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Divergent cAMP Signaling Pathways Regulate Growth and Pathogenesis in the Rice Blast Fungus *Magnaporthe grisea*

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cAMP is involved in signaling appressorium formation in the rice blast fungus *Magnaporthe grisea.* However, null mutations in a protein kinase A (PKA) catalytic subunit gene, *CPKA*, do not block appressorium formation, and mutations in the adenylate cyclase gene have pleiotropic effects on growth, conidiation, sexual development, and appressorium formation. Thus, cAMP signaling plays roles in both growth and morphogenesis as well as in appressorium formation. To clarify cAMP signaling in *M. grisea*, we have identified strains in which a null mutation in the adenylate cyclase gene (*MAC1*) has an unstable phenotype such that the bypass suppressors of the Mac1$^-$ phenotype (*sum*) could be identified. *sum* mutations completely restore growth and sexual and asexual morphogenesis and lead to an ability to form appressoria under conditions inhibitory to the wild type. PKA assays and molecular cloning showed that one suppressor mutation (*sum1-99*) alters a conserved amino acid in cAMP binding domain A of the regulatory subunit gene of PKA (*SUM1*), whereas other suppressor mutations act independently of PKA activity. PKA assays demonstrated that the catalytic subunit gene, *CPKA*, encodes the only detectable PKA activity in *M. grisea.* Because *CPKA* is dispensable for growth, morphogenesis, and appressorium formation, divergent catalytic subunit genes must play roles in these processes. These results suggest a model in which both saprophytic and pathogenic growth of *M. grisea* is regulated by adenylate cyclase but different effectors of cAMP mediate downstream effects specific for either cell morphogenesis or pathogenesis.

INTRODUCTION

cAMP is a ubiquitous secondary messenger in prokaryotic and eukaryotic cells. A well-characterized intracellular target of cAMP in eukaryotic cells is the regulatory subunit of protein kinase A (PKA; Taylor et al., 1990). PKA is a tetrameric holoenzyme that is composed of two regulatory subunits and two catalytic subunits. Binding of cAMP to the regulatory subunits releases the catalytic kinase subunits to phosphorylate target proteins involved in cAMP-regulated processes. In fungi, cAMP regulates both metabolism and morphogenesis (reviewed in Pall, 1981). For example, in *Saccharomyces cerevisiae*, cAMP regulates carbon metabolism (reviewed in Thevelein, 1994), cell cycle progression (Matsumoto et al., 1982; Tokiwa et al., 1994), and pseudohyphal growth (Lorenz and Heitman, 1997). In filamentous fungi, such as *Neurospora crassa*, cAMP signaling plays important roles in hyphal tip growth, conidiation, and carbon metabolism (Terenzi et al., 1974, 1979; Bruno et al., 1996). More recently, cAMP signaling also has been found to be important in fungal pathogenesis (Kronstad, 1997).

Chronic and widespread throughout most of the world's rice growing areas, rice blast disease, caused by *Magnaporthe grisea*, is a persistent threat to world rice production (Ou, 1980). Asexual spores (conidia) infect plant cells by germinating and differentiating a dome-shaped cell called an appressorium (reviewed in Talbot, 1995), which allows direct penetration of the plant cell wall. Germinating conidia also form appressoria efficiently on inert hydrophobic surfaces in a droplet of water (Hamer et al., 1988). In many strains, appressoria are much less efficiently formed on hydrophilic surfaces (Gilbert et al., 1996). The exact mechanism by which the germ tubes of *M. grisea* sense surface hydrophobicity is not known. However, the secretion and self-assembly of a hydrophobin protein encoded by the *MPG1* gene may provide a sensing mechanism for surface hydrophobicity (Talbot et al., 1993, 1996; Beckerman and Ebbole, 1996). Appressorium formation can be inhibited by yeast extract and by high concentrations of the *S. cerevisiae* α factor pheromone (Beckerman et al., 1997). Although the biochemical effects of these additions are not known, they may perturb signaling mechanisms that trigger appressorial differentiation. When germinated on a hydrophilic surface, appressorial differentiation can be induced by the addition of cAMP, its soluble analogs, or inhibitors of cAMP–phosphodiesterase (Lee and Dean, 1993). These findings suggest that increases in the intracellular concentration of cAMP may act as part of an early signaling event in appressorium formation.

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The role of the cAMP signaling pathway in appressorium formation in *M. grisea* has been investigated by assessing the phenotypes of gene knockout mutants in *CPKA*, a gene encoding a putative catalytic subunit gene (Mitchell and Dean, 1995; Xu et al., 1997), and *MAC1*, a gene encoding adenylate cyclase (Choi and Dean, 1997; this study). However, mutations in these genes give dramatically different phenotypes, neither of which specifically affects appressorium differentiation. For example, *cpkA* null mutants can still form appressoria but fail to penetrate plant cells. Thus, it is not clear whether cAMP mediates its effect on appressorium formation directly through PKA regulation. In addition to affecting appressorium formation, *mac1* null mutants have pleiotropic defects in growth, sporulation, and mating ability, whereas *cpkA* mutants have no effect on these processes. The observation that exogenously added cAMP can overcome Mac1$^-$ defects in appressorium formation continues to suggest a role for cAMP signaling in appressorium formation.

To clarify further the relationship of cAMP signaling to growth and pathogenesis, we performed additional studies on *MAC1* and PKA regulation in *M. grisea.* We functionally characterized *MAC1* and identified strains in which the Mac1$^-$ phenotype is unstable. Among the bypass suppressors of the *mac1* mutation, we identified a specific mutation in the regulatory subunit of PKA. This mutation completely restores the growth and morphology of *mac1* mutants. This mutation also allows appressorium formation on nonhydrophobic surfaces and in the presence of yeast extract. These findings, together with biochemical studies with *cpkA* mutants, allow us to conclude that cAMP mediates its effect on appressorium formation through PKA, most likely through a catalytic subunit highly divergent from CPKA. Thus, cAMP signaling for growth and pathogenesis in *M. grisea* diverges at the level of PKA regulation.

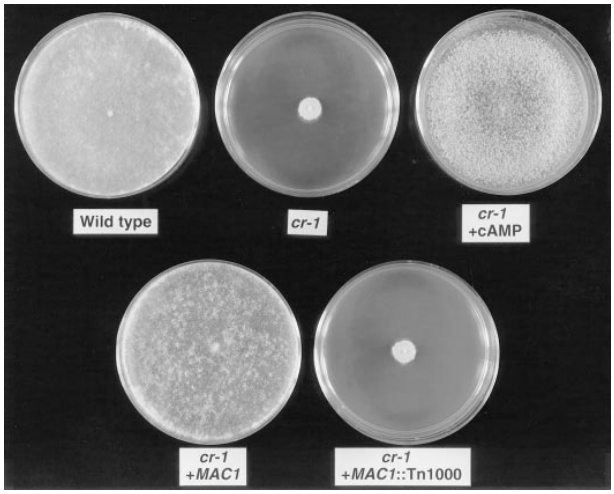

Figure 1. The *MAC1* Gene Complements Colonial Growth of the *N. crassa cr-1* Mutant.

The cultures were made on Vogel minimal agar medium (Davis and de Serres, 1970) and incubated at 25°C for 3 days. Compared with the wild type (top left), the *cr-1* mutant exhibits dramatically reduced growth with tight clusters of conidia (top middle). This abnormal morphology was restored by the addition of 5 mM cAMP in the medium (top right) and also by introduction of the *MAC1* gene (bottom left). The *MAC1* gene disrupted in the catalytic domain with Tn*1000* loses the ability to rescue the growth defect of the *cr-1* mutant (bottom right).

RESULTS

An Unstable Allele of *MAC1* in Strain Guy11

A candidate gene for the *M. grisea* adenylate cyclase was identified from a genomic cosmid library by using a probe generated by polymerase chain reaction (PCR) amplification with primers designed to amplify the conserved catalytic domain (see Methods). A 13.4-kb EcoRI fragment was subcloned, and sequencing of 8.7 kb of DNA around the putative catalytic site identified an open reading frame of 7151 nucleotides encoding a 2160–amino acid protein with significant similarity to fungal adenylate cyclases. The DNA sequence has GenBank accession number AF006827. A second *MAC1* sequence from strain 70-15 (GenBank accession number AF012921) and characteristic features of this gene have been published (Choi and Dean, 1997). This sequence differs from AF006827 at 13 nucleotide positions, resulting in three amino acid changes. All nucleotide polymorphisms in AF006827 were reconfirmed by sequencing.

To test whether *MAC1* could function in a well-characterized cAMP signaling pathway, we introduced *MAC1* into the *N. crassa crisp-1* (*cr-1*) mutant. cAMP signaling is essential for growth polarity (hyphal extension) in *N. crassa* (Bruno et al., 1996). *cr-1* mutants have a dramatically reduced colonial growth rate (Figure 1), which can be rescued by the application of exogenous cAMP. Introduction of the *MAC1* gene together with a plasmid conferring hygromycin B resistance efficiently rescued the *cr-1* phenotype, whereas disruption of *MAC1* in the conserved catalytic domain prevented complementation (Figure 1). DNA gel blot analysis was used to confirm integration events (data not shown). We conclude that *MAC1* encodes a typical membrane-associated fungal adenylate cyclase and can function in the polarity signaling pathway of *N. crassa.*

A *mac1* null mutant was created by one-step gene replacement (Figure 2A). Among 160 initial hygromycin-resistant transformants, spore PCR (Xu and Hamer, 1995) identified 26 putative null mutants. Δ*mac1*::*Hph* strains were confirmed by the absence of *MAC1* coding sequences (Figure 2B, probe 1), the alteration of flanking DNA sequences (Figure 2B, probe 2), and the introduction of the hygromycin B phosphotransferase gene (*HPH*) (Figure 2B, probe 3). One

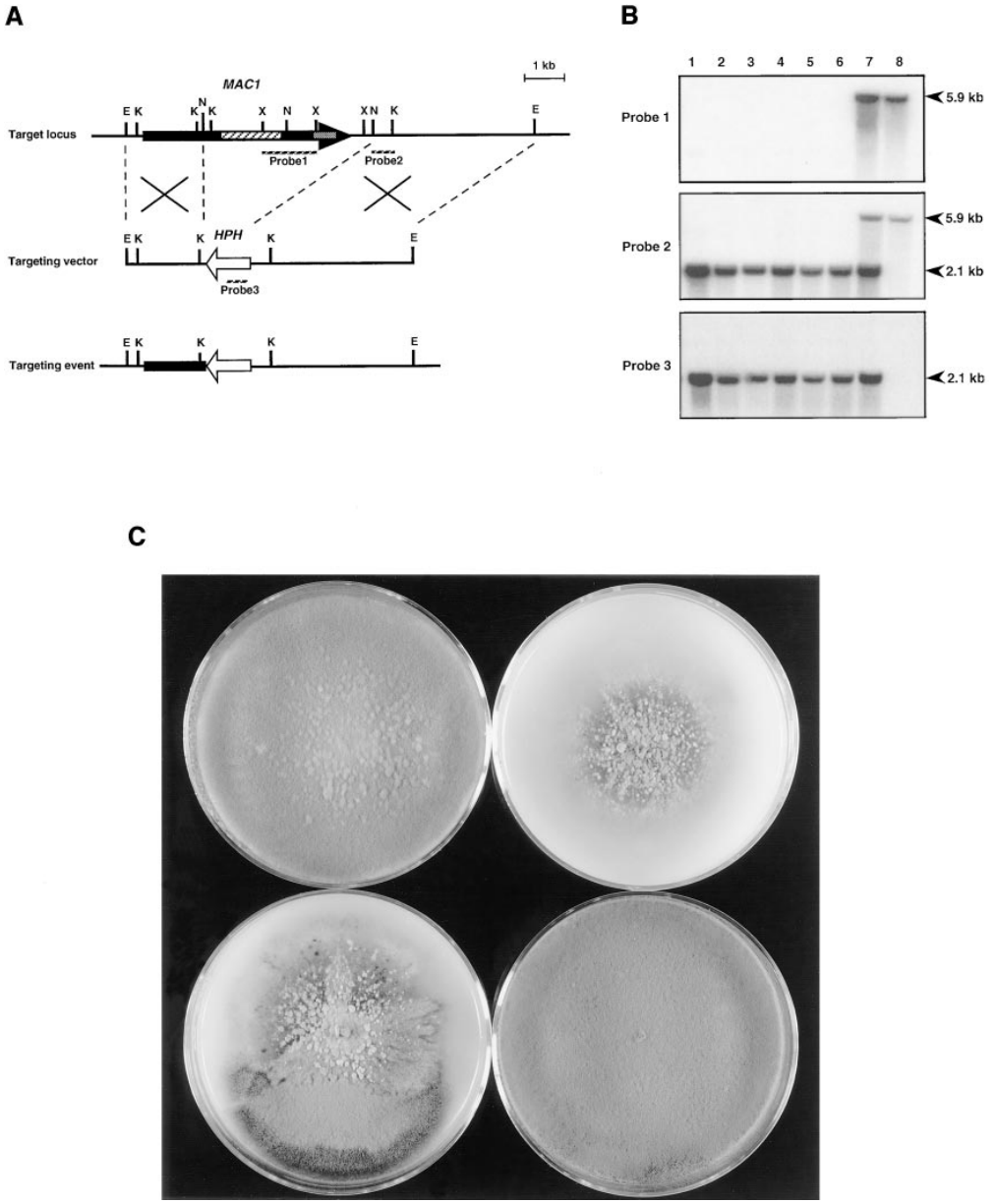

Figure 2. Disruption of the *M. grisea MAC1* Gene.

(A) The *MAC1* locus and targeting knockout vector. The black and white arrows indicate the positions and transcriptional directions of the *MAC1* and *HPH* genes, respectively. Restriction enzyme cleavage sites are abbreviated as follows: E, EcoRI; K, KpnI; N, NcoI; and X, XbaI. Integration by homologous recombination produces a mutated locus in which the 3′ two-thirds portion of the *MAC1* gene containing the leucine-rich repeats (amino acids 700 to 1374; hatched box) and catalytic domain (amino acids 1751 to 1994; shaded box) are deleted. The positions of three probes for DNA gel blot analysis are depicted with stippled lines.

(B) DNA gel blot analysis of *MAC1* knockout transformants. The genomic DNAs extracted from wild-type strain Guy11 (lane 8), *MAC1* knockout transformants DA127, DA128, DA133, DA138, DA154, and DA156 (lanes 1 to 6), and ectopic insertional mutant DA50 (lane 7) were digested with KpnI and separated in a 0.7% agarose gel. The blot was probed with a 1.8-kb XbaI fragment of the *MAC1* gene (Probe 1), stripped and reprobed with 600-bp NcoI-KpnI fragment in the right flanking region (Probe 2), and then stripped and reprobed with the 600-bp PCR product from the *HPH* gene (Probe 3). The *HPH* primers were described in Xu and Hamer (1996).

(C) Colony morphology of the *MAC1* knockout transformant and a suppressor mutant. Wild-type strain Guy11 (top left) and *MAC1* knockout transformant DA128 (top right) were cultured on oatmeal agar medium for 10 days. A sporulating sector arose after DA128 (bottom left) was cultured for 15 days. The sector was single-spore isolated on oatmeal agar medium and cultured for 10 days (bottom right).

Table 1. Appressorium Formation in *mac1* and Suppressor Mutants of *M. grisea*

Strain	Genotype	Surface Property	
		Hydrophobic	Hydrophilic
Guy11	Wild type	90.6 ± 4.0[a]	0.9 ± 1.3
DA128	Δ*mac1*::*Hph*	1.2 ± 1.8	0.4 ± 0.8
DA154	Δ*mac1*::*Hph*	1.7 ± 2.1	0.0 ± 0.0
DA156	Δ*mac1*::*Hph*	0.0 ± 0.0	0.3 ± 0.8
DA61	Δ*mac1*::*Hph sum-61*	98.3 ± 1.2	97.3 ± 0.6
DA67	Δ*mac1*::*Hph sum-67*	95.3 ± 0.6	95.0 ± 1.0
DA99	Δ*mac1*::*Hph sum1-99*	97.0 ± 1.0	95.7 ± 1.5

[a]The percentage of germinated conidia that formed appressoria was quantitated after 24 hr. The mean and standard deviation were calculated from three independent experiments. Each experiment consisted of three replicates, and 100 germinated conidia were examined per replicate.

transformant (DA50; Figure 2B, lane 7) contained an intact *MAC1* gene, and the targeting vector presumably inserted at an ectopic chromosomal site. As expected, loss of adenylate cyclase had a dramatic effect on growth, asexual morphogenesis, and mating ability. Δ*mac1*::*Hph* strains had reduced colonial growth rates, reduced conidiation, and a highly mosaic colony appearance on oatmeal agar medium (Figure 2C). Transformants with similar phenotypes were recovered when cAMP was included in the protoplast regeneration medium.

After 10 to 15 days of growth, the rapidly growing and evenly sporulating sectors from Δ*mac1*::*Hph* strains (Figure 2C) were recovered. Single-spore isolations from these sectors resulted in the growth of strains with wild-type patterns of sporulation, growth rate, and colony appearance. An independently generated collection of Δ*mac1*::*Hph* strains created with a different gene disruption vector produced identical results, that is, Δ*mac1*::*Hph* strains with slow-growing colony phenotypes that eventually produced more rapidly growing sectors (data not shown). Sectors were observed on a variety of culture media (oatmeal agar, V8 juice agar, and minimal and complete agar media). DNA gel blot analysis of several wild-type-like colonies derived from Δ*mac1*::*Hph* strains confirmed the presence of the gene disruption event (data not shown). Genetic crosses of one wild-type-like colony derived from a Δ*mac1*::*Hph* strain to a wild-type tester showed that the original Δ*mac1*::*Hph* colony phenotype could be recovered from segregating progeny. We conclude that these Δ*mac1*::*Hph* strains of strain Guy11 can give rise to spontaneous bypass suppressing mutations that restore wild-type-like growth. We designated these strains Δ*mac1*::*Hph sum* (for suppressors of Mac1$^-$). We selected three independent suppressor mutants designated DA61, DA67, and DA99 for further analysis. Each was from an independent Δ*mac1*::*Hph* transformant.

MAC1 Requirements for Appressorium Formation Are Suppressed by *sum* Mutations

Infection structure (appressorium) formation was assayed on hydrophobic plastic coverslips or the hydrophilic side of GelBond film (Table 1) in the absence or presence of different concentrations of exogenously added cAMP (Table 2). Appressorium formation was scored after 24 hr of incubation (see Methods). The wild-type strain Guy11 forms appressoria efficiently on hydrophobic surfaces but does not form appressoria efficiently on hydrophilic surfaces. As previously reported, *mac1* mutants are deficient for appressorium formation (Table 1) and produced germ tubes that failed to undergo appressorium formation and remained somewhat undifferentiated (Figure 3). However, the appressorium-deficient phenotype is reminiscent of mutations in the Mpg1 hydrophobin (Talbot et al., 1993). For both phenotypes, germ tubes curl and hook repeatedly and appear to grow wider but do not form appressoria. In contrast, when *mac1* mutants were incubated on a hydrophilic surface, the germ tubes were very similar to germ tubes of the wild-type strain Guy11. They were thin, grew straight, and remained undifferentiated.

The *mac1* mutants could be induced to form appressoria in the presence of increasing concentrations of cAMP (Table 2 and Figure 3). These concentrations of cAMP also restored appressorium formation to the wild-type strain when it was germinated on a hydrophilic surface (Table 2). Although appressorial assays were done with conidia from 10-day-old cultures, the small fraction of appressoria produced from the *mac1* mutants is likely due to the presence of spores containing suppressor mutations.

sum mutations dramatically restored the ability of *mac1* strains to form appressoria on hydrophobic surfaces (Table 1 and Figure 3). In addition, unlike wild-type strains, *mac1* strains carrying *sum* mutations also were able to form ap-

Table 2. cAMP Rescues the Appressorium Formation Defect in *mac1* Mutants of *M. grisea*[a]

Strain	No cAMP	10 mM cAMP	50 mM cAMP
Guy11	0.7 ± 1.2[b]	36.3 ± 8.0	90.7 ± 2.5
DA127	4.0 ± 1.0	61.3 ± 9.2	96.7 ± 2.5
DA128	0.7 ± 0.6	57.3 ± 4.0	95.7 ± 0.6
DA129	1.0 ± 1.0	69.3 ± 3.1	91.0 ± 3.6
DA154	0.3 ± 0.6	81.7 ± 6.8	89.0 ± 3.0
DA156	0.7 ± 0.6	76.7 ± 4.2	92.0 ± 2.6

[a]Hydrophobic plastic coverslips (Fisher) were used for the *mac1* mutants; pieces of the hydrophilic side of GelBond were used for wild-type strain Guy11.

[b]The percentage of germinated conidia that formed appressoria was quantitated after 24 hr. The mean and standard deviation were calculated using combined data of at least three replicates. One hundred germinated conidia were examined per replicate.

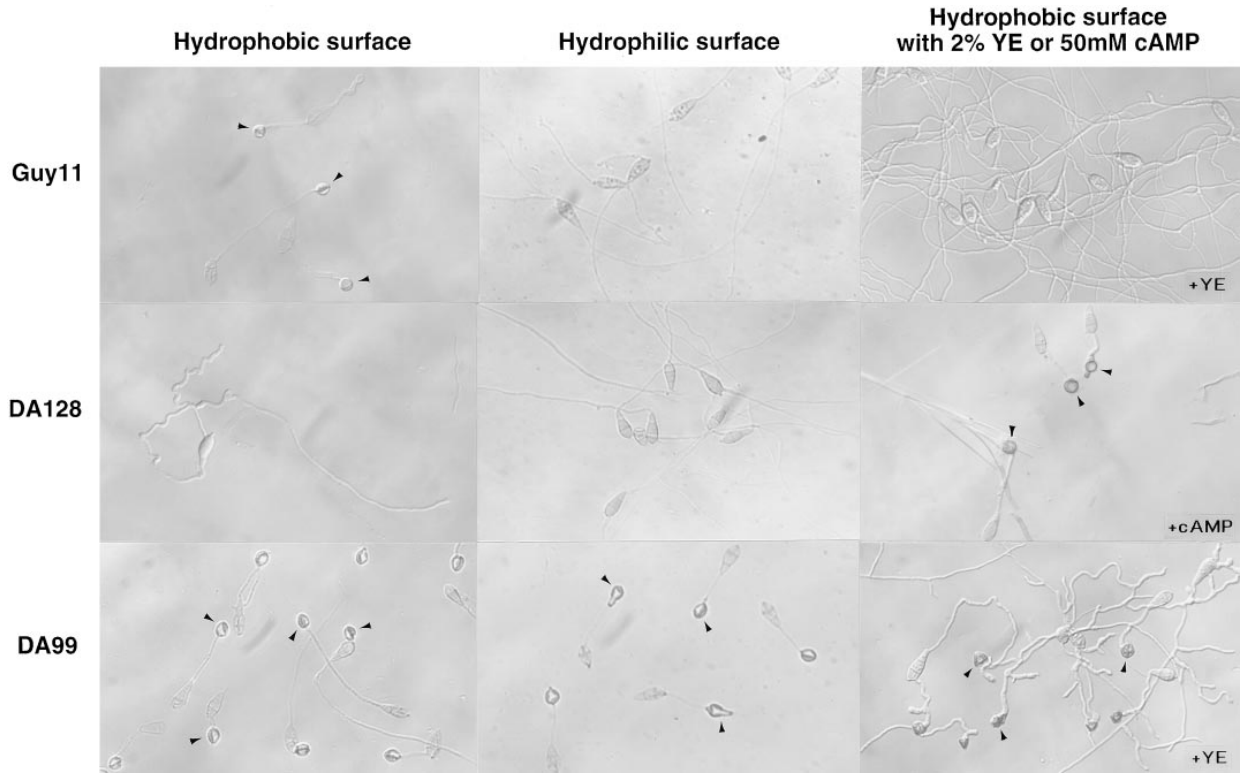

Figure 3. Appressorium Formation of Wild-Type Strain Guy11, Δ*mac1*::*Hph* Strain DA128, and Δ*mac1*::*Hph sum1-99* Strain DA99 on Hydrophobic and Hydrophilic Surfaces.

The effects of 2% yeast extract (+YE; for Guy11 and DA99) and 50 mM cAMP (+cAMP; for DA128) on appressorium formation on hydrophobic surfaces are indicated. The loss of appressorium formation in DA128 was restored by the *sum1-99* mutation in DA99. Appressorium formation assays performed with Δ*mac1*::*Hph sum* strains DA61 and DA67 gave identical results. Germinating conidia and appressoria were photographed using differential interference contrast microscopy. Arrowheads indicate appressorium formation.

pressoria efficiently on hydrophilic surfaces (Table 1 and Figure 3). Thus, *sum* mutations not only restore wild-type growth characteristics to *mac1* strains but also restore and enhance appressorium formation. These findings suggest that the suppression of the requirement for adenylate cyclase during appressorium formation leads to an inability to discriminate hydrophobic and hydrophilic surfaces. Adenylate cyclase–deficient strains containing *sum* mutations did not form appressoria when germinated on agar-solidified media and did not differentiate appressoria in liquid culture (data not shown).

Appressorium formation in *M. grisea* can be repressed by the addition of 2% yeast extract, and this suppression has been shown to be mating-type dependent (Table 3 and Figure 3). The suppression phenomenon is proposed to be mediated by small pheromone-like peptides present in yeast extract because the *S. cerevisiae* α factor also suppresses appressorium formation in a mating-type dependent manner (Beckerman et al., 1997). Two percent yeast extract effectively suppressed appressorium formation in strain Guy11 (*MAT1-2*) but did not suppress appressorium formation in strain AFTH3 (*MAT1-1*) (Table 3). Adenylate cyclase–deficient mutants containing *sum-99*, *sum-67*, and *sum-61* mutations (all strains are isogenic with Guy11 and therefore with *MAT1-2*) were no longer sensitive to mating-type-dependent inhibition of appressorium formation by yeast extract (Table 3 and Figure 3). In yeast extract, appressoria differentiated from the tips of highly branched germ tubes (Figure 3).

PKA Activity in *M. grisea*

PKA has been implicated in the regulation of appressorium formation in *M. grisea* (Lee and Dean, 1993; Mitchell and Dean, 1995); however, there have been no direct measurements of PKA activity. Null mutations in *CPKA* do not block appressorium formation and do not affect growth and sexual or asexual morphogenesis. Thus, the growth and appressorium

Table 3. Effect of Yeast Extract on Appressorium Formation of *mac1* Suppressor Mutants of *M. grisea*[a]

Strain	Mating Type	No Additive	Yeast Extract
Guy11	*MAT1-2*	92.3 ± 2.9[b]	0.0 ± 0.0
AFTH3	*MAT1-1*	96.3 ± 2.3	62.0 ± 4.5
DA61	*MAT1-2*	92.7 ± 3.1	90.0 ± 3.6
DA67	*MAT1-2*	97.0 ± 1.7	90.0 ± 1.0
DA99	*MAT1-2*	97.3 ± 1.2	92.7 ± 3.1

[a]Hydrophobic plastic coverslips (Fisher) were used for the assays.
[b]The percentage of germinated conidia that formed appressoria was quantitated after 24 hr. The mean and standard deviation were calculated using combined data of at least three replicates. One hundred germinated conidia were examined per replicate.

formation defects of *mac1* mutants may be mediated by catalytic subunit genes other than *CPKA.* Although multiple PKA catalytic subunit genes exist in fungi, only a single regulatory subunit occurs in *S. cerevisiae* (Toda et al., 1987a, 1987b). Thus, *sum* mutations may alleviate the effects of the loss of adenylate cyclase by altering the single regulatory subunit of PKA and affecting the activity of multiple catalytic subunits.

To test this hypothesis, we assayed PKA activity in protein extracts made from wild-type and mutant strains of *M. grisea* (see Methods). Figure 4A shows that exponentially growing hyphae from *M. grisea* contain a cAMP-stimulated protein kinase activity that phosphorylates a model PKA substrate (Kemptide). Assays with strains DA61 and DA67 showed that neither strain contained significant levels of cAMP-independent PKA activity, as might be expected from mutants in the regulatory subunit of PKA. In addition, both strains contained a cAMP-stimulated activity similar to that of the wild type. In contrast, strain DA99 containing the *sum-99* mutation showed elevated levels of cAMP-independent PKA activity. However, PKA activity in this strain still could be enhanced by the addition of cAMP. *sum-99* mutants consistently had lower levels of cAMP-stimulated PKA activity than did Guy11. Strikingly different results were obtained from extracts prepared from 8-hr germinated conidia (Figure 4B). PKA levels were undetectable for strain DA99, regardless of the presence or absence of cAMP. This suggests that the *sum-99* mutation inhibits PKA activity in germinating spores.

To confirm that *CPKA* encodes a biochemically active catalytic subunit and to search for additional PKA activities, we measured PKA levels in strains containing various alleles of *cpkA* in both germlings and actively growing mycelia. Two mutant alleles in strains I27 and DF51 were created by gene replacements and are presumed to be null (Mitchell and Dean, 1995; Xu et al., 1997). A third allele in strain pTH4 is an insertional mutation at the 3′ end of the gene that has phenotypes similar to the gene replacement alleles (Xu et al., 1997). All three *cpkA* mutants were dramatically deficient in PKA activity, and residual PKA activity could not be detected reliably in mycelia (Figure 4C). Similar results were obtained with germling extracts (data not shown). These results suggest that *CPKA* encodes the major PKA in *M. grisea* that could be assayed.

sum-99 Identifies a Mutation in the Regulatory Subunit of PKA

To determine whether *sum-99* contained a mutation in the regulatory subunit of PKA, we cloned the regulatory subunit gene by using PCR primers designed around conserved amino acid motifs in fungal regulatory subunit genes (see Methods). An amplified DNA fragment with significant homology to fungal PKA regulatory subunits was used to identify clones from cDNA and genomic libraries. Three full-length cDNAs and a genomic clone were sequenced. The gene (designated *SUM1*) structure is very similar to that of the *N. crassa* regulatory subunit gene *mcb* (Bruno et al., 1996). That is, one intron is in the coding region (the position identical to the intron of *mcb*), whereas two introns are in the 5′ untranslated region (Figure 5A). DNA gel blot analysis showed that *SUM1* is a single-copy gene (data not shown). The open reading frame encodes 390 amino acids and shows high similarity with regulatory subunits from other fungi (Figure 5B). Pairs of oligonucleotide primers were used to amplify the *SUM1* coding region from DA61, DA67, and DA99 (see Methods). As expected, DA61 and DA67 carry a wild-type *SUM1* gene. However, DA99 contains a single (T-to-G) mutation in the first cAMP binding domain (Figure 5C). The mutation changes an invariant leucine at position 211 to arginine.

Because a mutation in the regulatory subunit of PKA reverts the growth and appressorium-defective phenotypes in *mac1* mutants, an additional catalytic subunit gene must exist that cannot be detected in our PKA assays. Additional catalytic subunit genes could not be identified by DNA gel blot analysis (Mitchell and Dean, 1995). We performed an extensive PCR-based screen for additional catalytic subunit genes. Although many different protein kinase–related sequences were detected, we failed to detect additional PKA-related sequences in *CPKA*-deleted strain (data not shown). We conclude that a highly divergent and *sum1-99*–affected activity plays a role in cAMP-stimulated appressorium formation and growth morphogenesis in *M. grisea.*

sum Mutations Only Partially Suppress the Pathogenicity Defect in the Adenylate Cyclase Mutant

The *sum1-99* mutation and the undefined *sum-61* and *sum-67* mutations restore normal growth morphology and appressorium formation to adenylate cyclase–deficient mutants of *M. grisea.* To determine whether these suppressors fully restored pathogenicity, we conducted conidial spray inoculations and leaf injections of rice cultivars CO39 (Figure 6) and Sariceltik (data not shown) with wild-type strain

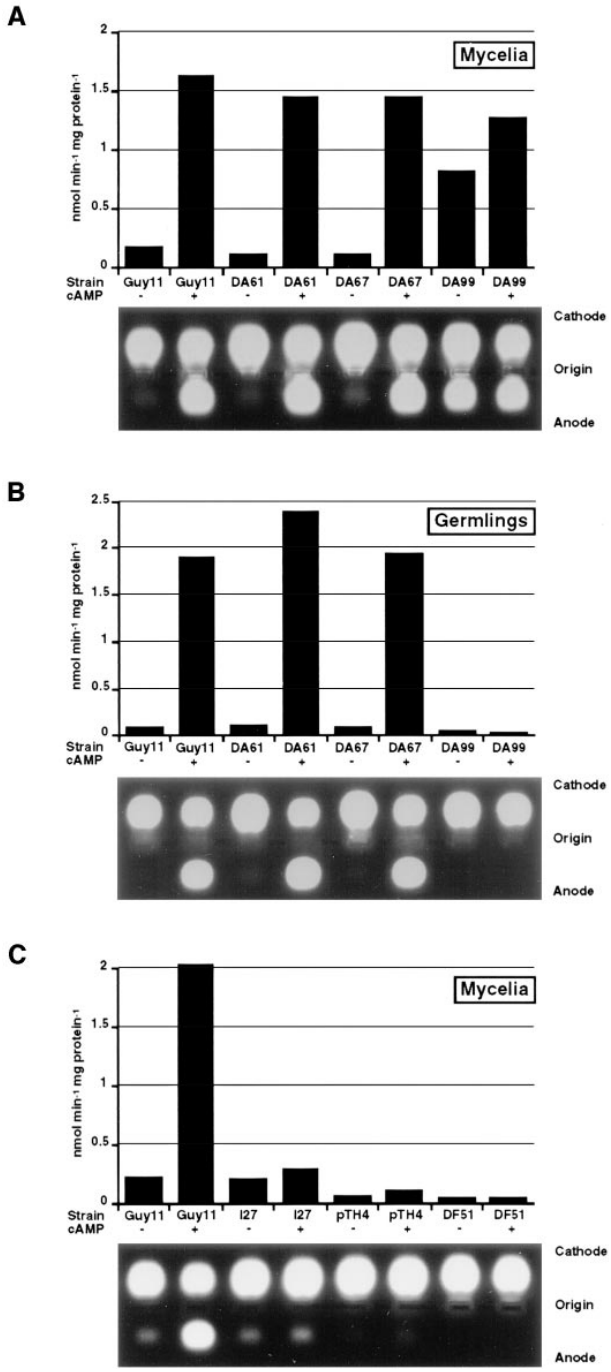

Figure 4. PKA Activity in Wild-Type and Mutant Strains of *M. grisea.*

PKA activity was monitored in Δ*mac1*::*Hph sum* mutants (**[A]** and **[B]**) and *cpkA* mutants **(C)**. Extracts were prepared from mycelia (**[A]** and **[C]**) and germlings **(B)**, and PKA activity was determined with (+) or without (−) the addition of 1 μM cAMP. Enzyme activity was monitored by gel electrophoresis (phosphorylated substrate migrating toward the anode) and quantitated using spectrofluorometry (see Methods). The gel was photographed on a UV transilluminator.

Guy11 and isogenic derivatives DA50 (ectopic integration event), DA154 (Δ*mac1*::*Hph*), DA99 (Δ*mac1*::*Hph sum1-99*), DA61 (Δ*mac1*::*Hph sum-61*), and DA67 (Δ*mac1*::*Hph sum-67*). As expected, adenylate cyclase–deficient strain DA154 was nonpathogenic when compared with control inoculations, and only rare pinpoint lesions were observed occasionally. Surprisingly, strain DA99 only showed a modest increase in pathogenicity (Figure 6). Fewer and smaller lesions in DA61, DA67, and DA99 were consistently observed compared with Guy11 and DA50. Lesion counts on second leaves of rice cultivar Sariceltik showed that Guy11 produced 10.1 lesions (SD = 9.8; $n = 58$) and DA50 produced 12.1 lesions (SD = 13.2; $n = 53$). In contrast, DA61 produced 2.4 lesions (SD = 3.3; $n = 59$), DA67 produced 0.8 lesions (SD = 1.2; $n = 57$), and DA99 produced 4.4 lesions (SD = 6.3; $n = 52$). Leaf sheath injection assays also showed that strains carrying *sum* mutations were still reduced in pathogenicity (data not shown).

DISCUSSION

Recent studies have demonstrated that fungal pathogens of plants and animals require genes encoding components of highly conserved signal transduction pathways for pathogenesis. These include components of heterotrimeric G proteins (Gao and Nuss, 1996; Alspaugh et al., 1997; Kasahara and Nuss, 1997; Liu and Dean, 1997; Regenfelder et al., 1997), MAP kinases (Banuett and Herskowitz, 1994; Xu and Hamer, 1996), and components of cAMP signal transduction pathways (Gold et al., 1994; Mitchell and Dean, 1995; Xu et al., 1997). Although these pathway components have been well studied in model genetic systems, such as *S. cerevisiae*, their role in fungal pathogenesis is novel and somewhat unexpected. Components of these pathways with specific effects on pathogenesis provide opportunities for the development of novel antifungal drugs and crop-protecting chemicals.

cAMP Signaling Pathways for Fungal Growth and Pathogenesis

A role for cAMP signaling during pathogenesis of the rice blast fungus has been confirmed by an analysis of phenotypes

PKA activity is expressed in nanomoles of phosphate transferred to a substrate per minute per milligram of protein. Data are representative of two independent experiments with nearly identical results.
(A) and **(B)** Guy11 (wild type), DA61 (Δ*mac1*::*Hph sum-61*), DA67 (Δ*mac1*::*Hph sum-67*), and DA99 (Δ*mac1*::*Hph sum1-99*) were tested.
(C) I27, pTH4, and DF51 are various *cpkA* mutants (see text for details).

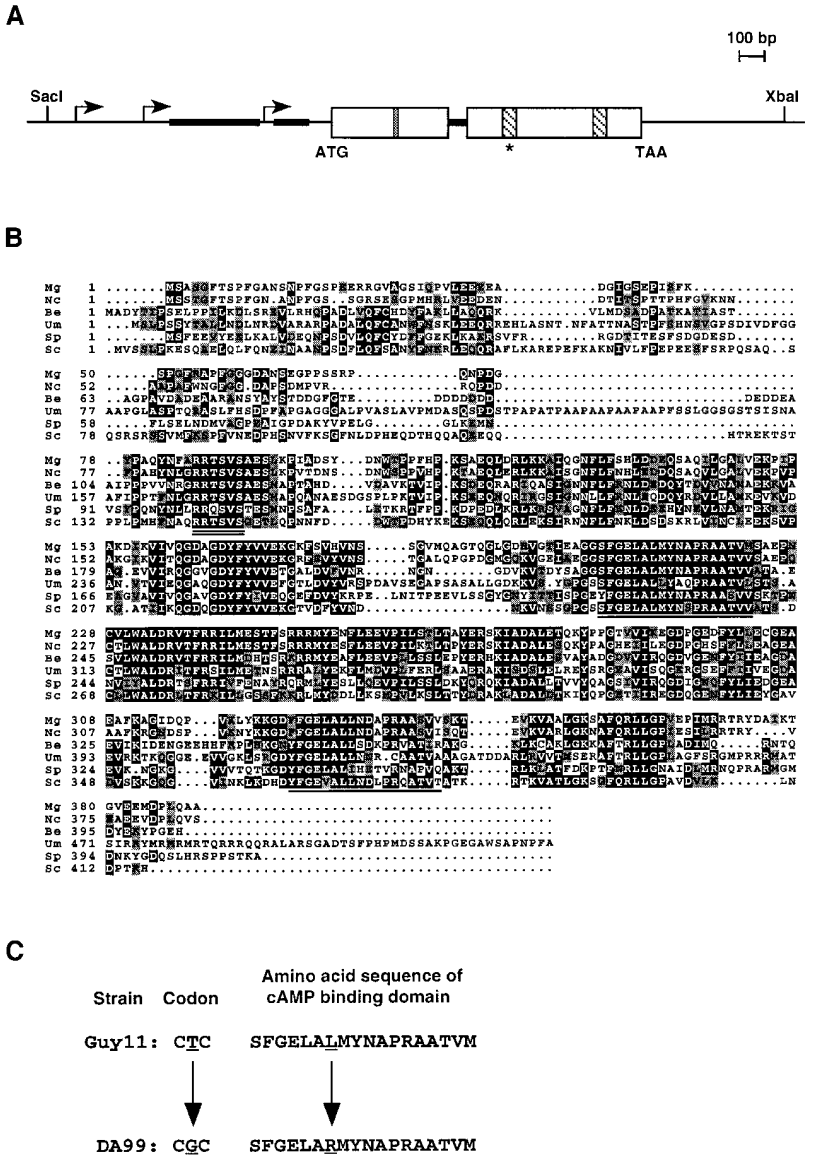

Figure 5. Molecular Cloning and Analysis of the PKA Regulatory Subunit Gene (*SUM1*) from *M. grisea.*

(A) Schematic diagram of the *SUM1* gene. The boxes and boldface lines indicate exons and introns, respectively. Nucleotide sequencing of three cDNA clones identified two introns within the 5′ untranslated region and one intron within the coding region. The start positions of the cDNA clones are marked by arrows. The two cAMP binding domains and the interaction site with the PKA catalytic subunit are represented by boxes with widely spaced and closely spaced diagonal lines, respectively (Taylor et al., 1990). The nucleotide sequence from SacI to XbaI has GenBank accession number AF024633.

(B) Amino acid sequence alignment of the PKA regulatory subunit genes from fungi. Sequences are aligned using the CLUSTAL W program (Thompson et al., 1994): Mg, *M. grisea*; Nc, *N. crassa* (Bruno et al., 1996); Be, *Blastocladiella emersonii* (Marques and Gomes, 1992); Um, *Ustilago maydis* (Gold et al., 1994); Sp, *Schizosaccharomyces pombe* (DeVoti et al., 1991); and Sc, *S. cerevisiae* (Toda et al., 1987a). Identical amino acids are highlighted on a black background; similar amino acids are shown on a gray background. Gaps introduced for the alignment are indicated by dots. The amino acid positions are indicated at left. The two cAMP binding domains are underlined. The interaction site with the PKA catalytic subunit is double underlined.

(C) Point mutation found in the PKA regulatory subunit gene of strain DA99 (Δ*mac1*::*Hph sum1-99*). The codon and amino acid substitutions are underlined. The position of the point mutation is indicated by an asterisk in **(A)**.

imparted by null mutations in the adenylate cyclase gene (*MAC1*) (Choi and Dean, 1997; this study). However, *mac1* mutants also display a wide variety of defects in growth and morphogenesis, demonstrating that cAMP has wider roles in growth regulation. The finding of mutations that suppress the growth defects of *mac1* mutants but do not restore full pathogenicity demonstrates that there are divergent cAMP signaling pathways for growth and pathogenesis. Two lines of evidence suggest that divergence occurs at the level of PKA regulation. First, mutations in a PKA catalytic subunit gene (*CPKA*) have specific effects on pathogenesis and, unlike mutations in *MAC1*, do not affect axenic growth and sexual or asexual morphogenesis (Mitchell and Dean, 1995; Xu et al., 1997). Second, a suppressor of the pleiotropic Mac1$^-$ phenotype, *sum1-99*, identifies the regulatory subunit of PKA. Although this mutation restores growth and conidiation, it alters appressorium formation and does not restore full pathogenicity.

Based on the phenotypes of *cpkA* null mutants, we suggested that an additional PKA(s) may exist in *M. grisea* (Xu and Hamer, 1996; Xu et al., 1997). For example, when assayed over 24 hr, *cpkA* mutants retained an ability to be stimulated to form appressoria by cAMP. However, we could find no other PKA activity in *cpkA* null mutants, and DNA gel blot analysis (Mitchell and Dean, 1995) failed to detect additional catalytic subunit genes. Studies with yeast suggest that these findings do not rule out the existence of additional PKA catalytic subunit genes. *S. cerevisiae* contains three catalytic subunit genes: *TPK1*, *TPK2*, and *TPK3* (Toda et al., 1987b). Tpk2 activity could not be detected in assays, even when overexpressed in a Δ*tpk1* Δ*tpk3* strain. Detecting low levels of Tpk3 activity requires overexpression in a Δ*tpk1* Δ*tpk2* background (Mazon et al., 1993). *TPK* genes are partially redundant for growth, and a triple *tpk* null mutant of *S. cerevisiae* is very slow growing. Overexpression of a PKA-related kinase gene, *SCH9*, can functionally rescue a triple *TPK* deletion mutant (Toda et al., 1988) and suppress many of the defects imparted by mutations in the Ras–adenylate cyclase signaling pathway. A related gene, *sck1*, exists in *Schizosaccharomyces pombe.* This gene can also rescue defects associated with the PKA signaling pathway and appears functionally related to the *pka1*-encoded catalytic subunit gene (Jin et al., 1995). Although other cAMP targets apart from PKA have been identified in yeast (e.g., Muller and Bandlow, 1994), the growth defects of the *mac1* mutants were suppressed by a mutation in the regulatory subunit of PKA. Thus, it remains possible that a divergent cAMP-dependent catalytic subunit gene(s) or PKA-related genes exist in *M. grisea* that are not functionally redundant with *CPKA.* A search for these genes is in progress.

Our findings are compatible with a model in which a surface-activated signal, such as assembly of the Mpg1 hydrophobin (Talbot et al., 1993, 1996; Beckerman and Ebbole, 1996), results in the activation of adenylate cyclase (Figure 7). The finding that *MAC1* can complement the *cr-1* mutation of *N. crassa* suggests that *MAC1* is membrane associated, similar to *N. crassa* adenylate cyclase (Flawia et al., 1977). Similar to mutations in *MAC1*, a null mutation in the *M. grisea* G protein α subunit gene *MAGB* has pleiotropic effects on growth, sporulation, appressorium formation, and pathogenicity, and appressorium formation can be restored in *magB* null mutants by the addition of cAMP (Liu and Dean, 1997). These findings are consistent with the possibility that adenylate cyclase in *M. grisea* may be activated by a membrane-associated heterotrimeric G protein.

Appressorium formation is inhibited in a mating-type-specific manner by yeast extract and α factor (Beckerman et al., 1997). This inhibition can be overcome by the *sum1-99* mutation, suggesting that the inhibition acts at the level of cAMP generation. Interestingly, the addition of the α factor also is known to repress adenylate cyclase in yeast (Liao and Thorner, 1980). The requirement for a hydrophobic surface for appressorium formation is also overcome by the *sum1-99* mutation. We propose that cAMP activates multiple PKAs through an interaction with the regulatory subunit of PKA. Specificity of cAMP signaling for either growth or pathogenesis is imparted by the action of activated catalytic subunits. The finding that the *sum1-99* mutation inhibits *CPKA*-encoded PKA activity in germlings but allows appressorium formation is consistent with our previous findings that *CPKA* is largely dispensable for appressorium formation but necessary for plant penetration (Xu et al., 1997). The observation that the *sum1-99* mutation restores normal growth

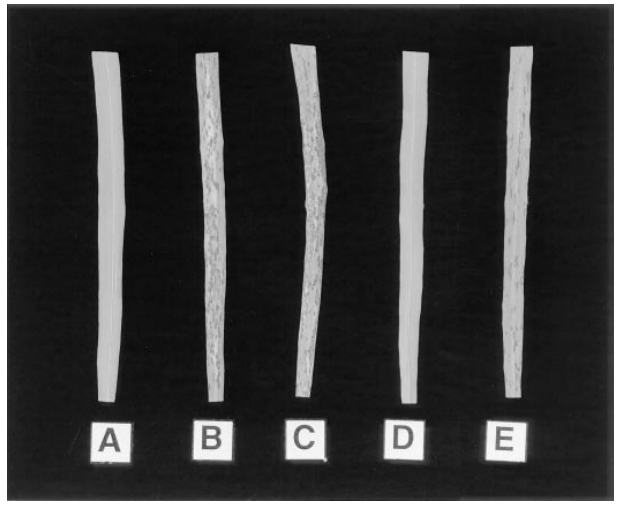

Figure 6. Rice Infection Assays.

Rice seedlings of cultivar CO39 were spray inoculated as indicated below. Typical leaves were collected 7 days after inoculation and photographed.

(A) Gelatin solution.

(B) Conidial suspension of wild-type strain Guy11.

(C) Conidial suspension of ectopic integration transformant DA50.

(D) Conidial suspension of Δ*mac1*::*Hph* mutant DA156.

(E) Conidial suspension of Δ*mac1*::*Hph sum 1-99* mutant DA99.

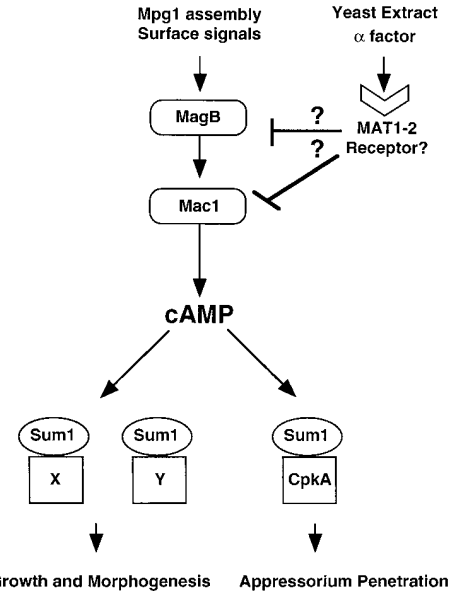

Figure 7. A Model for Divergent cAMP Signaling during Early Stages of Infection-Related Morphogenesis in *M. grisea*.

X and Y represent PKA catalytic subunits other than CPKA that are involved in growth and morphogenesis of *M. grisea.* Yeast extract and the α factor presumably interact with the membrane-associated MAT1-2 receptor and inhibit cAMP signaling upstream of cAMP generation.

and morphogenesis but not pathogenicity leads us to propose the existence of additional cAMP–Sum1—regulated activities required for growth, conidiation, and appressorium morphogenesis.

Suppressors of the *mac1* Mutation

Suppressors of adenylate cyclase mutations have also been identified in *S. cerevisiae* and *Ustilago maydis* (Matsumoto et al., 1982; Gold et al., 1994). In these studies, suppressor mutations frequently define the gene for the regulatory subunit of PKA. Surprisingly, suppressor mutations were not identified in *mac1* mutants generated by Choi and Dean (1997). One possibility is that the different genetic background employed in their studies may have masked the effects of suppressor mutations. We note that the *mac1* gene-replacement mutant created by Choi and Dean retained the highly conserved catalytic domain. Interestingly, studies with the homologous *CYR1* gene in *S. cerevisiae* showed that insertion/deletion mutations that did not inactivate the catalytic domain did not create null mutants (Suzuki et al., 1990). The deletion of the catalytic domain in our gene-replacement constructs may have provided a stronger growth selection for suppressor mutations.

In addition to mutations in the PKA regulatory subunit gene in yeast, suppressors of the Ras-dependent cAMP signaling pathway defects in *S. cerevisiae* include mutations in a cAMP phosphodiesterase gene *SRA5* (Wilson and Tatchell, 1988) and the overexpression of a protein kinase gene *SCK1*/*SCH9* (Toda et al., 1988) and a transcription factor gene *SOK2* (Ward et al., 1995). In *N. crassa*, mutations in the *hah* gene elevate the levels of intracellular cAMP and suppress the colonial growth of *cr-1* mutants (Murayama et al., 1995). Other suppressor mutations identified in our study remain undefined. Neither *sum-61* nor *sum-67* elevates the levels of PKA activity, and neither mutation occurs in the *SUM1* gene. These findings suggest that *sum-61* and *sum-67* act independently of PKA. Like *sum1-99*, both suppressors restore growth and morphogenesis but not pathogenicity to *mac1* mutants. Although *sum* mutations allow abundant appressorium formation on hydrophilic surfaces, none of the *sum* mutations permitted appressorium formation on agar surfaces or in liquid media. Studies have suggested that surface hardness may also be a signal for appressorial differentiation (Xiao et al., 1994). This hypothesis can be tested using the strains containing *sum* mutations.

The *sum1-99* mutation alters an invariant leucine to arginine in the first cAMP binding domain (site A) of the regulatory subunit of PKA. This mutation allows an increase in PKA activity in the absence of cAMP, but PKA activity still can be partially stimulated by the addition of cAMP. These biochemical findings suggest that the *sum1-99*–encoding regulatory subunit may be inefficient at inhibiting the catalytic subunit. Previous mutagenesis studies with other organisms did not identify this leucine residue (Su et al., 1995). Analysis of the crystal structure of the bovine regulatory subunit shows that the homologous leucine residue (L-203) is conserved in cAMP binding domain A but does not contact the cAMP molecule (Su et al., 1995). Rather, nearest-neighbor analysis shows that it aids in forming a network of contacts with conserved residues that make up the cAMP binding pocket in domain A.

The finding that the *sum1-99* mutation restores growth morphology and appressorium differentiation but not full pathogenicity suggests a hierarchical requirement for cAMP/PKA signaling in *M. grisea.* Low PKA activity may be sufficient for growth and morphogenesis but insufficient for pathogenicity. However, an extensive mutational analysis of the PKA regulatory subunit gene, *bcy1*, in *S. cerevisiae* shows that the severity of the physiological defect imparted by a regulatory subunit mutation does not correlate with the level of PKA activity (Cannon et al., 1990; Zaremberg and Moreno, 1996). These studies suggest that regulatory subunit mutations can impart a variety of phenotypes based on their binding affinity to catalytic subunits. For example, the *sum1-99*–encoding regulatory subunit may not separate efficiently from the catalytic subunit in the presence of cAMP, thus reducing PKA activity even in the presence of cAMP. In addition, the *sum1-99* mutation causes a dramatic loss of PKA activity in germinating spores. Although the basis for this inhibition is

not known, it highlights the diverse ways in which regulatory subunit mutations can affect PKA activity.

Finally, the *sum1-99* mutation may allow PKA activity in the holoenzyme complex and thereby alter PKA affinities for specific substrates. Thus, PKA substrates phosphorylated in the Δ*mac1*::*Hph sum1-99* background restore growth and appressorium formation but not full pathogenicity. The unfulfilled requirement for cAMP signaling in pathogenicity may reflect the need for increased carbohydrate mobilization for efficient penetration and/or alterations in fungal metabolism to accommodate growth in plant cells. In *U. maydis*, accurate PKA regulation imparted by the regulatory subunit of PKA is necessary for pathogenicity (Gold et al., 1997).

METHODS

Fungal Strains and Methods

Magnaporthe grisea wild-type strains Guy11 and its descendant 4375-R-26 were used for *MAC1* and *SUM1* cloning. Guy11 also was used for disruption of the *MAC1* gene. AFTH3, carrying the *MAT1-1* mating-type allele, was used for sexual crosses. Fungal maintenance, sexual crosses, transformation, and DNA isolations were performed as previously described (Crawford et al., 1986; Sweigard et al., 1992; Talbot et al., 1993). Rice infection assays were performed using cultivar CO39 and Sariceltik, as previously described (Xu and Hamer, 1996; Xu et al., 1997). For the quantitative analysis of pathogenicity, 2-week-old seedlings of cultivar Sariceltik were sprayed with 12 mL of conidial suspension (2×10^4 conidia per mL in 0.25% gelatin solution), and lesions on second leaves were counted 7 days after inoculation. *Neurospora crassa* strains were kindly provided by M. Plamann (University of Missouri, Kansas City). The culture methods for *Neurospora* strains are described in Davis and de Serres (1970), and transformations were performed as described by Vollmer and Yanofsky (1986).

MAC1 and *SUM1* Cloning

A nested polymerase chain reaction (PCR) strategy was used to clone the *MAC1* and *SUM1* genes. For *MAC1*, two degenerate primer sets were synthesized to match the conserved catalytic domain of fungal adenylate cyclases. The catalytic domain of *MAC1* was initially amplified with primers FP1 (5′-TNGTNTTYACNGAYATHAA-3′) and RP4 (5′-DATYTGNCCNCCRTCNGC-3′) by using Guy11 genomic DNA as a template. Nested PCR was performed with primers FP2 (5′-ACNGARGGNGAYGCNTTYATG-3′) and RP3 (5′-AYNGGNCCRWARTARTCCAT-3′). The PCR mixture was heated to 94°C for 3 min and amplified for 30 cycles under the following conditions: 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, with a final extention cycle at 72°C for 10 min. A 250-bp nested PCR product was cloned into pGEM-T (Promega) and used to identify cosmid clone 1F8 from a genomic cosmid library. A 13.4-kb EcoRI fragment from 1F8 was subcloned into pUC18 and designated pKA1. The 8.7-kb DNA region containing the full-length *MAC1* gene was sequenced. For *SUM1* cloning, three degenerate primers were designed based on the conserved domain of fungal protein kinase A (PKA) regulatory subunits. The primers RF1 (5′-GAYTAYTTYTAYGTNGTNGA-3′) and RR4 (5′-GCNARYTCNCCRAARWA-3′) were used with Guy11 genomic DNA as a template. Nested PCR was performed with primers RF2 (5′-GARYTNGCNYTNATGTA-3′) and RR4. Thermal cycling conditions were identical to those used for *MAC1* except that 45°C was used as an annealing temperature. *SUM1* cDNA and genomic clones were isolated from cDNA and genomic libraries (Xu and Hamer, 1996) by using a 370-bp nested PCR product as a probe. Three cDNA clones were sequenced. A 3.5-kb SacI fragment from a genomic clone was subcloned into pBluescript KS+ (Stratagene, La Jolla, CA), and the DNA sequence of the 2.9-kb SacI-XbaI region containing the full-length *SUM1* gene was determined.

All DNA sequencing was done using Tn*1000* mutagenesis to create nested primer sites (Strathmann et al., 1991), and dideoxy sequencing was performed on an ALF-Express DNA sequencer (Pharmacia) by using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham). To scan for mutations in the *SUM1* gene, two PCR primers, RPKA1 (5′-ACCGAGTGAACCCGTTTATC-3′) and RPKA4 (5′-CAGCATTCTTCAGCCTCTG-3′), were designed to amplify the 1.3-kb coding region. Thermocycling reactions were performed with Pfu polymerase (Stratagene) for high fidelity of DNA synthesis. The 1.3-kb products were cloned into the SmaI site of pBluescript KS+, and the DNA sequences were determined from at least two independently amplified PCR clones.

Disruption of the *MAC1* Gene

The gene disruption vector pKA2 was created as follows. The plasmid pKA1 was digested with NcoI to release 2.9- and 2.7-kb NcoI fragments containing the 3′ two-thirds of the *MAC1* coding region. The *HPH* gene cassette was prepared from plasmid pCB1003 (Carroll et al., 1994) by digestion with HpaI. After blunt-end modification of the NcoI sites of pKA1, the *HPH* cassette was inserted and designated as pKA2. pKA2 was linearized with EcoRI and transformed into Guy11. By using this construct, we expected complete disruption of both leucine-rich repeats and the predicted catalytic domain of the *MAC1* gene.

Appressorium Formation Assays

Conidia were collected from 10-day-old cultures on oatmeal agar medium, filtered once through Miracloth (Calbiochem-Novabiochem, La Jolla, CA), and adjusted to 2×10^4 conidia per mL in water or 2% yeast extract. Ninety microliters of conidial suspension was placed on a hydrophobic plastic coverslip (Fisher, Pittsburgh, PA), whereas 50 μL was used on a 1 × 1-cm piece of the hydrophilic side of GelBond (FMC, Rockland, ME). Counts were performed after a 24-hr incubation in a moist chamber at room temperature. For cAMP addition, we prepared 10 × concentrated stock solutions (100 and 500 mM) in water and added them to conidial suspension at appropriate dilutions.

PKA Assays

PKA assays were performed using a nonradioactive PKA assay kit (Promega) according to the manufacturer's suggestions. PKA activity was visualized by agarose gel electrophoresis of a fluorescent PKA model substrate (Kemptide). To quantify PKA activity, fluorescent

Kemptide was released from the agarose gel according to the manufacturer's suggestions, and the amount of fluorescent Kemptide was determined in a spectrofluorometer. Concentrations of the Kemptide were calculated from a standard curve generated with a Kemptide phosphorylated with purified PKA catalytic subunit from bovine heart. Hyphal extracts were prepared from mycelia grown in complete liquid medium (Talbot et al., 1993) for 2 days with vigorous shaking. Germling extracts were prepared from conidia germinated for 8 hr in sterile distilled water. The extraction procedure was described previously (Yang and Dickman, 1997). Protein concentrations were adjusted to 1.5 mg/mL by using the protein assay kit (Bio-Rad), with BSA as a standard.

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