Role of a Mitogen-Activated Protein Kinase Pathway during Conidial Germination and Hyphal Fusion in *Neurospora crassa*

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Mitogen-activated protein (MAP) kinase signaling pathways are ubiquitous and evolutionarily conserved in eukaryotic organisms. MAP kinase pathways are composed of a MAP kinase, a MAP kinase kinase, and a MAP kinase kinase kinase; activation is regulated by sequential phosphorylation. Components of three MAP kinase pathways have been identified by genome sequence analysis in the filamentous fungus *Neurospora crassa*. One of the predicted MAP kinases in *N. crassa*, MAK-2, shows similarity to Fus3p and Kss1p of *Saccharomyces cerevisiae*, which are involved in sexual reproduction and filamentation, respectively. In this study, we show that an *N. crassa* mutant disrupted in *mak-2* exhibits a pleiotropic phenotype: derepressed conidiation, shortened aerial hyphae, lack of vegetative hyphal fusion, female sterility, and autonomous ascospore lethality. We assessed the phosphorylation of MAK-2 during conidial germination and early colony development. Peak levels of MAK-2 phosphorylation were most closely associated with germ tube elongation, branching, and hyphal fusion events between conidial germlings. A MAP kinase kinase kinase (NRC-1) is the predicted product of *N. crassa nrc-1* locus and is a homologue of STE11 in *S. cerevisiae*. An *nrc-1* mutant shares many of the same phenotypic traits as the *mak-2* mutant and, in particular, is a hyphal fusion mutant. We show that MAK-2 phosphorylation during early colony development is dependent upon the presence of NRC-1 and postulate that phosphorylation of MAK-2 is required for hyphal fusion events that occur during conidial germination.
TABLE 1. N. crassa strains

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| Xa-2   | het-c24 arg-5; pan-2 a | A. J. F. Griffiths |
| 1-1-83 | ad3A his-3 A | FGSC* |
| 9-1-5  | ptr-4 A | FGSC |
| FGSC 4347 | fl A | FGSC |
| FGSC 4317 | fl A | FGSC |
| R1-08  | a | R. L. Metzenberg |
| RLM 40-27 | 74 ORS A | R. L. Metzenberg |
| FGSC 4564 | ad-3B cyhl 1 a1 | FGSC |
| FGSC 1814 | al-1 aro-8 A | FGSC |
| DJ19-99 | arg-1; trp-4 A | This study |
| RLM 32-27 | cyhl-1 nic-2 lys-4 A | R. L. Metzenberg |
| PB-1   | mak-2 a | P. Bobrowicz and D. Ebbole |
| 1822   | ad3A nic-2 a | A. J. F. Griffiths |
| 12-21-388 | ad3B al-2; cot-1; pan-2 A | A. J. F. Griffiths |
| API-2   | al-1; mak-2 a | This study |
| API-4   | lys-4 nic-2; mak-2 A | This study |
| API-3   | arg-1; trp-4; mak-2 a | This study |
| API-5   | lys-4 nic-2; mak-2 A | This study |
| API-1   | al-2; mak-2 pan-2 a | This study |
| rnc-1   | al-2; aro-9; rnc-1; avq; qa-2 a (qa-2) | 31 |

* FGSC, Fungal Genetic Stock Center.

wilt pathogen Fusarium oxysporum, also result in mutants that are unable to effectively colonize plants (15, 68).

The aim of this study was to analyze the phenotype of a mutant in a FUS3/KSS1 ortholog, mak-2, in the saprotrophic filamentous fungus N. crassa. We observed that phosphorylation of MAK-2 was associated with morphological events occurring during early colony development. In addition, we examined the phosphorylation of MAK-2 in an N. crassa nrc-1 mutant (31); nrc-1 encodes a STE11 ortholog and is predicted to be in the same MAP kinase pathway as mak-2. We show that mak-2 and nrc-1 are hyphal fusion mutants and that phosphorylation of MAK-2 is correlated with germ tube elongation, branching, and fusion events between conidial germings.

MATERIALS AND METHODS

Strains and growth conditions. N. crassa strains used in this study are listed in Table 1. The Δmak-2 strain PB-1 was constructed by gene replacement of the entire mak-2 open reading frame (ORF) with the gene for hygromycin phosphotransferase (P. Bobrowicz and D. J. Ebbole, unpublished data) (see Fig. 2), including 25 bp upstream of the mak-2 ATG and 278 bp downstream of the stop codon for the mak-2 ORF (accession number AF348490). All strains were grown on Vogel's medium (57) with or without supplements depending on the strains used. The nrc-1 mutant (31) was grown on Vogel's dextrose medium (the nrc-1 strain contains an inveratase mutation, inv). All crosses were performed on Westergaard's synthetic cross medium (60), in some cases with limiting amounts of nutritional supplements. The Δmak-2 strain PB-1 is male sterile but male fertile and was therefore used as a male in crosses. The helper strain, Δ[nuc] (FGSC 4564) was used in heterokaryons for crosses where complementation of auxotrophic markers was required in the female parent (43).

To isolate the API-1 Δmak-2 progeny, a heterokaryon between (FGSC 4564 + 12-21-388) (Table 1) was used as a female and fertilized with conidia from PB-1. One-tenth of the normally added supplement of adenine was added to mating medium, since both strains used as a female were auxotrophic for adenine. The Δmak-2 API-2 progeny was obtained by crossing (FGSC 4564 + FGSC 1814) with PB-1 conidia. API-3 was obtained by crossing (FGSC 4564 + DJ19-99) as a female, which was fertilized with PB-1 conidia. Ascospores were heat shocked at 60°C for 40 min to induce germination followed by plating on Bdes plates (60) with or without 20 μg/ml of hygromycin/mL plus various supplements, as required.

Escherichia coli strain DH5α (F endA1 hsdR17 supE44 lacZM15) (Bethesda Research Laboratory, Gaithersburg, Md.) was used for all DNA manipulations (46).

PCR and Southern blot analysis. The genotype of the Δmak-2 progeny was confirmed by PCR amplification of genomic DNA from putative Δmak-2 progeny using mak-2 specific primers. Genomic DNA was isolated as described previously (35). The primer for the hygromycin phosphotransferase gene was 5′-TATAGAAGATCTCCGGCGGTTAAGGGA-3′, and the primer for the 5′ flanks of mak-2 was 5′-CTCTATTCTCTCTCCTTCATC-3′. The PCR amplicons were sequenced from the reverse strand and aligned with the Δmak-2 strain. The original Δmak-2 strain was digested overnight with EcoRV and BgIII and subsequently used for Southern blot analysis (46). A 1.4-kbp hygromycin phosphotransferase fragment (isolated from pCB1004 by restriction digestion with HpaII) (10) and a Δmak-2 PCR fragment (see above for primer sequences) were used as probes for Southern blot analyses. RNA isolation and Northern analyses were done according to the method described in reference 40.

Heterokaryon tests. Heterokaryons were forced by spotting approximately 106 conidia from two auxotrophic strains onto a petri plate containing Vogel's minimal medium. In hyphal fusion-competent strains, vigorous heterokaryotic growth is observed within 24 h. A modified heterokaryon test was performed for hyphal fusion mutants (63). Briefly, conidia of two strains with different nutritional requirements were spotted 1 to 2 cm apart on plates containing Vogel's minimal medium. One-hundredth of the normal concentration of the required supplement was co-applied with the conidia. If the conidial growth is sufficient for conidial germination and sparse growth, successful heterokaryon formation was observed by the formation of a dense patch of mycelia at the intersection of the two sparse colonies.

Preparation of protein extracts and immunoblot analysis. Four-day-old conidia (107) were suspended in 300 μl of sterile H2O and were spotted onto sterile cellulose that had been placed onto duplicate plates containing Vogel's medium plus any required supplements. All plates were incubated at 24°C. One set of plates was examined microscopically for development during conidial germination, and the second set of plates was used for protein extraction. Fungal tissue, along with the cellulose membrane, was harvested at various time points, depending on the experimental design. For protein extraction, cellulose containing conidia, germings, or hyphae (depending upon the stage of growth) was peeled off the plate and plunged into liquid nitrogen. The samples were subsequently ground in liquid nitrogen, and extraction buffer (50 mM HEPES [pH 7.5], 2 mM EGTA, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), protease inhibitor cocktail (Roche), and phosphatase inhibitor (1 mM sodium orthovanadate and 1 mM sodium fluoride) were added. After centrifugation at 2,390 × g for 30 min, supernatants were transferred to clean tubes. All extraction and centrifugation steps were carried out at 4°C. The concentration of protein extract was determined by using a Bio-Rad protein assay kit with bovine serum albumin (Sigma) as the standard.

For immunoblot analysis, 30 μg of total protein (per lane) was separated on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel by electrophoresis. Protein was transferred onto nitrocellulose membranes by wet electrophoretic blotting (Bio-Rad). Transferred protein was assayed with reversible Ponceau-S dye (Fluka Biochemica). Blocking of membranes was performed for 1 h in Tris-buffered saline (50 mM Tris [pH 7.5], 150 mM NaCl, 0.2% Tween 20, and 5% nonfat dried milk (Nestle Carnation) at room temperature. The membranes were subsequently incubated with either anti-p44/42 MAP kinase (1:3,000 dilution) or anti-phospho p44/p42 MAP kinase antibodies (1:3,000 dilution) (PhosphoPlus antibody kit; Cell Signaling Technology) in Tris-buffered saline (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20, and 0.1% nonfat dried milk, NaCl) at 4°C. The anti-phospho p44/p42 antibodies recognize phosphorylated threonine (amino acid 202, Erk1) and tyrosine (amino acid 204, Erk1) residues in Erk1/2. The TEY sites are highly conserved in MAK-2 (Fig. 1). The anti-p44/p42 antibodies were produced by immunizing rabbits with a synthetic peptide derived from the carboxy terminus sequence of Erk2; the exact epitope used for production of anti-phospho p44/p42 antibody is proprietary (Cell Signaling Technology). Following hybridization, blots were washed three times for 5 min each in Tris-buffered saline and 0.2% Tween 20 and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution) (PhosphoPlus antibody kit; Cell Signaling Technology) for 1 h at room temperature. The antibody complex was visualized by using an enhanced chemiluminescence PhosphoPlus antibody kit (Cell Signaling Technology) and an ECL kit (Amersham) according to the manufacturer's instructions.

For equal protein loading controls, blots were stripped and probed with monoclonal antibody (clone TU27) against β-tubulin (dilution 1:2,000, BabCo).
FIG. 1. Alignment of MAK-2, Kss1p, Erk1, and Fus3p. All four of the MAP kinases are aligned by Clustal W with identity-based alignment. The regions presented are the catalytic core (63 amino acids, P+1 to L14) and the substrate interaction and docking domain (L16). All four of the MAP kinases are activated by phosphorylation at T*EY* in the P+1 loop in the catalytic domain. The phosphorylation lip starts from amino acids Asp-Phe-Gly (DFG) in subdomain VII and ends at TEY in subdomain VIII. Structural domains (L, loop; α, α-helix) are designated according to established nomenclature (1). National Center for Biotechnology Information accession numbers are as follows: mak-2, AF348490; FUS3, Z35777; KSS1, Z72825; Erk1, P27361. Asterisks indicate phosphorylated residues.

Transformation assays and protoplast fusion experiments. Protoplasts were prepared as described previously (47). Strain AP1-1 (Table 1) was used as a recipient for transformation assays with pOKE103 constructs (62). Neurospora strains were transformed as described previously (62). Transformation plates were incubated at 30°C for 2 days. Pantothenate prototrophic transformants were identified and transferred to Vogel’s plates (57) and incubated at 24°C, and the phenotypic and growth characteristics of transformants were monitored.

For protoplast fusion experiments, an equal amount of protoplasts from two strains with different auxotrophic markers were incubated with 30% (wt/vol) polyethylene glycol 6000, 50 mM CaCl2, and 10 mM morpholinepropanesulfonic acid (MOPS) at 30°C for 40 min. The protoplasts were then mixed with prewarmed top agar and poured onto plates containing FIGS minimal medium (47). The plates were incubated at 30°C for 2 to 3 days. Heterokaryotic colonies were subsequently transferred to minimal medium and incubated at 24°C. Growth characteristics of the colonies were monitored for a week.

For complementation experiments, a 2,385-bp fragment was ligated into an EcoRI linearized vector. An EcoRI fragment was then ligated into the pGEM-T vector. An EcoRI mak-2 fragment was ligated into an EcoRI linearized plasmid vector pOKE103 (62) (containing the panthothenate gene, pan1Δ, gift from R. L. Metzenberg). This plasmid, pOKEmak-2, was used for complementation experiments of Δmak-2 strains via transformation.

Microscopy. For light microscopy, conidia, germinating conidia, and hyphae were observed on a Zeiss Axioskop 2 fluorescence microscope with bright field or differential interference contrast (DIC) optics and under identical growth conditions used for protein extraction (see above). Conidal germination, branching, and hyphal fusion events were quantitated at hourly intervals over a 12-h period with a Nikon TE2000 microscope with an oil immersion 40× (N.A. 1.0) plan fluor objective. At each time point, >100 conidia or germ tubes were analyzed on each of 8 replicate slides taken from two petri plates sampled throughout the time course. The percentage of germinated conidia and germ tubes involved in hyphal fusion events was calculated.

Confocal laser scanning microscopy was performed with a Nikon PCM2000 confocal microscope equipped with an argon ion laser. Cells were stained with FM4-64 as described previously (26) and excited at 514 nm, and fluorescence was detected at >550 nm. Oil immersion 60× (N.A. 1.4) or dry 20× (N.A. 0.75) plan apo objectives were used for imaging. Captured images were finally processed with Photoshop software (version 6.0; Adobe).

RESULTS

Alignment of MAK-2 with Fus3p, Kss1p, and Erk1 shows conservation of catalytic residues. A putative ortholog of KSS1/FUS3, called mak-2 (Bobrowicz and Ebbole, unpublished) encodes a 352-amino-acid protein in N. crassa. This protein is predicted to encode a MAP kinase on the basis of sequence alignment and the presence of a highly conserved catalytic core, i.e., threonine and tyrosine residues T*EY* (Fig. 1). MAK-2 is predicted to phosphorylate the T and Y residues in the catalytic core of MAK-2, resulting in activation. The predicted MAK-2 amino acid sequence shows high sequence identity to predicted MAP kinases from other filamentous fungi, such as PMK1 from M. grisea (65), CMKI from C. lagenarium (51), BMP1 from B. cinerea (68), fim1 in F. oxysporum (15), and CHK1 in C. heterostrophus (37) (>90% identity). The MAP kinases in these plant pathogens are required for appressorium formation and/or colonization of host tissues. MAK-2 was also highly similar to Erk1 (extracellular signal-regulated kinase 1) (73% identity), Erk2 (55% identity), and S. cerevisiae Fus3p (59% identity) and Kss1p (59% identity). Erk1 and Erk2 are MAP kinases involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated mammalian cells (11, 42). All known kinases have a regulatory and a catalytic domain; the former is absent in MAP kinases. The catalytic domain is composed of 11 major subdomains, and the catalytic core lies in subdomain VIII. As evident from the alignment data (Fig. 1), all four kinases (i.e., MAK-2, Erk1, Kss1p, and Fus3p) have identical amino acid residues in the catalytic core Thr-Glu-Tyr (TEY). A mak-2 deletion mutant exhibits a pleiotropic phenotype. The Δmak-2 mutant strain PB-1 was isolated by the replace-
ment of the predicted mak-2 ORF with the gene for hygromycin phosphotransferase (hph) (Bobrowicz and Ebbole, unpublished). We confirmed the disruption of mak-2 and the linkage of the hph gene to sequences flanking the mak-2 locus in PB-1 by PCR with one primer specific for hph and the other for sequences flanking mak-2; amplification of an ~500-bp band was apparent in PB-1 but was absent in wild-type strains (data not shown). Southern blot analysis of genomic DNA from the wild type (RLM 40-27) and PB-1 showed the presence of a single copy of the hph gene in PB-1 (Fig. 2A). A band corresponding to mak-2 was detected only in the wild type; PB-1 lacks mak-2 hybridizing sequences (Fig. 2A). By Northern analysis, a transcript from mak-2 was identified in RLM 40-27 but not in the Δmak-2 strain PB-1 (Fig. 2B). We were successful in obtaining a number of hygromycin-resistant Δmak-2 progeny from a variety of crosses (APJ-1, APJ-2, APJ-3, APJ-4, and APJ-5) (see Materials and Methods and below). The molecular analysis of the mak-2 gene disruption in these progeny gave a pattern identical to that of the original Δmak-2 strain PB-1 (Fig. 2A).

The Δmak-2 deletion strain PB-1 and its Δmak-2 progeny displayed a pleiotropic phenotype. A wild-type strain (RLM 40-27) had a growth rate of 7 ± 0.5 cm/day compared to 2.3 ± 0.5 cm/day (24°C) for strains containing the Δmak-2 mutation. N. crassa normally sporulates upon carbon depletion (55) and conidiates at the edges of a petri dish approximately 2 days postinoculation (Fig. 2C). By contrast, the Δmak-2 mutant has stunted aerial hyphae and conidiates profusely as it grows across the petri plate (Fig. 2C). In addition to vegetative growth and conidiation phenotypes, the Δmak-2 mutant and its progeny failed to make female reproductive structures, perithecia, and are therefore female sterile.

To assess whether female sterility, growth rate, and conidiation defects of Δmak-2 can be complemented by heterokaryon formation with a mak-2" strain, we attempted to force heterokaryons between Δmak-2 progeny and mak-2" strains containing different auxotrophic markers by using mixed conidial suspensions. In all cases, recovery of heterokaryons failed. This observation suggested that the Δmak-2 mutants could be defective in hyphal fusion. We therefore used a modified heterokaryon test (63) to assess the ability of the Δmak-2 strains to form a heterokaryon with a wild-type strain by using complementing auxotrophic markers. Conidial suspensions from auxotrophic strains were inoculated 1 cm apart on a plate with limiting amounts of supplements. Successful heterokaryon formation between Δmak-2 auxotrophic strains (Table 1) and mak-2" auxotrophic strains was visualized by vigorous growth occurring in the region of contact between Δmak-2 and mak-2" strains. Such heterokaryons showed a wild-type growth and conidiation phenotype, indicating that the mutation in mak-2 is recessive. By contrast, we were unable to recover heterokaryons between Δmak-2 strains with different nutritional requirements by using either conidial suspensions or modified heterokaryon tests.

Δmak-2 mutants are hyphal fusion defective. Filamentous fungi normally form an interconnected mycelium network during growth, a process that is mediated by hyphal fusion events (8, 26). Our inability to recover (Δmak-2 and Δmak-2) heterokaryons suggested that mak-2 might be required for hyphal fusion. We therefore assessed the ability of Δmak-2 mutants to undergo self-fusion by bright field, DIC, and confocal microscopy. The hyphae at the periphery of the Δmak-2 colony (PB-1) showed some differences in apical extension and branching frequency compared to the hyphae in the periphery of a wild-type colony (Fig. 3A). The hyphal density in the Δmak-2 mutant was reduced, and individual hyphae were typically thinner and exhibited a more meandering growth pattern. Although hyphae at the periphery of a wild-type colony of
N. crassa undergo apical extension and branching, these hyphae are refractory for hyphal fusion (8, 26). The interior hyphae of a wild-type colony also undergo apical extension and branching, but they additionally undergo numerous hyphal fusion events (26).

The interior of the Δmak-2 colony was significantly different from that of a wild-type colony (Fig. 3). In the Δmak-2 mutant, hyphal fusion in the colony interior did not take place, although hyphae frequently made contact with each other. These data are consistent with our previous observation that heterokaryons between two Δmak-2 strains with forcing auxotrophic markers were unrecoverable and indicate that the mak-2 MAP kinase pathway is required for hyphal fusion in N. crassa.

Δmak-2 mutants show an ascospore autonomous lethal phenotype. We assessed sexual phenotypes of Δmak-2 strains as a female by forcing a heterokaryon between a Δmak-2 strain containing auxotrophic markers and a strain containing a mutation at the mating type locus am1 (21, 43) by using a modified heterokaryon procedure (see Materials and Methods). The (am1 + Δmak-2) heterokaryon formed female reproductive structures and protoperithecia and was fertilized with conidia

![Colony periphery and Colony interior](image)
from the opposite mating type. Normal numbers of perithecia and ascospores were produced. However, very few of the heat-shocked ascospores germinated on plates containing 200 μg of hygromycin/ml. Similarly, normal numbers of perithecia and ascospores were produced when conidia Δmak-2 mutants were used as the fertilizing parent; very few hygromycin-resistant progeny were recovered. In all, we performed 5 replicate crosses and screened many thousands of ascospore progeny, but we successfully isolated only 5 mak-2 progeny containing auxotrophic markers (Table 1). When these five Δmak-2 hygromycin-resistant progeny (APJ-1 to -5) were crossed as either males or females to a wild-type strain, they all displayed the ascospore autonomous lethal phenotype of the original Δmak-2 mutant. These data suggest that a functional mak-2 MAP kinase pathway is essential for ascospore germination and/or maturation.

The Δmak-2 vegetative phenotypes were complemented by the introduction of a wild-type copy of mak-2 into APJ-1 by selection for Pan+ transformants (pOKEmak-2) (see Material and Methods and reference 62). The growth rate of the mak-2-complemented transformants was almost equal to that of RLM 40-27 (wild-type strain, approximately 7 cm/day). Aerial hyphae in the mak-2-complemented transformants were still short but not as short as Δmak-2 mutants, and the conidiation pattern was mostly like that of the wild type, although some sporulation still occurred at the plate center. Hyphal fusion was restored in all analyzed transformants (Fig. 4D). Complemented transformants also formed abundant protoperithecia when grown on synthetic crossing medium. Complementation for ascospore maturation and germination was not performed due to the occurrence of genome surveillance mechanisms in N. crassa that effectively silence or mutate ectopic copies of genes (48, 49).

Δmak-2 mutants lack a protein that cross-reacts with anti-p44/42 (Erk1/Erk2) antibodies. Because of the similarity of MAK-2 to mammalian Erk1 and Erk2, we used commercially available antibodies against mammalian Erk1/Erk2 (anti-p44/42 antibodies, PhosphoPlus antibody kit; Cell Signaling Technology) to assess cross-reactivity with MAK-2. When anti-p44/42 antibodies were used as a probe in Western blots of protein extracts from a wild-type N. crassa strains, the anti-p44/42 antibodies hybridized to a protein of approximately 43 kDa (Fig. 4A, lanes 5 and 6). The predicted molecular mass of MAK-2 is approximately 41 kDa. In the Δmak-2 mutant PB-1 and in Δmak-2 progeny (APJ-1 to -4), this ~43 kDa band was not detectable (Fig. 4A, lanes 2 to 4 and 7). To determine whether the introduction of mak-2 into PB-1 restored the presence of a cross-reacting ~43-kDa protein, we subjected protein extracts from Δmak-2-complemented transformants carrying pOKEmak-2 to Western analysis with anti-p44/42 antibodies (Fig. 4B, lanes 3 to 5). Varying levels of an ~43-kDa protein was observed in the Δmak-2-complemented transformants.

Activation of MAK-2 is correlated with germ tube elongation, branching, and fusion between conidial germings. The phenotype of the Δmak-2 mutant suggested that activation of MAK-2 via phosphorylation could be both temporally and spatially regulated in a growing colony. Phospho-specific anti-p44/42 (phospho-p44/42) antibody is available that recognizes phosphorylated threonine (T) and tyrosine (Y) residues in Erk1/Erk2. In MAK-2, these residues are highly conserved; the predicted phosphorylation sites occur at amino acid positions 180 (T) and 182 (Y) (Fig. 1). As with anti-p44/42 antibody, the Δmak-2 mutants lack a protein that is recognized by the phospho-p44/42 antibody (Fig. 4C, lanes 2 to 4), although a protein was identified in extracts from RLM 40-27 (Fig. 4C, lane 5). We therefore assessed whether variations in phosphorylation...
of MAK-2 in a wild-type strain (RLM 40-27) occurred during conidial germination and early colony development. As shown in Fig. 5A, MAK-2 phosphorylation was evident at 4 h postinoculation. Phosphorylation of MAK-2 increased until 8 h, followed by a decrease in phosphorylation at 12, 16, 20, and 24 h (Fig. 5A, I). The blot was stripped and reprobed with anti-tubulin and anti-p44/42 antibodies (Fig. 5A, II and III); an approximately equal level of protein is present at each time point. A similar pattern of MAK-2 phosphorylation during germination was observed in replicate trials with conidia from RLM 40-27 and also when equal amounts of conidia from 1-1-83 (ad3A his-3 A) and 9-1-5 (pyr-4 A) (Table 1) were coinoculated onto minimal medium to force heterokaryon formation (8 separate trials) (data not shown). These observations suggest a role for the mak-2 MAP kinase pathway in events associated with conidial germination and/or early colony development in N. crassa.

We microscopically examined wild-type conidia during germination and early colony development, focusing on events that occurred with the increase in phosphorylation of MAK-2, approximately 4 to 12 h postinoculation. Conidia began to form germ tubes between 2 and 3 h postinoculation; the majority of conidia had germinated by the 8-h time point (Fig. 5B and C). From 2 to 8 h postinoculation, germ tubes elongated and branched. After 5 h postinoculation, many of the germ tubes and germling branches had fused with other branches or germ tubes. After 8 h, 62% ± 1.9% of the conidial germlings were involved in hyphal fusion events with other germlings (Fig. 5B and C). At the 12-h time point, the interconnected hyphal network was too dense to accurately measure the percentage of germlings involved in fusion. The increase in MAK-2 phosphorylation observed between the 4- and 8-h time points was associated with a period in which germ tubes were elongating and branching. During this same time period, the number of hyphal fusion events rapidly increased.

We evaluated the germination of Δnrc-1 conidia compared with wild-type at time points similar to those for RLM 40-27. Germination of Δnrc-1 conidia was initiated at a similar time point (between 2 and 3 h) to that of wild-type conidia (but occurred over a longer period [2 to 12 h] than the 2 to 8 h in the case of the wild type) (Fig. 5C). Following germination, Δmak-2 germ tubes grew at a much slower rate than the wild-type and branching of the germ tube was delayed (Fig. 6A). At the 4-h time point, the average germ tube length of Δmak-2 conidia was only 3.5 ± 0.1 μm; for the wild-type, the average germ tube length at this time point was 6.45 ± 0.5 μm. Most significantly, hyphal fusion events between conidial germlings were completely absent in the Δmak mutant.

**nrc-1 is an upstream component of the mak-2 pathway.** N. crassa strains that have mutations in a MEKK gene, nrc-1, display a phenotypic similar to that of Δmak-2 mutants, i.e., slow growth, nonrepressed conidiation, lack of aerial hyphae and protoperithecia, and an autonomous ascosporal lethal phenotype (31). nrc-1 encodes a protein that is an ortholog of STE11 of S. cerevisiae; Ste11p phosphorylates the MEK Ste7p, which subsequently phosphorylates Fus3p or Kss1p (2, 25). Therefore, NRC-1 is predicted to be in the same phosphorylation cascade as MAK-2. We assessed hyphal fusion capability in the nrc-1 mutant and observed that it failed to form heterokaryons with a wild-type strain under all conditions tested (data not shown). Conidial germination in nrc-1 was delayed (cf. nrc-1 in Fig. 6A, which was imaged 8 h after hydration, to a wild-type strain), as was germ tube elongation and branching. As with the Δmak-2 mutant, hyphal fusion during conidial germination and colony establishment was absent in the nrc-1 mutant (Fig. 6A).

MAK-2 was detected in protein extracts from a 20-h culture of nrc-1 with anti-p44/42 antibody (Fig. 4A, lane 1); phosphorylated MAK-2 was not detectable in protein extracts from the nrc-1 mutant at the same time point (Fig. 4C, lane 1). These data also supported the hypothesis that NRC-1 is in the same signaling pathway and upstream of MAK-2. We therefore assessed phosphorylation of MAK-2 during conidial germination to colony establishment in an nrc-1 mutant. As shown in Fig. 6B, MAK-2 was not phosphorylated at any time point over a 24-h time period in the nrc-1 mutant. However, when the same blot was probed with anti-p44/42 antibodies, MAK-2 protein was detected at all time points. Thus, mutations in nrc-1 affect phosphorylation levels of MAK-2 during conidial germination and colony establishment. These results suggest that mak-2 and nrc-1 belong to a common signal transduction pathway in N. crassa which is involved in early germination events, including germ tube elongation, branching, and hyphal fusion.

**DISCUSSION**

In Neurospora, hyphal fusion occurs at two stages during colony development: (i) between germlings during the establishment of fungal colonies (29, 33) and (ii) between hyphae in subapical parts of mature colonies (8, 26). In this paper, we provide evidence that a MAP kinase pathway in N. crassa, involving MAK-2 and NRC-1, regulates hyphal fusion between germlings and hyphae in mature fungal colonies. These results are based on an analysis of mak-2 and nrc-1 mutants, time course studies of phosphorylation of MAK-2, and quantitation of the cytological events occurring during early colony development. Fusions between conidia and germlings is not unique to Neurospora but has also been described for other fungi (23, 29, 33, 45). We observed numerous hyphal fusion events between germlings during early stages of colony development, which correlated with a peak of MAK-2 phosphorylation. Our hypothesis is that, during conidial germination, the MAK-2 MAP kinase pathway is induced by a diffusible substance elaborated by germinating conidia, which triggers hyphal fusion between conidial germlings. We suggest that the decreased, yet significant, level of MAK-2 phosphorylation after 12 h postinoculation may reflect the nonsynchronous and lower rate of hyphal fusion events in the colony as it grows older. In this study, it was not possible to quantify the rate of hyphal fusion in a mature colony because the hyphal density became too great to obtain reliable measurements.

Live cell imaging of hyphal fusion events in N. crassa (26) and microscopic analyses in a number of filamentous fungi (8, 18, 29, 33) show that attraction between hyphae involved in fusion events is mediated by diffusible substances. At present, there are no clues as to what controls the frequency or the spatial and temporal distribution of hyphal fusion within a fungal colony. It is generally assumed that hyphal fusion, by networking hyphae, is important for intrahyphal communication, translocation of water and nutrients, and general ho-
FIG. 5. MAK-2 phosphorylation is associated with germ tube elongation and hyphal fusion between germlings. (A) Conidia from a wild-type strain (RLM 40-27) were inoculated onto a cellophane membrane layered on Vogel's minimal medium plates and incubated at 24°C. Total protein (30 μg) isolated at 0, 4, 8, 12, 16, 20, and 24 h postinoculation of conidia was used for Western blot analysis. (I) Protein extracts from the different time points probed with anti-phospho p44/42 antibodies (PhosphoPlus antibody kit; Cell Signaling Technology). (II) The blot in panel I was stripped and reprobed with anti-β-tubulin monoclonal antibodies (clone TU27; BabCo). (III) The blot in panel II was restriped and probed with anti-p44/42 antibodies. The anti-phospho p44/42 antibodies (Cell Signaling Technology) recognize highly conserved phosphorylated T and Y residues in MAP kinases (residues 202 to 204 in Erk1 and residues 180 to 182 in MAK-2) (Fig. 1). The anti-p44/42 antibodies were raised to a peptide synthesized based on the C-terminal amino acid sequence of Erk1. The phospho-p44/42 antibody gave a stronger signal on Western blots than the anti-p44/42 antibody, presumably because of the highly conserved TEY site in MAK-2. The same experiment was repeated with unstripped blots for anti-p44/42 antibodies, and identical results were obtained. (B) DIC images of conidia and conidial germlings from the wild type (RLM 40-27) at 0, 4, 8, and 12 h postinoculation. Bar, 30 μm. (C) Quantitation of germination and hyphal fusion in conidial germlings in the wild type (RLM 40-27) over the first 8 h postinoculation. Error bars represent standard errors.
It is also possible that hyphal fusion is required to appropriately regulate developmental processes, such as conidiation and protoperithecial development. Hyphal fusion events between germlings could be a way to optimize resource exploitation by increasing the biomass of genetically identical individuals.

Because conidial germlings and hyphae within a single colony are genetically identical, hyphal fusion is a self-signaling phenomenon. The nature of diffusible substances in filamentous fungi that mediate self-signaling during either hyphal attraction or hyphal avoidance (53) is unknown. During sexual reproduction, pheromones are involved in the attraction of hyphae to each other or between specialized structures of different genotypes and are well characterized for many fungi (3, 28, 32, 50). The mating type locus of *N. crassa*, which regulates the expression of mating type-specific pheromone genes (4), apparently does not play a role in hyphal fusion; *mat* mutants are hyphal fusion competent (13, 43). However, many of the processes required for hyphal fusion in filamentous fungi are also required by *S. cerevisiae* cells during mating, a process that has been well characterized, i.e., signaling by diffusible substances, redirected growth of fusing cells, attachment, production and targeting of wall-degrading enzymes to the attachment site, fusion of the plasma membrane, and cytoplasmic mixing (2, 19, 32). Significantly, these components include MAP kinase pathways.

In this paper, we show that during conidial germination, NRC-1 is required for phosphorylation of MAK-2. The *nrc-1* mutant has many of the same phenotypic traits as the *mak-2* mutant (31), including an inability to undergo hyphal fusion. Our data support the hypothesis that NRC-1 and MAK-2 are components of the same signal transduction pathway. Mutations in an *A. nidulans* *nrc-1* homolog, *steC*, resulted in the isolation of a pleiotropic mutant that also failed to form heterokaryons (58). By using phosphospecific antibodies, the authors showed that phosphorylation of two MAP kinases were

![FIG. 6. *nrc-1* is required for phosphorylation of MAK-2 during conidial germination.](image)
associated with conidiation in *A. nidulans* and require functional SteCp. However, neither of these two kinases were in the 40-kDa range, in the size range predicted for the KSS1/FUS3 ortholog in *A. nidulans*. A downstream component of the pheromone response pathway in *S. cerevisiae* is STE12, which encodes a transcription factor (16). Mutations in STE12 homologs in filamentous fungi result in mutants that are affected in sexual or pathogenic development on host plants (5, 41, 56). An *N. crassa* strain containing a mutation in the STE12 ortholog has recently been identified (Bobrowicz and Ebbole, unpublished). The *N. crassa* ste12 mutant is similar in phenotype to *nrc-1* and mak-2 mutants and also fails to undergo hyphal fusion (D. J. Jacobson and N. L. Glass, unpublished data). These data suggest that other components of the pheromone response pathway that are present in the genomes of filamentous fungi may also be involved in hyphal fusion and colony establishment.

It has recently been shown that the SLT2 cell wall integrity MAP kinase pathway may be important for heterokaryon formation in filamentous fungi. In *Fusarium graminearum*, disruption of the SLT2 (MAP kinase) ortholog resulted in mutants affected in cell wall integrity. These mutants also failed to form heterokaryons (27). During mating in *S. cerevisiae*, the SLT2 MAP kinase pathway is downstream of the FUS3 MAP kinase pathway and is required for remodeling of the cell wall during shmoo formation (7). These observations suggest that a similar relationship between the orthologous FUS3 and SLT2 MAP kinase pathways may also exist in filamentous fungi and may be important for hyphal fusion.

Conidial germination in filamentous fungi depends on various factors such as nutrient deprivation, hydration, physical stimuli, contact stimulation, and proximity to other conidia (14). Mutational analysis of genes in filamentous fungi that are orthologous to those required in *S. cerevisiae* for budding indicate that many of the functions involved in polarization and bud site selection affect germ tube emergence in filamentous fungi (for reviews, see references 39 and 59). Mutations in mak-2 did not significantly affect the timing of the initiation of germination or germ tube formation and, thus, MAK-2 does not play an essential role in breaking conidial dormancy or formation of the germ tube. However, mak-2 mutants showed a much slower rate of germ tube elongation, indicating that a functional MAK-2 MAP kinase pathway is required for optimal apical extension of a hypha, a process apparently unrelated to hyphal fusion. In addition, the *nrc-1* and mak-2 mutants are female sterile and show nonrepressible conidiation. It is unclear whether these phenotypes are due to an inability to form an interconnected fungal colony or if the MAK-2 MAP kinase pathway has a role in these developmental processes that is separable from its role in hyphal fusion. All hyphal fusion mutants so far described for *N. crassa* have pleiotropic phenotypes that share some, but not all, of the developmental abnormalities of mak-2 and *nrc-1* mutants (31, 61, 63).

Plant pathogens containing mutations in *mak-2* homologs, such as *M. grisea pmk1* (65), *C. lagenarium cmk1* (51), and *C. heterosporus chk1* (37) affect appressorium formation and host colonization. We predict that all of these MAP kinase mutants are also hyphal fusion mutants, which may affect the ability of these organisms to colonize host tissue. Hyphal fusion and appressorium formation typically involve cell adhesion, swelling, and cell wall digestion (26, 54). It may be that these two processes between pathogenic and saprotrophic fungi use common signal transduction mechanisms and may be evolutionarily related. Further comparisons between pathogenic and saprotrophic fungi with regard to requirements for signal transduction, hyphal fusion, and pathogenicity may reveal the answer.

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