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The Nuclear Dbf2-Related Kinase COT1 and the Mitogen-Activated Protein Kinases MAK1 and MAK2 Genetically Interact to Regulate Filamentous Growth, Hyphal Fusion and Sexual Development in *Neurospora crassa*

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ABSTRACT

Ndr kinases, such as *Neurospora crassa* COT1, are important for cell differentiation and polar morphogenesis, yet their input signals as well as their integration into a cellular signaling context are still elusive. Here, we identify the *cot-1* suppressor *gul-4* as *mak-2* and show that mutants of the *gul-4/mak-2* mitogen-activated protein (MAP) kinase pathway suppress *cot-1* phenotypes along with a concomitant reduction in protein kinase A (PKA) activity. Furthermore, *mak-2* pathway defects are partially overcome in a *cot-1* background and are associated with increased MAK1 MAPK signaling. A comparative characterization of *N. crassa* MAPKs revealed that they act as three distinct modules during vegetative growth and asexual development. In addition, common functions of MAK1 and MAK2 signaling during maintenance of cell-wall integrity distinguished the two ERK-type pathways from the p38-type OS2 osmosensing pathway. In contrast to separate functions during vegetative growth, the concerted activity of the three MAPK pathways is essential for cell fusion and for the subsequent formation of multicellular structures that are required for sexual development. Taken together, our data indicate a functional link between COT1 and MAPK signaling in regulating filamentous growth, hyphal fusion, and sexual development.

APICAL tip extension is the hallmark of filamentous fungi, and fungal hyphae share, along with neurons and pollen tubes, the distinction of being among the most highly polarized cells found (Palanivelu and Preuss 2000; Borkovich *et al.* 2004; Harris 2006). Polarized growth is a complex multifactorial property, which is coordinated by numerous signals. These pathways, such as the cAMP-dependent protein kinase A (PKA), the mitogen-activated protein kinase (MAPK), or the nuclear Dbf2-related (Ndr) kinase pathways, are highly conserved and regulate numerous aspects of growth and development, including cell proliferation, differentiation, motility, and survival, among many others (Lewis *et al.* 1998; Lengeler *et al.* 2000; Hergovich *et al.* 2006). In fungal systems, they are important for maintaining polarity, pathogenicity, and development (Xu 2000; D'Souza and Heitman 2001; Monge *et al.* 2006; Xu *et al.* 2007).

MAPKs are modular signaling units composed of three-tiered kinase cascades, in which a series of three protein kinases phosphorylate and activate one another (Qi and Elion 2005). Frequently, a fourth kinase of the Ste20/PAK group acts upstream of the MAPK-signaling pathways [therefore also called MAPKKKK (Dan *et al.* 2001)]. Numerous reports have revealed that distinct MAPK pathways are tightly regulated by cross-communication with each other and other signaling pathways (summarized in Lengeler *et al.* 2000; Stork and Schmitt 2002). Both the functional modules of each MAPK pathway and the interplay between the different signaling routes are best understood in the unicellular ascomycete *Saccharomyces cerevisiae* and summarized in several recent reviews (Madhani and Fink 1998; Lengeler *et al.* 2000; Pan *et al.* 2000; Bahn *et al.* 2007). In the budding yeast, the MAPKs constitute five partially overlapping pathways regulating mating, filamentation, cell integrity, response to high osmolarity, and ascospore formation.

In filamentous fungi that undergo highly complex and multicellular developmental phases [*e.g.*, *Neurospora crassa* has been shown to differentiate into at least 28 different cell types (Bistis *et al.* 2003)], the situation is much less clear. Three basic MAPK modules have been identified, but, so far, only the kinase cascade homologous to the *S. cerevisiae* osmosensing/stress pathway has been fully characterized in the filamentous ascomycetes *N. crassa* and *Aspergillus nidulans* (Zhang *et al.* 2002; Fujimura *et al.* 2003; Jones *et al.* 2007; Noguchi *et al.* 2007).

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Osmostress signaling in *N. crassa* is transduced through the OS1 histidine kinase to the OS4, OS5, and OS2 MAPK cascade. *os* mutants are unable to grow on high-osmolarity media and are resistant to phenylpyrrole fungicides. Furthermore, lysis and increased pigmentation of asexually derived spores (macroconidia) and female sterility due to the lack of protoperithecia has been reported, yet the cellular or developmental defects involved have not been analyzed in depth (Zhang *et al.* 2002; Fujimura *et al.* 2003; Jones *et al.* 2007; Noguchi *et al.* 2007). *A. nidulans* HOG pathway mutants are similarly growth inhibited under high-osmolarity conditions and are sensitive to oxidative stress (Kawasaki *et al.* 2002; Furukawa *et al.* 2005). In contrast to the yeast HOG pathway, which depends on two upstream osmosensing branches [the Sln1p transmembrane hybrid-type histidine kinase and a putative seven-transmembrane osmosensor kinase (Maeda *et al.* 1995; Posas and Saito 1998)], activation of this pathway in *A. nidulans* and *N. crassa* depends solely on the two-component signaling system (Furukawa *et al.* 2005; Noguchi *et al.* 2007).

Several MAPK components homologous to the yeast pheromone/filamentation pathway have been found in *N. crassa*. The MAPKKK NRC1 was first identified as a repressor of the conidiation program, but was later shown to be also involved in hyphal fusion and in the activation of the MAPK MAK2 (Kothe and Free 1998; Pandey *et al.* 2004; Li *et al.* 2005). Mutants in *mak-2* and *pp-1* (the downstream transcription factor homologous to yeast Ste12p that is activated by the MAPK Fus3p/Kss1p) display reduced growth rates, the inability to undergo hyphal fusion, shortened aerial hyphae formation, and derepressed conidiation. Furthermore, they fail to develop protoperithecia, and ascospores carrying null mutations of either gene are autonomous lethal (Pandey *et al.* 2004; Li *et al.* 2005). A similar pleiotrophic phenotype has been observed in SteC MAPKKK mutants in *A. nidulans*, which result in slower growth rates, more branched hyphae, altered conidiophore morphology, inhibition of heterokaryon formation, and inhibited sexual development (Wei *et al.* 2003). Additional homologs of budding yeast Fus3p/Kss1p have been characterized in several pathogenic fungi and have been shown to play key roles in appressorium formation and host colonization (Xu 2000).

Even though mutants in the MAPK homologous to yeast Slt2 have been generated in *A. nidulans*, and in several phytopathogenic fungi (Xu *et al.* 1998; Bussink and Osmani 1999; Hou *et al.* 2002; Kojima *et al.* 2002; Mey *et al.* 2002), information concerning this third MAPK pathway in filamentous fungi is still limited. Common phenotypes of Slt2-like kinase mutants included altered cell walls and defects in conidial germination (which could be remedied by high-osmolarity media) and autolysis in central areas of the colony, suggesting the involvement of a cell-integrity-type MAPK pathway in filamentous fungi. Furthermore, the *Fusarium graminearum* Slt2 homolog MGV1 is required for female fertility, heterokaryon formation, and plant infection (Hou *et al.* 2002).

The functional analysis of Ndr kinases has gained much interest in recent years. They are important for normal cell differentiation and polar morphogenesis in various organisms, yet their specific functions are still elusive (Yarden *et al.* 1992; Geng *et al.* 2000; Racki *et al.* 2000; Zallen *et al.* 2000; summarized in Hergovich *et al.* 2006). An interesting connection between Ste20/PAK (= MAPKKKK) and Ndr kinase signaling was provided through the analysis of the *Schizosaccharomyces pombe* Ndr kinase mutant *orb-6* (Verde *et al.* 1998). *orb-6* and *pak-1* share similar phenotypes, double mutants are synthetically lethal, and the overexpression of ORB6 in *pak-1* partially suppressed the *pak-1* defect, suggesting that PAK1 acts upstream of ORB6. Furthermore, members of the MST2 and MST3 groups of Ste20 kinases have recently been described as upstream regulators of Ndr kinases (Nelson *et al.* 2003; Kanai *et al.* 2005; Stegert *et al.* 2005; Emoto *et al.* 2006; Praskova *et al.* 2008).

The MST3 and Ndr kinases POD6 and COT1 of *N. crassa* are essential for hyphal tip extension and coordinated branch formation. Both kinases have been shown to interact, and they share common suppressors and are localized in a kinesin/dynein-dependent manner (Seiler *et al.* 2006). We have provided evidence indicating that COT1/POD6 and PKA act in parallel pathways that regulate polarity formation in a positive or negative manner, respectively, in *N. crassa* (Seiler *et al.* 2006). However, the input and outcome components of the Ndr kinase network as well as its integration into a cellular signaling context have not been described in any system. This information is critical for elucidation of the mechanistic involvement of Ndr kinases in cell growth and polarity.

The described differences between the MAPK pathways in various filamentous fungi and yeasts highlight the need for a comparative analysis of MAPK modules during vegetative growth and the multiple developmental decisions made in a filamentous fungus. Here, we describe three MAPK cascades, which function as distinct modules during vegetative growth of *N. crassa*, but whose joint activity is necessary for hyphal fusion and the development of complex multicellular sexual structures. Furthermore, we provide evidence for cross talk between COT1 and the MAK1 and MAK2 pathways.

MATERIALS AND METHODS

Strains, media, and growth conditions: General genetic procedures and media used in the handling of *N. crassa* have been described (Davis and Deserres 1970) or are available through the Fungal Genetic Stock Center (http://www.fgsc.net), with the exception of genetic crosses, which were performed on 2% cornmeal agar (Sigma) supplemented with

0.1% glucose. This complex, low-nitrogen-containing media increased the success rate of crosses with strains that are difficult or impossible to cross on standard synthetic crossing media such as *gul-4* and most MAPK mutants, and the hyg^R and *cot-1* markers segregate perfectly in crosses that produce viable spores. Also, the terminal phenotype of mutants defective in sexual reproduction could be determined in a more reliable manner on this media compared to synthetic crossing media (Muller *et al.* 1995). Strains were grown in either liquid or solid (supplemented with 1.5% agar) Vogel's minimal media with 2% (w/v) sucrose, unless otherwise stated. When required, 5 μM KT5720, 500 μM Br-cAMP, or 5 mg/ml lysing enzymes, all purchased from Sigma, were added. Gradient plates contained solid Vogel's minimal media with 1% sucrose (w/v) and 1% sorbose (w/v) to restrict the radial growth rate. Inhibitors were added at 50°, the plates slanted during the solidification of the agar and then overlaid with an equal volume of the same medium lacking additives in horizontal position, and incubated for 1 day to allow equal diffusion of the additive. To induce stress-dependent MAPK signaling, H_2O_2 (7 mM) or NaCl (1 M) were added to liquid cultures of the relevant strains 2 hr prior to harvesting. Stress induction by temperature shift was achieved by germinating the strains for 15 hr on cellophane-covered agar plates, followed by a shift to 37° for 10 hr. For protein extraction, the mycelial sheet was peeled off the cellophane and plunged into liquid nitrogen.

The *gul-4/mak-2* complementation construct was generated by amplifying the *mak-2* ORF using the primers 2393-Not-5′ (ATC GGC GGC CGC CAT GAG CAG CGC ACA AAG AGG CG) and 52393-Not-3′ (ATC GGC GGC CGC TCA CCT CAT AAT CTC CTG GTA GAT C) designed to introduce *Not*I restriction sites. The *Not*I-digested PCR product was cloned into the expression vector pEHN1nat (kindly provided by Stephanie Poeggler), which allowed the expression of mak-2 via *A. nidulans gpd* promotor and *trpC* terminator sequences. DNA-mediated transformation of *N. crassa* protoplasts was carried out as described (Vollmer and Yanofsky 1986). The nourseothricin concentration was adjusted to 30 μg/ml to select for transformants.

Strains used in this study are listed in Table 1 (see also McCluskey 2003). *gul-4* was mapped by introducing the auxotrophic markers *arg-10*, *arg-11*, and *met-7* into the *cot-1(ts)* background and subsequently crossing the obtained double mutants with *gul-4;cot-1(ts)*. Progeny were plated on Vogel's minimal media containing 0.005% sucrose and 2% sorbose at 25°, overlaid with Vogel's minimal media containing 2% sucrose after 2 days, and incubated for an additional 5–10 days at 37°. The ratio between *cot-1* and *cot-1;gul-4* progeny was scored by stereomicroscopy and indicated the linkage of *gul-4* with the auxotrophic marker.

Protein extraction, immunoblotting, and PKA activity measurement: Western blot analysis was performed as previously described (Gorovits and Yarden 2003). Briefly, *N. crassa* mycelial samples were frozen in liquid nitrogen, pulverized, and suspended in lysis buffer [1 M sorbitol, 10 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1 M KCl, 0.2% Triton X-100, and complete protease inhibitor mixture (Roche Applied Science)]. The samples were homogenized by 10 strokes of pestle A in a Dounce homogenizer. The homogenates were centrifuged for 40 min at 10,000 × *g* and the supernatant recovered and stored at −70° until analysis. Proteins were separated by 7.5 or 10% SDS–PAGE and subsequently blotted onto nitrocellulose membranes. Antibodies used throughout this study included anti-COT1 (Gorovits *et al.* 1999), anti-PhosphoMAPK (Cell Signaling Technology), monoclonal 9E10 anti-cMYC (Santa Cruz), and goat peroxidase-coupled secondary antibody (Amersham Biosciences).

PKA assays were performed as previously described (Ziv *et al.* 2008) with minor modifications. Specifically, 10^6 conidia/ml were shaken for 11 hr in prewarmed (36°) Vogel's sucrose minimal medium. The cultures were harvested by centrifugation (10 min, 3000 × *g*, 4°) and immediately assayed for PKA activity. Differences in kemptide phosphorylation were determined by densitometry and subjected to paired two-sample *t*-test analyses.

Microscopy: Samples were viewed with an ORCA ER digital camera (Hamamatsu) mounted on an Axiovert S100 microscope (Zeiss). Image acquisition was done using the Openlab 5.01 software (Improvision) and images were further processed using Photoshop CS2 (Adobe). Low-magnification documentation of fungal hyphae or colonies was performed with an SZX12 stereomicroscope (Olympus) and a PS30 camera (Kappa).

RESULTS

Mutants of the MAK2 MAP kinase pathway suppress *cot-1* growth defects: The phenotypic characteristics of the conditional *cot-1(ts)* mutant, which forms tight colonies with growth-arrested needle-shaped hyphal tips when germinated at restrictive temperature, facilitates the easy identification of *cot-1* suppressors. This efficient procedure makes *N. crassa* ideal for the genetic dissection of Ndr signaling. Several mutants designated "*gulliver*" that act as modifiers of the compact *cot-1(ts)* morphology at restrictive temperature have been described (Terenzi and Reissig 1967; Bruno *et al.* 1996b; Seiler *et al.* 2006). *gul-4* has been mapped to *nic-3* (17%) on linkage group VII (Perkins *et al.* 2001). Using additional auxotropic markers, we determined that *gul-4* is closely linked with *arg-10*, *arg-11*, and *met-7* (<1%, <1%, and <0.1% recombination frequencies, respectively). This information and the available genome sequence identified several candidate genes for *gul-4*. By sequencing potential ORFs as well as their 5′ and 3′ untranslated regions, we identified a 12-bp insertion (CAA CAA CAA CAA) in the *mak-2* promotor at position −270/271 upstream of the start ATG as a potential cause for the suppression of *cot-1(ts)*. To test if *gul-4* is allelic to *mak-2*, we generated a *cot-1(ts);*Δ*mak-2* double mutant. When tested at restrictive temperature, the Δ*mak-2* deletion partially suppressed the *cot-1(ts)* defect in a manner identical to that observed in the original *gul-4* background (Figure 1A; Southern blot analyses confirming the genetic nature of the double mutants generated throughout this report are available as supplemental Figure 1). Microscopic analysis of the hyphal apex revealed that, in contrast to the extension-arrested pointed tips of *cot-1(ts)* grown at restrictive temperature, the *cot-1(ts);gul-4* and *cot-1(ts);*Δ*mak-2* strains generated a dome-shaped apex, typical of a normal (although slow) growing tip (Figure 1B). The presence of a tight genetic linkage between *mak-2* and *gul-4* was made evident by the analysis of crosses between *cot-1(ts);gul-4* and *cot-1;*Δ*mak-2*. Of >2000 progeny screened, no *cot-1(ts);gul*$^+$ strains were obtained. To confirm that *gul-4*

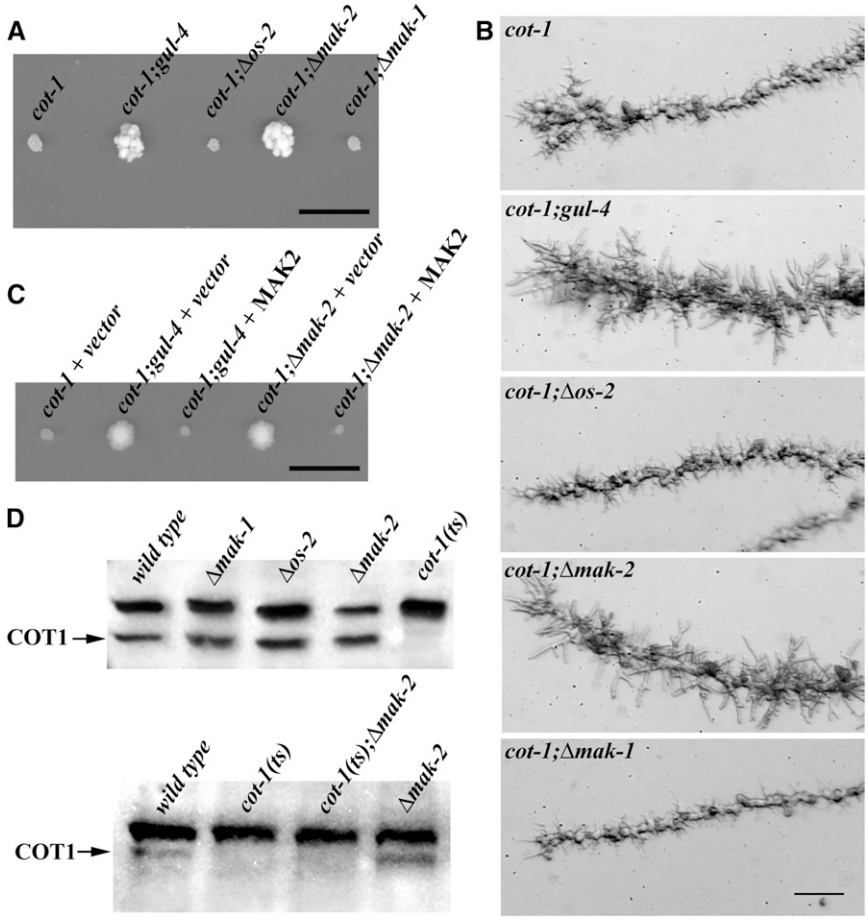

FIGURE 1.—*gul-4*/Δ*mak-2* strains suppress the *cot-1(ts)* growth defects. (A) The indicated strains were germinated and grown on minimal media plates for 3 days at 37°. Note the increased colony diameters of *cot-1(ts);gul-4* and *cot-1(ts);*Δ*mak-2* compared to *cot-1(ts)*. Bar, 1 cm. (B) Results of temperature-shift experiments, in which strains grown at 25° and shifted to 37° for 8 hr illustrate pointed growth-arrested tips of *cot-1(ts)*, *cot-1(ts);*Δ*os-2*, and *cot-1(ts);*Δ*mak-1*, while dome-shaped slow-growing apices are visible in *cot-1(ts),gul-4* and *cot-1(ts);*Δ*mak-2*. Bar, 20 μm. (C) The indicated strains were transformed with *mak-2* expression vector or the empty vector as control and grown on minimal media plates supplemented with 30 μg/ml nourseothricin for 3 days at 37°. Bar, 1 cm. (D) Western blot analysis of cell extracts probed with anti-COT1 antibodies indicate that deleting any of the three MAPKs does not affect COT1 expression (top) and that the *gulliver*-like suppression of the *cot-1(1)* phenotype by Δ*mak-2* at restrictive temperature is independent of the presence of the COT1 67-kDa band (arrow on bottom).

is allelic to *mak-2*, we expressed MAK2 in *gul-4* and Δ*mak-2* and found that it complemented the growth defects of both mutants (data not shown). Furthermore, when we expressed MAK2 in *cot-1(ts);gul-4* and *cot-1(ts);*Δ*mak-2*, the suppression of the *cot-1(ts)* growth defect was abolished at the restrictive temperature (Figure 1C).

To determine if the suppression of *cot-1(ts)* is specific to the MAK2 MAPK pathway, we generated double mutants of *cot-1(ts)* with loss-of-function mutants in *os-2* and *mak-1*, the other two MAPK genes present in the *N. crassa* genome (BORKOVICH *et al.* 2004). When we introduced the three MAPK mutations into the *cot-1(ts)* background, only Δ*mak-2* suppressed the *cot-1(ts)* growth defects, indicating a specific interaction between COT1 and MAK2 kinase signaling (Figure 1, A and B).

Western analyses were performed to determine if deletion of any one of the three MAPKs affected the pattern of COT1 expression (Figure 1D). The typical 67-kDa COT1 band was clearly evident in protein extracts of all three MAPK mutants. Furthermore, loss of MAK2 function in *cot-1(ts);*Δ*mak-2* did not confer quantitative or qualitative alterations in the COT1 protein expression pattern, indicating that the improved growth of *cot-1(ts)* by deleting *mak-2* was not dependent on the presence of COT1. On the basis of these results, we concluded that COT1 and MAK2 act in independent pathways and that the suppression of the *cot-1(ts)* defect was indirect.

Deletion of *mak2* is accompanied by a reduction in PKA activity: The suppression of *cot-1(ts)* by Δ*mak-2* resembled the previously described environmental suppression of *cot-1(ts)* and *pod-6(ts)* by external stresses (GOROVITS and YARDEN 2003; SEILER *et al.* 2006). As environmental suppression of both kinases was correlated with reduced PKA activity levels, we analyzed PKA activity in the Δ*mak*-2 strain and found several lines of evidence for reduced PKA activity. *mcb* is a temperature-sensitive mutant defective in the regulatory subunit of PKA, which displays elevated PKA activity levels at restrictive temperature, resulting in apolar growth and irregular chains of spherical cells (BRUNO *et al.* 1996a; SEILER *et al.* 2006; ZIV *et al.* 2008). Genetic analysis of a Δ*mak-2;mcb(14-4)* double mutant demonstrated that Δ*mak-2;mcb(14-4)* grew slower than the parental strains at permissive temperature, suggesting a genetic interaction between MAK2 and PKA signaling. Nevertheless, the Δ*mak-2* background partially suppressed the polarity defect of *mcb(14-4)* at restrictive temperature, suggesting that PKA activity levels are reduced in Δ*mak-2* (Figure 2A). To test this hypothesis, we increased the cellular PKA activity in *cot-1(ts);*Δ*mak-2* grown at 37° by culturing the strain in the presence of 500 μM 8-Br-cAMP, which

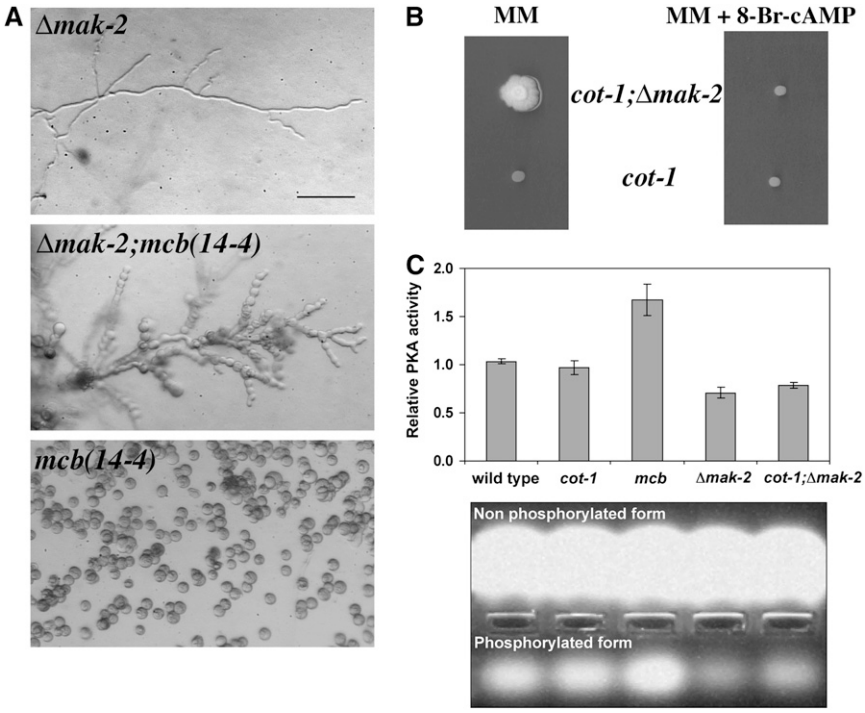

FIGURE 2.—PKA activity is reduced in Δ*mak-2*. (A) Morphology of Δ*mak-2; mcb(14-4)* and *mcb(14-4);*Δ*mak-2* germinated for 12 hr at 37°. Bar, 20 μm. (B) Growth of *cot-1(ts)* and *cot-1(ts);*Δ*mak-2* on minimal media and media supplemented with 500 μM 8-Br-cAMP at restrictive temerature. (C) PKA activity in extracts of germinating conidia of wild type, *cot-1(ts)*, *mcb(14-4)*, Δ*mak-2*, and *cot-1(ts);*Δ*mak-2*, 11 hr post-inoculation, relative to wild type. Cultures were incubated in prewarmed liquid Vogel's minimal medium at 36° and were assayed for PKA activity. Data presented in the graph are means of at least four independent experiments with two replicates each. Standard errors are shown. (Bottom) A selected experiment demonstrating the nonphosphorylated and phosphorylated (indicating PKA activity) fluorescent Kemptide substrates, migrated to the anode and cathode of the agarose gel, respectively. The PepTag assays utilize fluorescent peptide substrates specific for PKA. Phosphorylation of the substrate by PKA alters the peptide's net charge from +1 to −1, allowing separation of the phosphorylated substrate from the nonphosphorylated on the agarose gel.

mimics increased levels of cAMP, and found that the suppressive effect of Δ*mak-2* on *cot-1(ts)* at restrictive temperature was abolished (Figure 2B), while it had only a minor effect on the growth rate of *cot-1(ts)* or wild type (data not shown). Finally, we directly measured PKA activity in Δ*mak-2* single and Δ*mak-2;cot-1(ts)* double mutants and found that a significant ($P < 0.001$; paired two-sample *t*-test) reduction in PKA activity could be detected in these strains (Figure 2C). Several measurements (with independent cultures) detected a consistent 30–35% decrease in kinase activity in the Δ*mak-2* and *cot-1(ts);*Δ*mak-2* strains in comparison to wild type. An ~70% increase ($P < 0.001$) in PKA activity was measured in the *mcb(14-4)* control, as expected (ZIV *et al.* 2008). Thus, we suggest that the suppression of *cot-1* by the deletion of *mak-2* is part of a bypass mechanism, which includes a reduction in PKA activity levels.

The three *N. crassa* MAP kinases act as three distinct modules during growth and development: To further dissect the cross-communication between the MAK2 pathway and COT1 signaling, we carried out a comparative characterization of the three *N. crassa* MAPKs (Figure 3). Δ*os-2* displayed growth rates that were similar to wild type, but frequent dichotomous branching events suggested minor defects at the hyphal apex. The conidial lysis defect and its sensitivity to sorbitol have already been described (ZHANG *et al.* 2002; NOGUCHI *et al.* 2007), and these phenotypes clearly distinguished Δ*os-2* from Δ*mak-1* and Δ*mak-2*. Δ*mak-1* was the most drastically growth-impaired MAPK mutant with tip extension rates of <15% of wild type forming a rosetta-like colony (Figure 3A). The mutant was almost devoid of aerial hyphae and produced few conidia, and the conidial population was highly enriched with arthroconidia (86% compared with 5% in wild type). Abnormal and apolar branching events indicated a major defect during polarity establishment of newly formed branches. Polarity establishment was also affected during germination. Only 15% of Δ*mak-1* conidia produced germ tubes after 7 hr in liquid minimal medium in contrast to 85% of wild-type conidia. Δ*mak-1* and Δ*mak-2* exhibited a cell-wall defect, as protoplast production was approximately four- and twofold, respectively, enhanced in comparison to wild type in the presence of Novozyme. In addition, their growth behavior on plates containing 1% sucrose and 1% sorbose in the presence of a concentration gradient of lysing enzymes indicated that both Δ*mak-1* and Δ*mak-2* have altered cell walls, with Δ*mak-1* being more sensitive than Δ*mak-2* (Figure 3B; note that the effect of sorbose on tip extension and the cell wall is not compensated by the addition of lysing enzymes in a manner similar to the wild type and Δ*os-2* strains). In addition to their common cell-wall defect, the two strains displayed additional similarities such as their conidial sensitivity to high temperature, which could be overcome by the addition of 1 M sorbitol prior to the heat shock. In addition, Δ*mak-1* is sensitive to formamide, a general stress-inducing agent, which is readily taken up by fungi yet is not metabolized (HAMPSEY 1997). A unique defect of Δ*mak-2* is its highly irregular zig-zagging growth, which suggested *Spitzenkörper* positioning defects, but no altered sensitivity to

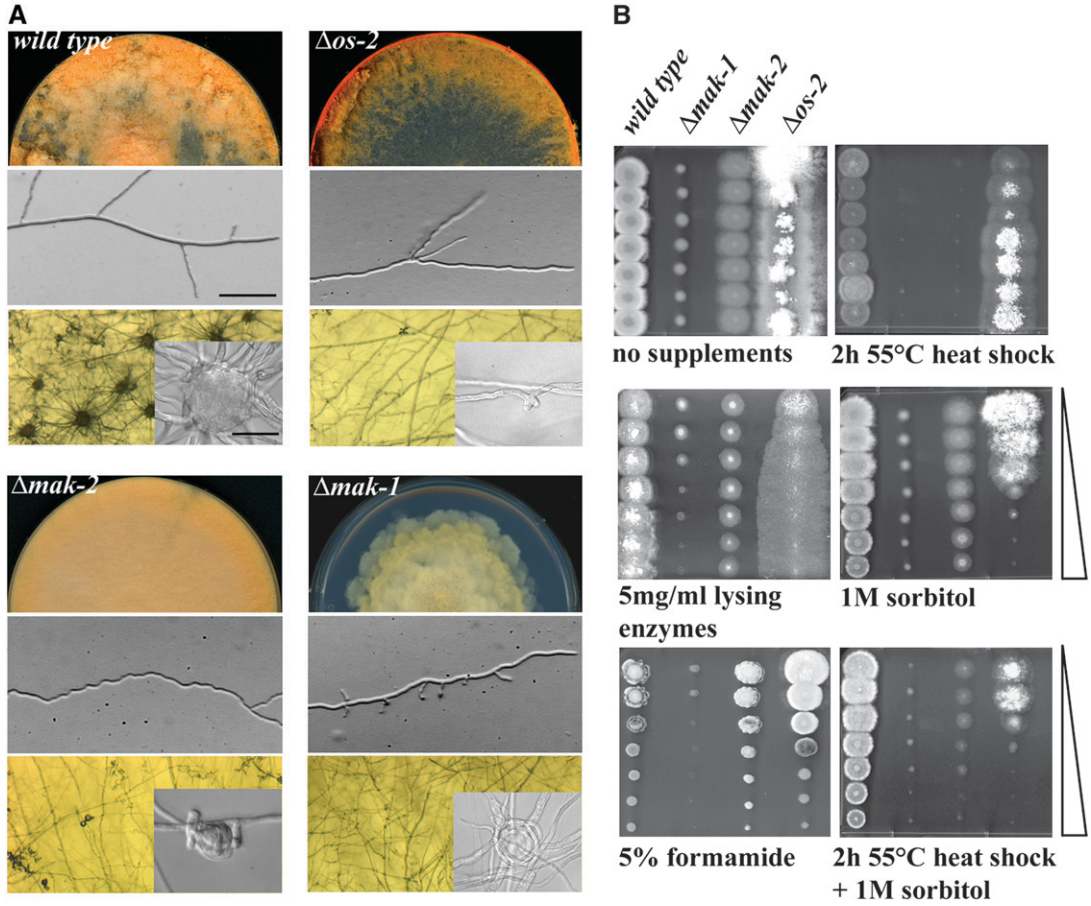

FIGURE 3.—Comparative characterization of the *N. crassa* MAP kinase mutants. (A) Colony morphology, asexual development, and hyphal morphology (top and middle, respectively; bar, 20 μm) of the indicated strains grown on minimal media plates. Sexual development (bottom) was induced by growth for 5 days on cornmeal agar. The insets illustrate the terminal morphology of the female reproductive structures (protoperithecia). Bar, 10 μm. (B) Growth of the three MAPK mutants on gradient plates supplemented with the indicated additives. Conidia (5×10^3) were inoculated for each spot. Wedges denote the compound gradient. To restrict the radial growth rates of the strains, all plates were supplemented with 1% sorbose in addition to the indicated additives.

the microtubule inhibitors Benomyl or Nocodazol were observed (data not shown).

Δ*mak-2* has been described as female sterile (PANDEY *et al.* 2004; LI *et al.* 2005), but the exact developmental block in sexual development has not been reported. Inspection of Δ*mak-2* grown on cornmeal agar plates for 10 days revealed no mature and fertilization-competent protoperithecia (female sexual structures in *N. crassa*), but did reveal an ~50-fold reduced number of protoperithecia-like structures in comparison to wild type (Figure 3A). Furthermore, the protoperithecia-like structures produced in the Δ*mak-2* strain were smaller, less developed, and nonfertile, but morphologically resembled immature protoperithecia of wild type (*e.g.*, POGGELER and KUCK 2004; POGGELER *et al.* 2006). This indicated that loss of the MAK2 pathway function does not abolish the capability of initiating protoperithecia formation, but rather affects their abundance and, more importantly, their maturation into fertile structures. Interestingly, when we tested Δ*os-2* and Δ*mak-1*, we found them to also be female sterile yet they produced no protoperithecia at all. Thus, the other two MAPK mutants were blocked at an earlier developmental stage. In Δ*mak-1*, we observed only lasso-like structures embedded in the agar, suggesting failed attempts of hyphae to coil and fuse during ascogonia formation. In Δ*os-2*, we detected the presence of small, curled side branches, typical of early stages during ascogonia formation, suggesting that both strains are blocked at, or even prior to, the initiation of ascogonia formation.

To better characterize the modularity of the upstream MAPKs (GALAGAN *et al.* 2003; BORKOVICH *et al.* 2004), we extended this analysis to include the respective MAPKK and MAPKKK components. Three distinct MAP kinase cascades were previously found by *in silico* analyses in several fungal genomes (GALAGAN *et al.* 2003; BORKOVICH *et al.* 2004) but a comparative functional characterization is still lacking. Several of the mutants provided by the genome project (DUNLAP *et al.* 2007) were available only as heterokaryons and were therefore backcrossed to wild

TABLE 1

***N. crassa* strains used in this study**

Strain	Genotype	Source
Wild type	*74-OR23-1A*	FGSC 987
cot-1(ts)	*cot-1(C102t)*	FGSC 4066
gul-4;pe;fl;cot-1;inl	*gul-4 pe fl cot-1 inl*	FGSC 1173
cot-1;gul-4	*gul-4 cot-1*	This study
Δ*os-4 (heterokaryon)*	*hph::os-4Δ bar::mus-51 + bar::mus-51*	FGSC 11479
Δ*os-4 (microconidia)*	*hph::os-4Δ bar::mus-51*	This study
Δ*nrc-1 (heterokaryon)*	*hph::nrc-1Δ bar::mus-51 + bar::mus-51*	FGSC 11466
Δ*nrc-1*	*hph::nrc-1*	This study
Δ*mik-1*	*hph::mik-1Δ*	FGSC 11326
Δ*os-5 (heterokaryon)*	*hph::os-5Δ bar::mus-51 + bar::mus-51*	FGSC 11480
Δ*os-5*	*hph::os-5Δ*	This study
Δ*mek-2 (heterokaryon)*	*hph::mek-2Δ bar::mus-51 + bar::mus-51*	FGSC 11481
Δ*mek-2 (microconidia)*	*hph::mek-2Δ bar::mus-51*	This study
Δ*mek-1*	*hph::mek-1Δ*	FGSC 11318
Δ*os-2*	*hph::os-2Δ*	FGSC 11436
Δ*mak-2*	*hph::mak-2Δ*	Li *et al.* (2005)
Δ*mak-1*	*hph::mak-1Δ*	FGSC 11321
Δ*nrc-1;cot-1(ts)*	*hph::nrc-1 cot-1(C102t)*	This study
Δ*os-5;cot-1(ts)*	*hph::os-5Δ cot-1(C102t)*	This study
Δ*mek-1;cot-1(ts)*	*hph::mek-1Δ cot-1(C102t)*	This study
Δ*os-2;cot-1(ts)*	*hph::os-2Δ cot-1(C102t)*	This study
Δ*mak-2;cot-1(ts)*	*hph::mak-2Δ cot-1(C102t)*	This study
Δ*mak-1;cot-1(ts)*	*hph::mak-1Δ cot-1(C102t)*	This study
mcb(14-4)	*mcb(14-4)*	Seiler and Plamann (2003)
Δ*mak-2;mcb(14-4)*	*hph::mak-1Δ mcb(14-4)*	This study

type to isolate homokaryotic deletions or, if crosses were not successful, the heterokaryons were colony purified several times and their homokaryotic status confirmed by Southern analysis (Table 1). A detailed phenotypic analysis of the mutants confirmed the phylogenetic comparison and supported the existence of three functional modules (Table 2), each consisting of a kinase, a kinase–kinase, and a kinase–kinase–kinase, each of which displayed identical phenotypes on the basis of growth rate, hyphal morphology, conidiation pattern, sexual development, and behavior with respect to inhibitors. The only exception was Δ*mik-1*, which displayed a slightly better growth rate and produced more conidia than the respective MAPKK and MAPK mutants of the MAK1 pathway. Furthermore, double-mutant analysis of *cot-1(ts)* with the available MAPKK and MAPKKK mutants corroborated that the suppression of the *cot-1* defect was specific for *mak-2* pathway deletions (Table 2).

An increase in MAK1 activity in a *cot-1* background bypasses the *mak-2* pathway defects: In a more detailed analysis of the MAPK deletions and in comparison with respective *cot-1(ts)* double mutants, we determined that *cot-1(ts);*Δ*mak-2* and *cot-1(ts);*Δ*nrc-1* double mutants had an intermediate growth rate when compared to that of the parental strains when grown at permissive conditions (Figure 4A; Table 2). Additional *mak-2* pathway defects, such as their shortened aerial hyphae, the derepression of their conidial production, and the female sterility, were also suppressed in the *cot-1(ts)* background (Figure 4, B and C). As Δ*mak-2* and Δ*nrc-1* have been described as hyphal fusion defective mutants (Pandey *et al.* 2004), we also tested if *cot-1(ts)* has any effect on the fusion of vegetative hyphae. *cot-1(ts)* grown at permissive temperature is fusion competent, and we did not observe any qualitative differences when compared with wild type. When we analyzed the *cot-1(ts);*Δ*mak-2* and *cot-1(ts);*Δ*nrc-1* double mutants, we observed a suppression of the fusion defect of the *mak-2* pathway deletions (Figure 5; Table 2). The resulting interconnected, syncycial mycelium could increase the efficiency of nutrient flow and organelle distribution throughout the colony. This, in turn, may explain the increased growth rate, the enhanced formation of aerial hyphae, the better conidiation rates, and the restored female fertility of the double mutants in comparison to the *mak-2* pathway deletions.

To confirm that this *cot-1*-dependent suppression is specific for the MAK2 pathway, we analyzed the involvement of the other two MAPK modules in the hyphal fusion process. When we tested Δ*os-2* and Δ*mak-1*, we found that both mutants were also defective in vegetative fusion, but determined that the *cot-1(ts);*Δ*os-2* and the *cot-1(ts);*Δ*mak-1* double mutants did not regain their fusion competence (Figure 5). We also tested the re-

TABLE 2

Phenotypic characteristics of *N. crassa* MAPK pathway single and respective *cot-1;MAPK* double mutants

Strain	Growth rates[a]		Vegetative fusion		Major hyphal defects of MAP kinase mutants[b]	Asexual development of MAP kinase mutants[b]	Female fertility[c]	
	mapk	*cot-1(ts); mapk*	*mapk*	*cot-1(ts); mapk*			*mapk*	*cot-1(ts); mapk*
Wild type	3.5		Yes				Yes	
cot-1(ts)	3.2		Yes				Yes	
Osmosensing pathway								
Δ*os-4* (NCU03071)	3.2	ND[d]	No	ND[d]	± Wild type; frequent tip splitting	Conidial lysis	No	ND[d]
Δ*os-5* (NCU00587)	3.1	3.0	No	No			No	No
Δ*os-2* (NCU07024)	3.2	3.2	No	No			No	No
Cell fusion/fertility pathway								
Δ*nrc-1* (NCU06182)	1.2	2.5	No	Yes	Highly irregular growth axis[b]	Reduced aerial hyphae and conidia formation[b]	No	Yes
Δ*mek-2* (NCU04612)	1.1	ND[d]	No	ND[d]			No	ND[d]
Δ*mak-2* (NCU02393)	1.2	2.6	No	Yes			No	Yes
Cell-wall integrity pathway								
Δ*mik-1* (NCU02234)	0.6	ND[d]	No	ND[d]	Polarity defect; branch formation abnormal	Arthroconidiation for *mak-1* and *mek-1*	No	ND[d]
Δ*mek-1* (NCU06419)	0.5	0.6	No	No			No	No
Δ*mak-1* (NCU11376)	0.6	0.5	No	No			No	No

[a] In centimeters/day at 20° ($n = 3$) as determined by radial hyphal growth experiments.

[b] No differences in hyphal morphology and asexual development were observed for the single and respective *cot-1* double mutants grown at 25° except for a better condidation rate of the *cot-1;mak-2* and *cot-1;nrc-1* double mutants compared to *mak-2* and *nrc-1*.

[c] Protoperithecia formation after 7 days at room temperature on 2% cornmeal agar supplemented with 0.1% glucose and viable ascospore formation when fertilized with wild-type conidia.

[d] Not determined, as we were not able to obtain viable hygromycin-resistant ascospores in crosses with wild type or *cot-1* as the female partner.

maining MAPKK and MAPKKK deletions and found them to be fusion defective. Thus, the *cot-1(ts)*-dependent suppression was specific for *mak-2* pathway components (Table 2). Taken together, these data indicate that the activity of all three MAPK pathways is essential for hyphal fusion. However, on the basis of the specificity of *cot-1(ts)* suppression of *mak-2* pathway deletion strains, this also indicates the presence of different mechanistic functions of the three MAP pathways during cell fusion.

The characterization of the MAPK mutants has revealed phenotypic similarities between the *mak-1* and *mak-2* pathway deletion strains, indicating a potential functional overlap between the two signaling cascades. Therefore, the loss of one pathway may affect the MAPK activity of one or two of the others. We tested the activity of the three MAPKs and found it to increase under various stress conditions, as determined by the use of phospho-specific antibodies against activated MAPKs (Figure 6A, top). Nevertheless, we detected a similar phospho-activation pattern in the two remaining MAPK pathways when one MAPK was deleted, suggesting that there is no compensatory activation of the other MAPK pathways under normal stress-sensing conditions (Figure 6A, bottom). However, in *cot-1(ts)*, we detected a marked increase of MAK1 phosphorylation as measured 8 hr after the shift to restrictive temperatures while MAK2 activity remained constant (Figure 6B). As mentioned before, *cot-1(ts);* Δ*mak-1* pathway double mutants did not display any synthetic characteristics. Thus, these results identified COT1 as a potential negative regulator of MAK1 activity.

DISCUSSION

Molecular understanding of fungal morphogenesis is still a major challenge. Phylogenetic analyses and the comparison of *S. cerevisiae* morphogenetic data with the limited results from various filamentous asco- and basidiomycetes have established that a core set of "polarity factors," including the existence of most signal transduction pathway components, are conserved between unicellular and filamentous fungi (Borkovich *et al.* 2004). Nevertheless, it is becoming increasingly evident that differences in the wiring of these conserved components and the presence of additional proteins that are absent in unicellular fungi result in dramatically different morphogenetic outcomes that range from

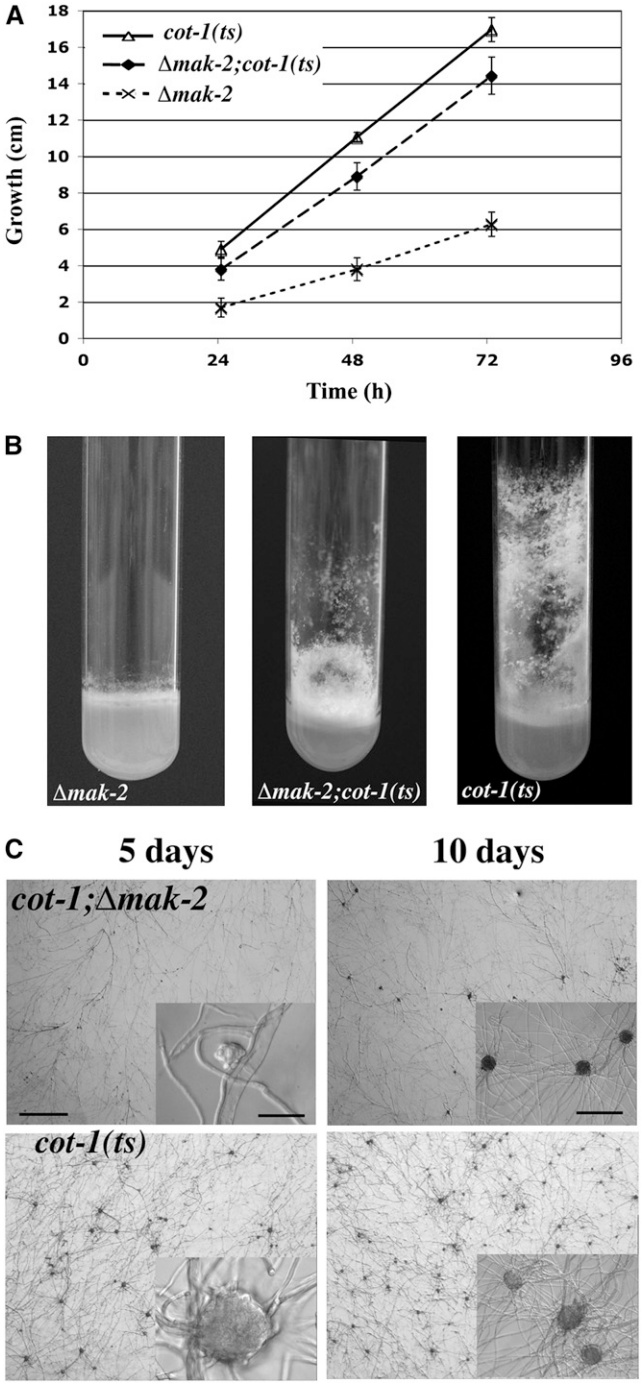

FIGURE 4.—*mak-2* pathway defects are suppressed when COT1 activity is reduced. (A) *cot-1(ts);*Δ*mak-2* grown at 25° in race tubes has an intermediate tip-extension rate (A) and generates intermediate amounts of aerial hyphae and conidia (B) when compared to the parental strains. (C) Time course of protoperithecia formation by *cot-1(ts);*Δ*mak-2*. Bars, 100 μm (5 and 10 days overview), 10 μm (5-day inset), and 25 μm (10-day inset).

unicellular to true filamentous growth and multicellular differentiation.

The recent advent of available genome sequences for several filamentous fungi (GALAGAN *et al.* 2003, 2005; DEAN *et al.* 2005) has provided the MAPK toolbox present in filamentous ascomycetes. In this report, we comparatively characterized the nine components of three MAPK modules of *N. crassa* and provided evidence that they act as three distinct modules during vegetative growth and asexual development, but also that the joined activity of the three pathways is required for hyphal fusion and for the formation of more complex multicellular structures necessary to undergo sexual development. Furthermore, we suggest a partial overlap of MAK1- and MAK2-dependent signaling for maintaining the functions of the cell wall on the basis of the shared phenotypes and similar sensitivities against cell-wall drugs, which distinguished the two ERK-type MAPK routes from the p38-type OS2-dependent osmosensing pathway (Figure 7).

Despite their common phenotype as female sterile mutants, we observed distinct terminal phenotypes of the MAPK mutants during the development of female reproductive structures. Thus, the three MAPK pathways seem to act by different mechanisms in regulating sexual development. The function of the MAK2 pathway was not necessary for the initial steps during the formation of ascogonia, but was required for the maturation of young protoperithecia. In contrast, mutants in the other two pathways are blocked prior to the formation of ascogonia. The coiling of Δ*mak-1* may indicate defects in cell–cell contact formation due to an altered cell wall or may suggest cell–cell signaling defects, while in Δ*os-2* we observed only small, bent side branches, suggesting that even the initial attempts of hyphal curling during ascogonia formation are defective. On the basis of the relative late block in the formation of female reproductive structures in *mak-2* pathway deletions, we speculate that the MAK2 pathway is an integral part of sexual development and that blocking either of the other two pathways impairs the sexual cycle by preventing the initiation of fruiting-body development or as part of the pleiotropic consequences of their inactivation.

We found it interesting that all mutants described here and in the literature that lack female reproductive structures are also cell-fusion defective. This is best documented in mutants characterized in *N. crassa* (WILSON and DEMPSEY 1999; PERKINS *et al.* 2001; XIANG *et al.* 2002; FLEISSNER *et al.* 2005) and the closely related fungus *Sordaria macrospora* (POGGELER and KUCK 2004; ENGH *et al.* 2007), but was also observed in *A. nidulans* (WEI *et al.* 2003). The hypothesis that hyphal fusion is functionally linked with sexual fruiting-body formation is also supported by our characterization of the suppression of the *mak-2* pathway by *cot-1(ts)*: the lack or delay of hyphal fusion correlated with defects in the formation of protoperithecia. Furthermore, hyphal fusion has been shown to occur in the fruiting bodies of basidiomycete species (WILLIAMS 1985). However, it is currently still unclear whether hyphal

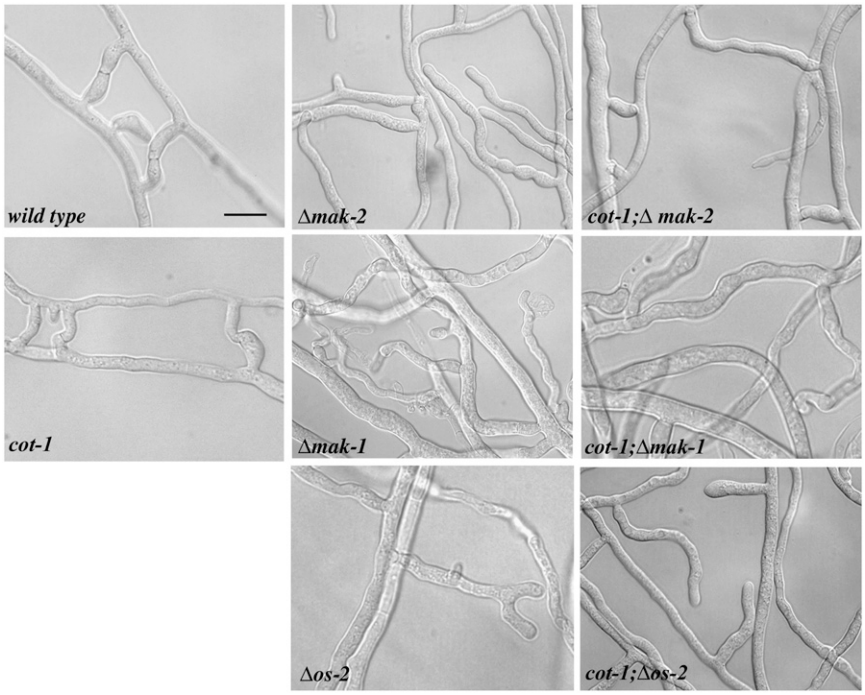

Figure 5.—Hyphal fusion is dependent on the three MAP kinase modules. Microscopic analysis of the indicated strains grown for 2 days on minimal media plates at 25°. Note that the three MAPK mutants show extended cell–cell contacts, but no distinct fusion bridges, which are clearly visible in wild type, *cot-1(ts)*, and *cot-1(ts)*;Δ*mak-2*. Bar, 5 μm.

fusion is a prerequisite for the formation of female reproductive structures (Glass *et al.* 2004; Poggeler *et al.* 2006).

Our genetic analysis suggests that the MAK1, MAK2, and COT1 signaling pathways in *N. crassa* are linked (Figure 7). This is best characterized by the *gulliver*-type suppression of the *cot-1(ts)* growth defects at restrictive conditions observed in mutants that harbor *mak-2* pathway deletions. We have recently presented evidence indicating that inhibiting PKA activity can suppress the *cot-1(ts)* phenotype (Gorovits and Yarden 2003; Seiler *et al.* 2006). Here, we demonstrate that the loss of MAK2 activity can also partially suppress the *cot-1(ts)* phenotype. It is tempting to speculate that the observed reduction in PKA activity in Δ*mak-2* may be involved in the suppression mechanism, thus establishing a potential MAK2-PKA interaction in *N. crassa*. Extensive literature supports the occurrence of direct cross talk between PKA and MAPK signaling in various organisms (Mosch *et al.* 1999; Lengeler *et al.* 2000; Pan *et al.* 2000; Stork and Schmitt 2002). However, cross talk between these two pathways is generally directed from PKA toward the MAPK pathway and not vice versa. One of the few examples of MAPK-to-PKA signaling is the phosphorylation of the phospho-diesterase RegA by Erk2 in *Dictyostelium discoideum* that results in the degradation of the cAMP-specific diesterase and thereby the activation of PKA (Loomis 1998; Mohanty *et al.* 2001). Alternatively, a common upstream link between MAPK and PKA (*e.g.*, via the small GTPase RAS) may be responsible for coordinating the activity intensities of the MAPK and PKA pathways in a manner that confers the observed phenotypes. If this is the case, additional *gulliver*-type suppressors may serve as a tool to further define the MAK2/PKA pathways in *N. crassa*.

Another example of the link between COT1 and MAPK signaling is the suppression of *mak-2* pathway defects by *cot-1(ts)*. A candidate component of this link is MAK1, whose activity was increased in *cot-1(ts)*. On the basis of the phenotypic similarities of *mak-1* and *mak-2* pathway deletions, we suggest that both pathways have partially overlapping functions and that the increase in phospho-MAK1 in *cot-1(ts)* can compensate, at least in part, for the loss of *mak-2* pathway functions. An interesting open question is, Why is this compensation mechanism specific for Δ*mak-2*? One possible explanation may be that the primary interaction between COT1 and MAPKs is via MAK1. This is supported by studies in yeasts and animals indicating the presence of a link between Ndr kinases and Rho-type GTPase. Genetic data in *S. cerevisiae* suggest that the COT1 homolog Cbk1p may negatively regulate the small GTPase Rho1p, which in turn activates the cell-wall integrity pathway that is most similar to the *N. crassa* MAK1 pathway (Versele and Thevelein 2001; Jorgensen *et al.* 2002; Schneper *et al.* 2004). A physical interaction has also been shown to exist between the Ndr kinase ORB6 and the Rho-GTPase-activating protein RGA4 in fission yeast (Das *et al.* 2007). An indication that this connection may be conserved between fungi and animals has been provided by studies in *Drosophila melanogaster* and *Caenorhabditis elegans*, which also describe genetic interactions between Ndr kinases and RhoA (Zallen *et al.* 2000; Emoto *et al.* 2004). Thus, the connections among

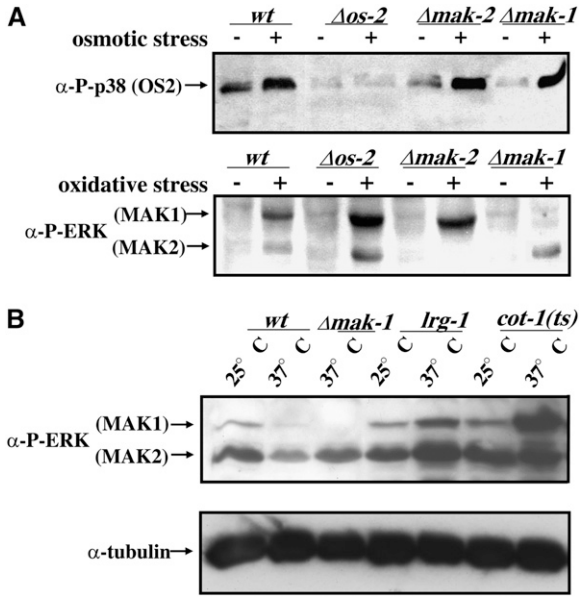

FIGURE 6.—MAK1 activity is increased in *cot-1(ts)*. (A) Total soluble protein (100 μg/lane) was extracted from the indicated strains grown in the presence or absence of stress inducers (1 M NaCl, 7 mM H_2O_2). The blot was probed with anti-phospho-ERK (α-P-ERK) and anti-phospho-p38 (α-P-p38) antibodies to detect activated MAK1, MAK2, and OS2 kinase. (B) For the temperature-shift experiments, total soluble protein (50 μg/lane) of the indicated strains grown at 25° and shifted to 37° for 12 hr was extracted and the blot was probed with anti-phospho-ERK (α-P-ERK) antibody (top). To confirm equal loading, the blot was stripped and reprobed with α-tubulin antibody (bottom). *lrg-1* is an unrelated temperature-sensitive hyperbranching mutant used as a control.

COT1, MAK1, and MAK2 signaling during hyphal growth may provide insights into the regulation of morphogenesis in other highly polar cells such as neurons or pollen tubes.

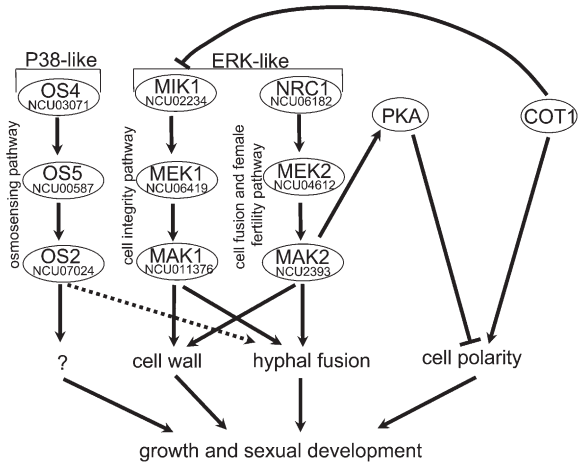

FIGURE 7.—Model summarizing the components and functions of the three *N. crassa* MAPK modules and cross-communication between COT1, MAP kinase, and PKA signaling pathways. Details are discussed in the text.

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