ability of cAMP to reverse the Δdcc-1-dependent phenotypes was tested. It was found that addition of cAMP to solid VGM and standing liquid cultures resulted in a drastic reduction of premature conidiation (Fig. 6A and B) and improved aerial hypha elongation of the Δdcc-1 mutant (Fig. 6C). It should be mentioned that, as has been reported for the Δgna-3 strain (19), cAMP addition to submerged cultures does not reverse the conidiation phenotype of the Δdcc-1 mutant strain (data not shown). Furthermore, carotenoid accumulation in the mutant strain was reduced to wild-type levels (Fig. 6D) whereas conidiation was decreased approximately 2-fold when the liquid VSM containing 0.2% Tween 80 as a wetting agent was supplemented with cAMP (data not shown). It may therefore be concluded that DCC-1 participates in the regulation of asexual sporaulation and carotenogenesis most likely by affecting cAMP metabolism in a pathway that may include GNA-3 and CR-1.

Finally, the effect of cAMP in circadianly regulated conidiation could not be tested because endogenous oscillations of the level of cAMP have been shown to be critical for clock-controlled conidiation (15). Also, as has been described for the Δgna-3 mutant (19), supplementation with cAMP did not restore the sexual defects (formation of submerged perithecia) of the Δdcc-1 mutant (data not shown).

Taken together, our findings strongly suggest that the DCC-1 protein of *N. crassa* plays a pivotal role in the pathways that regulate developmental processes such as conidiation and perithecial development and in the one responsible for masking circadianly regulated conidiation during vegetative growth and also, to a certain degree, affects carotenoid accumulation.

**Deletion of dcc-1 results in unmasking circadianly regulated conidiation of *N. crassa*.** Circadian oscillators, which respond to environmental signals such as light and temperature, are known to control the expression of various genes whose products are associated with cellular processes, including development (42). The most obvious and prominent circadianly controlled rhythm in *N. crassa* is that of conidiation, which forms a characteristic conidial banding-pattern on plates or in long glass culture tubes (race tubes). Also, it has been previously reported that RRG-1 of *N. crassa* functions in an output pathway to regulate circadian rhythmicity, although the upstream HK was not identified (41). Because of the intimate association of the DCC-1 sensor HK with the process of conidiation and the involvement of a two-component system protein (RRG-1) in the regulation of rhythmicity (2), we asked whether DCC-1 also affects this process. To test this, we assayed the Δdcc-1 deletion strain for the formation of conidiation bands in race tubes with glucose-arginine medium. It has to be mentioned that the wild-type strain does not form the conidial banding pattern. This is because CO2 levels become elevated during vegetative growth, and as a consequence, conidiation becomes suppressed, thereby masking the rhythmic banding pattern. Therefore, a nas-1 mutant strain (FGSC catalog no. 1858), carrying a T79I substitution and referred to as a bd mutant (34), which is about 200-fold less sensitive to the CO2 masking effect, is used for circadian rhythm studies.

Exponentially grown mycelia of the wild-type, Δdcc-1, bd, and Δdcc-1 bd mutant strains were inoculated at one side of the race tubes and incubated with illumination at 25°C for 24 h to entrain the cultures before a shift to constant darkness. The periods of the Δdcc-1, bd, and Δdcc-1 bd mutant strains were tested in race tubes. To entrain the cultures, tubes were kept for 24 h in constant light. Tubes were then shifted to constant darkness, and growth was monitored every 24 h. The periods of the Δdcc-1, bd, and Δdcc-1 bd mutant strains are expressed in hours. The wild-type strain period was not determined (ND).

**FIG. 5.** DCC-1 deletion unmasks circadianly regulated conidiation of *N. crassa*. Circadianly regulated conidiation patterns of the wild-type (wt) and Δdcc-1, bd, and Δdcc-1 bd mutant strains were tested in race tubes. To entrain the cultures, tubes were kept for 24 h in constant light. Tubes were then shifted to constant darkness, and growth was monitored every 24 h. The periods of the Δdcc-1, bd, and Δdcc-1 bd mutant strains are expressed in hours. The wild-type strain period was not determined (ND).

---

Vol. 10, 2011 NOTE 1737

**Vol. 10, 2011 NOTE 1737**

Downloaded from http://ec.asm.org/ on October 16, 2017 by guest
Conclusions. The *N. crassa* NCU00939 gene encodes a putative hybrid HK consisting of GAF and PAS/PAC as input domains, a transmitter domain with a conserved histidine residue at position 1031, and a receiver domain with a conserved aspartate residue at position 1464. Phenotypic analyses of a DCC-1 mutant strain (FGSC catalog no. 13652) indicate that this putative sensor HK negatively regulates the processes of conidiation and, to a certain degree, carotenogenesis and also has pronounced effects on the development of perithecia during the sexual life cycle. Moreover, DCC-1 appears to exert its effect by affecting cAMP metabolism in a pathway that includes GNA-3 and CR-1, thereby placing DCC-1 within the context of a signaling pathway that operates during conidiation and sexual development. Although this study provides some insight into the processes regulated by DCC-1, the scope of its control remains to be clarified and the other components participating in the same signaling cascade(s) have yet to be determined.

We thank Jorge Nieto-Sotelo for critically reading the manuscript and Claudia Rodriguez Rangel for technical assistance.

This work was supported by grants 80684 from the Consejo Nacional de Ciencia y Tecnología (CONACyT); IN219709-3 from DGAPA, UNAM; and CRPMEX008-02 from ICGEB.

**ADDENDUM IN PROOF**

The observed phenotypes were found to segregate with the hygromycin B resistance gene (*hph*), indicating that they are the result of the deletion of the *dcc-1* gene.

**REFERENCES**


