cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*

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The fungal pathogen *Ustilago maydis* exhibits a dimorphic switch from budding to filamentous growth in response to mating interactions and environmental conditions. We have found that disruption of the *uac1* gene, encoding adenylate cyclase, results in a constitutively filamentous phenotype. Budding is restored to the *uac1* mutant upon growth in the presence of cAMP or by extragenic suppression because of a mutation in the *ubc1* gene. The *ubc1* gene encodes a type II regulatory subunit of cAMP-dependent protein kinase (PKA); defects in this gene attenuate the filamentous growth that normally occurs in response to mating and exposure to air. Growth of wild-type cells in cAMP and mutation of the *ubc1* gene also cause defects in the separation of mother and daughter cells (cytokinesis) and alter bud site selection. These results indicate a key role for cAMP and PKA in morphogenesis in *U. maydis*; this role may be common among dimorphic fungal pathogens.

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Many fungi, including saprophytes and pathogens of animals and plants, alternate between filamentous and budding growth morphologies (dimorphism) in response to environmental conditions. For example, *Histoplasma capsulatum*, the causative agent of human histoplasmosis, has a filamentous form in soil and grows as a budding yeast in host tissue (Maresca and Kobayashi 1989). *Candida albicans*, which causes candidiasis, exists as hyphal and yeast-like cells in lesions; in vitro, the transition between the two morphologies is influenced by culture conditions, for example, temperature (Cutler 1991). *Ustilago maydis*, the basidiomycete smut pathogen of maize (*Zea mays*), exhibits dimorphism in which mating between haploid budding cells establishes a pathogenic, filamentous cell type (Christensen 1963; Holliday 1974). In addition, cells of *U. maydis* will switch between budding and filamentous forms in response to environmental conditions such as nutrient availability (Kernkamp 1939) and exposure to air (S. Gold, this study). The study described here takes advantage of the dimorphic transition in *U. maydis* to identify genes involved in the regulation of budding versus filamentous growth.

The filamentous cell type in *U. maydis* is established primarily through mating interactions governed by two genetic loci called *a* and *b*. Budding haploid cells are compatible to fuse and to form the filamentous dikaryon

only if they carry genes with different specificities at both the *a* and *b* loci. Cell fusion is controlled by the *a* mating-type locus, which has two alternative forms, *a1* and *a2*. The *a1* and *a2* sequences have recently been shown to be idiomorphs (Froeliger and Leong 1991) and to encode pheromones and pheromone receptors (Bölker et al. 1992; Spellig et al. 1994). The *b* locus appears to control events after cell fusion necessary for establishment of the filamentous, pathogenic cell type (termed the infectious dikaryon). The *b* locus encodes two products, *bE* and *bW*; the interaction of the *bE* product from one form of the *b* locus with the *bW* product from another is believed to establish a novel regulatory protein that triggers formation of the infectious dikaryon (Gillissen et al. 1992). The alignment of the predicted amino acid sequences of several alleles of the *bE* or *bW* genes revealed that each contains a variable amino-terminal region, a central homeo domain-like motif, and a conserved carboxy-terminal region (Kronstad and Leong 1990; Schulz et al. 1990; Gillissen et al. 1992). Recognition mediated by the products of the *b* genes is of particular interest because there are at least 25 different specificities at the *b* locus (Puhalla 1970; Silva 1972). Specificity is mediated by the variable amino-terminal region of *bE* as demonstrated by the construction of chimeric alleles of the *bE1* and *bE2* genes (Yee and Kronstad 1993). These experiments identified an ~40-amino-acid region in the amino-proximal region that is believed to determine the specificity of interaction between the *bE* and *bW* gene products. That is, the specificity region may control whether the heterodimer of *bE* and *bW* poly-

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peptides is active in maintaining filamentous growth (Gillissen et al. 1992; Yee and Kronstad 1993).

The filamentous cell type established by the action of the *a*- and *b*-encoded functions is the only infectious stage of the life cycle. This cell type causes a localized infection on maize plants typified by the formation of large tumors or galls on any above-ground part of the plant (Christensen 1963). The fungus proliferates within gall tissue, and as galls mature, the nuclei of the dikaryon fuse and diploid teliospores are formed. Upon dispersal from the host plant, the diploid teliospores may germinate, form promycelia, and undergo meiosis to yield haploid budding cells.

Recently, we described mutants in *U. maydis* that display a constitutively filamentous phenotype (Barrett et al. 1993). Colonies of these mutants become covered with white aerial mycelium in contrast with the flat gray morphology of colonies formed by wild-type parental cells. Interestingly, one of the mutations (*rem1-1*) was found to block pathogenicity, even though the cells displayed a filamentous morphology similar to that of the infectious dikaryon. Complementation of the *rem1-1* mutation with a cosmid library and subsequent disruption experiments with the cloned gene also yielded mutants with filamentous growth morphologies. Sequence analysis of the complementing region, reported in this paper, reveals that the cosmid carries a homolog of adenylate cyclase. Therefore, we have replaced the *rem1* designation for the gene with *uac1* for *Ustilago* adenylate cyclase.

In addition to the characterization of the *uac1* gene that encodes adenylate cyclase, we describe the influence of exogenous cAMP on the morphogenesis of wild-type cells and on a filamentous mutant carrying a disruption in the *uac1* gene. We find that cAMP restores budding growth to the filamentous mutant and alters bud site selection and mother-daughter separation (cytokinesis) in wild-type cells. The latter phenotype is also found in mutants that contain an extragenic suppressor of the defect in adenylate cyclase. The defective gene in one of these mutants was found to have sequence similarity to the regulatory subunit of cAMP-dependent protein kinase (PKA). The mutation in this gene, which has been designated *ubc1* (*Ustilago* bypass of cyclase), also attenuates filamentous growth resulting from mating interactions and from exposure to air. Overall, our results suggest a general role for cAMP and PKA in the regulation of dimorphism for *U. maydis*.

Results

Filamentous growth results from a defect in adenylate cyclase

Wild-type haploid cells of *U. maydis* divide by the formation of an elongated bud at an oblique angle at or near one end of the mother cell. In contrast, and as described by Barrett et al. (1993), haploid strains carrying a disruption of the *uac1* (*rem1*) gene grow exclusively by hyphal extension. Figure 1 shows a comparison of the morphol-

ogies displayed by wild-type budding cells (A,B), cells carrying a disruption of the *uac1* gene (C,D), and cells of the disruption mutant carrying a complementing cosmid, pFuz60 (E,F). It is clear that a mutation in the *uac1* gene dramatically alters the morphology of *U. maydis* and results in a constitutively filamentous phenotype.

The nucleotide sequence of ~10 kb of genomic DNA from the complementing region of cosmid pFuz60 (identified by subcloning) was determined and found to contain one long open reading frame of 7479 bp. The amino acid sequence of the predicted polypeptide (2493 amino acids) showed extensive similarity to adenylate cyclases from other fungal species. The size of the predicted polypeptide is similar to that of the adenylate cyclases from *Neurospora crassa* (2300 amino acids; Kore-eda et al. 1991) and *Saccharomyces cerevisiae* (2026 amino acids; Kataoka et al. 1985). In addition, the predicted polypeptide contains leucine-rich tandem repeats in the central portion of the sequence (between codons 1219 and 1517); these leucine-rich motifs are similar in sequence and organization to the leucine-rich repeats identified in the adenylate cyclases from *S. cerevisiae* and *N. crassa* (Kataoka et al. 1985; Kore-eda et al. 1991). An alignment of a portion of the predicted amino acid sequence of *uac1* with the sequences encompassing the catalytic domains of adenylate cyclases from *N. crassa* (Kore-eda et al. 1991) and *S. cerevisiae* (Kataoka et al. 1985) is shown in Figure 2. The disruption mutation in the *uac1* gene was constructed by insertion of a selectable marker for phleomycin resistance at codon 899 (Fig. 2); insertion at this position would block expression of the carboxy-terminal two-thirds of the polypeptide including the predicted catalytic domain. Overall, the finding that *uac1* encodes a homolog of adenylate cyclase and that a mutation in *uac1* results in filamentous growth provides strong evidence for a role for cAMP in the control of morphogenesis in *U. maydis*.

cAMP suppresses the defect in adenylate cyclase and promotes a multiple-budding phenotype

The discovery of sequence homology of the *U. maydis* *uac1* gene to adenylate cyclase prompted an analysis of the response of the *uac1* disruption strain to exogenous cAMP. As shown in Figure 1 (G,H), addition of 25 mM cAMP to the culture medium caused the cells of the *uac1* mutant to display a budding phenotype. Addition of 25 mM AMP (Fig. 1G, inset) or cGMP (data not shown) failed to influence the filamentous phenotype of the mutant cells. We conclude that cAMP influences budding growth in *U. maydis*; the phenotype of the mutant in the presence of cAMP suggests that high levels of cAMP promote budding growth and low levels condition filamentous growth.

An interesting phenotype is observed when wild-type cells are exposed to the same level of cAMP employed to restore budding growth to the *uac1* disruption strain. That is, wild-type cells grown in 25 mM cAMP show a distinctive phenotype in which mother and daughter

Figure 1. Influence of cAMP and mutations in *uac1* and *ubc1* on the morphology of *U. maydis*. (A,C,E,G,I,K,M) Photographs of the colony morphology of *U. maydis* strains grown on PDA (bar, 0.5 mm). (B,D,F,H,J,L,N) Scanning electron micrographs of cells grown in PDB (bar, 10 μ m). The following strains are shown: (A,B) 521 (wild type); (C,D) C002P, *uac1::ble*; (E,F) C002P carrying the pFuz60 cosmid; (G,H) C002P grown on medium containing 25 mM cAMP (inset in G is a control in which 25 mM AMP is present in the medium); (I,J) 521 grown with 25 mM cAMP; (K,L) C002a (*uac1::ble ubc1-1*; inset in L shows DAPI-stained nuclei); (M,N) C002a (*uac1::ble ubc1-1*) carrying the pUBC1 cosmid. Note that all of the mutants are derivatives of strain 521.

cells remain attached in clusters of more than two cells and lateral as well as apical sites are used for bud formation (Fig. 1I,J). This result is striking because it is rare to observe clusters of more than two cells or the use of lateral bud sites in cultures of wild-type cells. The phenotype of cells exposed to cAMP is referred to as multiple budding (Fig. 1J) and apparently results from failure of cytokinesis of mother and daughter cells prior to the formation of subsequent buds. It appears that daughter cells can begin to bud prior to separation from mother cells (Fig. 1, cf. B with J). It should be noted that the multiple-budding phenotype can also be observed with the *uac1* disruption mutant grown in the presence of 25 mM cAMP (Fig. 1H). Lower concentrations (e.g., 1 mM cAMP) generally cause cells of the disruption mutant to partially return to a budding phenotype, without the appearance of cells with multiple buds (S. Gold, unpubl.).

Filamentous growth is suppressed by a mutation in the ubc1 gene

Suppressor mutations that restored budding growth to the *uac1* disruption mutant were readily obtained. These mutations cause cells to form flat yeast-like colonies

(Fig. 1K) that are easily distinguished from the mycelial colonies of the parental *uac1::ble* strain (Fig. 1C). A collection of 46 suppressor mutants with various cellular morphologies was obtained and the mutations in these strains were designated *ubc* for *Ustilago* bypass of *cy*-clase. The double mutant shown in Figure 1 (K,L) carries the *ubc1-1* allele and displays a multiple-budding phenotype similar to that observed upon treatment of cells of a wild-type strain, or the *uac1* disruption strain, with cAMP. Staining with DAPI revealed that in general, each cell of the *ubc1-1* strain contains a single nucleus (Fig. 1L, inset), indicating that the newly formed cells are actual buds.

The *ubc1-1* mutation was complemented with a cosmid library by screening for transformants that regained the filamentous phenotype of the *uac1* disruption strain (Fig. 1M,N). Sequence analysis of a 3-kb genomic region that complemented the *ubc1-1* defect (identified by subcloning) revealed a 1566-bp open reading frame (Fig. 3), and RNA blot hybridization analysis using a probe from this region identified an $\sim$ 2.1-kb transcript (data not shown). The amino acid sequence of the predicted *ubc1* polypeptide (522 amino acids) shows significant similarity to the regulatory subunit of PKA. In addition, the presence of a serine residue at position 170

Figure 2. Map of the *uac1* open reading frame and homology among catalytic domains of three fungal adenylate cyclases. (A) The open reading frame is shown for the *uac1* gene along with the position of insertion of the phleomycin (*ble*) resistance gene, the leucine-rich repeats, the catalytic domain region employed in the sequence alignment, and the partial cDNA for the *uac1* gene. (B) Sequence alignment of the predicted catalytic domain (codons 1985–2290) encoded by the *U. maydis* *uac1* gene (Um) with the corresponding domains from the *S. cerevisiae* (Sc) and *N. crassa* (Nc) genes. The *uac1* region shows 47% sequence identity with the corresponding region of the *S. cerevisiae* adenylate cyclase sequence and 52% identity with the *N. crassa* sequence. The overall identity between the entire predicted polypeptides for *S. cerevisiae* and *U. maydis* is 33%; the *U. maydis* and *N. crassa* polypeptides are 34% identical. The absence of introns in the region used for alignments (encoding amino acids 1646–2493) was confirmed by sequence analysis of the cDNA and comparisons of restriction fragment sizes between cDNA and genomic versions of the gene. Sequence inspection of the remaining portion of the coding region revealed only one region that could potentially contain an intron. Splicing of this putative intron (between codons 1448 and 1503) would not change the reading frame of the polypeptide but would remove 54 amino acids from the coding region. Sequence analysis was performed with the GAP program from the Genetics Computer Group, University of Wisconsin (Devereux et al. 1984).

indicated that the *ubc1* gene encodes a type II regulatory subunit (Taylor et al. 1990). Overall, the finding that the *ubc1* gene encodes a regulatory subunit of PKA provides strong additional evidence that the cAMP pathway plays an important role in the regulation of dimorphic growth in *U. maydis*. We infer from these results that the catalytic subunit of PKA, when dissociated from the regulatory subunit in the presence of cAMP, is required for budding growth, establishment of a polar bud position, and completion of cytokinesis.

Cytokinesis and bud site selection require the *ubc1* gene

The multiple-budding phenotype resulting from mutation in the *ubc1* gene and from growth on medium containing cAMP has been examined in more detail in terms of the appearance of cells with multiple buds at different stages of growth, the number of cells per cluster, and the frequency of lateral buds (Fig. 4; Table 1). As shown in Figure 4A, wild-type cells (strain 521) generally showed single apical buds. This budding phenotype was evident throughout growth (Table 1). In addition, wild-type strains generally appeared as single cells or pairs of cells (mother and daughter). In contrast, wild-type cells grown in the presence of 25 mM cAMP displayed the multiple-budding phenotype in which clusters of cells were observed, particularly at high cell densities, in actively growing cultures (Fig. 4B; Table 1). These cells also showed a high percentage of lateral buds (Table 1). In general, the average number of cells per group was lower in the cultures at an early time after inoculation (8 hr) and in cells that had been in culture for extended periods (e.g., 48 hr). The average number of cells per group was highest in 22-hr cultures; this time corresponds to late exponential growth.

U. maydis strains carrying mutations in both the *uac1* and *ubc1* genes (C002a, Fig. 4C), or in the *ubc1* gene alone (111, Fig. 4D), had phenotypes similar to wild-type cells grown in cAMP (Figure 4B) at each of the sampling times (Table 1). That is, these cells displayed the multiple-budding phenotype with frequent misplacement of buds to lateral positions. The cellular morphology of strain C002a, which carries the disruption of *uac1* gene as well as the *ubc1-1* mutation, had a slightly different phenotype in that many of the cells were somewhat elongated (Figs. 4C and 1K,L). These cells appeared to be partially filamentous compared with cells that only carry the *ubc1-1* mutation. This phenotype may result from the opposing influences of a defect in the *uac1* gene, which would result in reduced cAMP levels and low PKA activity, and a defect in the *ubc1* gene, which would alleviate normal regulation of PKA and cause elevated activity.

The disruption mutation (*ubc1::hyg*; strain 521d) was constructed in *ubc1* (Fig. 3) to confirm that a defect in the regulatory subunit of PKA would result in the phenotype observed with a *ubc1-1* mutant. The insertion mutation in strain 521d (*ubc1::hyg*) is at codon 360, a site that is between the two cAMP-binding domains [Fig.

Figure 3. Map of the *ubc1* open reading frame and sequence similarity among three fungal regulatory subunits of PKA. (A) The open reading frame of the *ubc1* gene and the positions of key features of type II regulatory subunit genes such as the autoinhibition site (phosphorylation site) and the cAMP-binding sites (Taylor et al. 1990). The presence of the serine residue at codon 170, which is the target of phosphorylation by the catalytic subunit, indicates that *ubc1* encodes a type II regulatory subunit. The 6-amino-acid sequence shown is identical to that found in the yeast *BCY1* gene (Toda et al. 1987). The sequences at the cAMP-binding sites are also shown (aligned at the conserved glutamic acid residues at codons 294 and 416). The position of insertion of the hygromycin resistance gene is also shown (codon 360); this insertion mutation is between the the cAMP-binding domains and was used to construct the gene disruption in strain 521d (*ubc1::hyg*). (B) Sequence alignment of the predicted product of the *U. maydis ubc1* gene (Um) with the polypeptides from *Blastocladiella emersonii* (Be; Marques and Gomes 1992) and *S. cerevisiae* (Sc; Toda et al. 1987). The polypeptide predicted for *ubc1* is 38% identical to that of the *BCY1* gene of *S. cerevisiae* and 51% identical to the regulatory subunit of *B. emersonii*. The region between codons 48 and 155 of the *ubc1* polypeptide shows extensive gaps when aligned with the other sequences; an intron is found in this region in many PKA regulatory subunit genes. The absence of an intron in this region (codons 12–246) for the *ubc1* gene was confirmed by RT-PCR. That is, identical size PCR fragments from this region were obtained from genomic and cDNA templates (data not shown).

3). Given the position of the insertion, it is possible that the *ubc1::hyg* allele encodes a regulatory subunit of PKA that retains partial activity, assuming that a polypeptide is produced from the disrupted gene and that the remaining cAMP-binding site is functional. However, strain 521d (*ubc1::hyg*) displayed a similar pattern of multiple budding and lateral bud site selection (Table 1; Fig. 4E) when compared with strain 111 (*ubc1-1*), a phleomycin-sensitive, haploid segregant from a cross between strain C002a (*a1 b1 uac1::ble ubc1-1*) and the wild-type strain

518 (*a2 b2*) (Fig. 4D). It is possible that the *ubc1-1* allele might also encode a polypeptide truncated at a similar position to the one encoded by the *ubc1::hyg* allele. Overall, the results with the disruption mutant confirm that a defect in the gene encoding the regulatory subunit of PKA results in cells with a multiple-budding phenotype. In combination with the results for the *uac1* gene, these data indicate a role for cAMP and regulated PKA activity in cytokinesis and in the positioning of the bud site in *U. maydis*.

Figure 4. Multiple-budding phenotype of the PKA regulatory subunit mutants. Phase-contrast micrographs are shown for cells grown in PDB for 22 hr (Table 1). Individual panels show the following strains: (A) 521; (B) 521 with 25 mM cAMP; (C) C002a (*uac1::ble ubc1-1*); (D) 111 *ubc1-1*; (E) 521d *ubc1::hyg*. Bar, 20 μ m.

Filamentous growth in response to mating and air requires *ubc1*

The ability of *U. maydis* to form the infectious, filamentous cell type depends on mating interactions between compatible haploid strains of opposite mating type. Compatible interactions result in the formation of mycelial colonies on culture medium containing activated charcoal (Fig. 5A). This mating assay was employed to determine whether strains carrying the *ubc1-1* allele or both the *ubc1-1* allele and the *uac1::ble* allele were capable of forming mycelial colonies when paired with wild-type strains or with similar mutants of opposite mating type. As shown in Figure 5A, a mixture of compatible mutant strains (each of genotype *uac1::ble ubc1-1*) resulted in colonies with a greatly attenuated mycelial phenotype. The same result was obtained with mixtures of compatible strains that each carry only the *ubc1-1* allele. In contrast, each strain, whether a double mutant

(*uac1::ble ubc1-1*) or only carrying the *ubc1-1* allele, was capable of forming strongly mycelial colonies when paired with wild-type strains of opposite mating type (Fig. 5A).

These results indicate that the formation of aerial hyphae on mating colonies, which is a measure of formation of both mating hyphae (conjugation tubes) and infection hyphae (the infectious dikaryon; Snetselaar and Mims 1992), is at least partially dependent on the *ubc1* gene and, by inference, on correctly regulated PKA activity. We have also found that inoculation of maize seedlings with strains that carry the *ubc1-1* mutation results in greatly attenuated disease symptoms, although some filamentous cells are observed in plant tissue (S. Gold, D. Mills, and J. Kronstad, unpubl.). These observations indicate that *ubc1* mutants are defective in an as yet unidentified step in the formation or maintenance of the infectious dikaryon. The implication is that there may be an interaction between the cAMP pathway and

Table 1. Quantitation of multiple and lateral budding

Strain	Time (hr)	Cell density ^a	Cells counted ^b	Total units	Average cells/unit	Lateral buds	Maximum cells/unit
521	8	7.5×10^6	150	93	1.6	1	5
	22	1.9×10^8	153	129	1.2	0	2
	48	2.6×10^8	103	98	1.1	0	2
521 + cAMP ^c	8	8.7×10^6	174	50	3.5	25	19
	22	2.1×10^8	171	24	7.1	62	13
	48	3.4×10^8	205	35	5.9	62	17
C002a	8	8.7×10^5	121	43	2.8	23	9
	22	1.3×10^8	102	39	2.6	16	7
	48	2.5×10^8	147	110	1.3	5	5
111	8	2.0×10^6	195	47	4.1	49	10
	22	1.4×10^8	230	34	6.8	63	47
	48	2.2×10^8	219	93	2.4	69	12
521d	8	7.7×10^5	131	28	4.7	36	19
	22	9.4×10^7	151	29	5.2	36	14
	48	1.1×10^8	112	20	5.6	40	26

^aThe initial cell densities (cells/ml) for the cultures were as follows: 521, 3.2×10^6 ; 521 + cAMP, 3.3×10^6 ; C002a, 2.8×10^6 ; 111, 2.9×10^6 ; 521d, 1.4×10^6 .

^bCells in a random sample were counted, and the number of clusters of cells (units) was determined. For each unit, lateral buds were counted and the number of cells in the largest cluster was determined.

^cStrain 521 was grown in 1 ml of PDB containing 25 mM cAMP; all other cultures were grown in 5 ml of PDB. The results of one experiment are presented; similar results were obtained in two additional experiments.

Figure 5. Response of *ubc1* mutants to mating and air. (A) Colony morphology resulting from mating reactions on medium containing charcoal. The strains employed are listed at left; their genotypes are given in Table 2. The reaction between wild-type strains 518 (*a2 b2*) and 521 (*a1 b1*) in the center shows the white aerial mycelium indicative of the filamentous growth resulting from a positive mating. Mating interactions between compatible wild-type and mutant strains yielded reactions of wild-type intensity. (B) Colony morphology of wild-type and mutant strains grown on medium in petri plates sealed with parafilm (left) and in unsealed plates (right). The mating reaction between wild-type strains 518 and 521 gives a mycelial colony phenotype; strain C002P shows this phenotype by itself (Fig. 1C). In contrast with wild-type strains, strains 111 (*ubc1-1*) and C002a (*uac1::ble ubc1-1*) fail to become filamentous in response to free exchange of air. (C) Photomicrographs of cells of strains 521 (left) and 521d (*ubc1::hyg*; right) grown for 48 hr on water agar. The magnification is the same for both photographs. Bar, 10 μ m.

the mating pathway leading to formation of the infectious dikaryon.

Haploid cells of *U. maydis* grown on solid culture medium with free exchange of air eventually form colonies that are covered with aerial mycelium (Fig. 5B). This response to air (presumably O_2 or CO_2 levels) does not occur when colonies form on culture medium in petri plates sealed with parafilm. Unlike wild-type cells, strains carrying the *ubc1-1* or the *ubc1::hyg* alleles were unable to respond to air by forming aerial hyphae on colonies. Other dimorphic fungi, for example, *Mucor rouxii*, also appear to alter their morphology in response to air (Orlowski 1991). Microscopic observations of *U. maydis* wild-type and mutant cells (Fig. 5C) on water

agar in unsealed plates also confirmed the inability of *ubc1* mutants to form filaments. Under these conditions, wild-type cells formed small colonies and grew with a predominantly filamentous morphology. The *ubc1* mutants also formed small colonies, but the cells retained the yeast-like morphology of wild-type strains. Interestingly, the *ubc1* mutants often formed chains of cells (Fig. 5C), in addition to the clusters of cells resulting from multiple budding. The persistent attachment of the cells in the chains is similar to the phenotype displayed by *S. cerevisiae* during pseudohyphal growth (Gimeno et al. 1992).

Discussion

Defects in adenylate cyclase affect growth and morphogenesis

We have shown that a mutation in the *uac1* gene encoding adenylate cyclase results in filamentous growth in *U. maydis* and that cAMP restores budding growth to a *uac1* mutant. Defects in adenylate cyclase result in a variety of phenotypes in other fungi. For example, mutations in the *CYR1* gene of *S. cerevisiae* cause cells to arrest in the G_1 phase of the cell cycle and exogenous cAMP restores growth (Casperson et al. 1985; Kataoka et al. 1985; Ishikawa et al. 1988). Disruption of the gene encoding adenylate cyclase in *Schizosaccharomyces pombe* results in a reduced growth rate, compared with wild-type cells, and a tendency to enter the sexual reproduction pathway in rich medium (Maeda et al. 1990). In *N. crassa*, mutation in the *cr-1* gene results in reduced adenylate cyclase activity and lower levels of cAMP (Terenzi et al. 1976; Pall et al. 1981). Mutants carrying the defective *cr-1* allele form small compact colonies that lack extensive hyphal development and display short aerial hyphae with tight clusters of dense conidia. Growth in the presence of cAMP restores a colony morphology more like that of wild-type cells. In general, it appears that adenylate cyclase and cAMP play roles in both growth and morphogenesis in fungi. Our results provide genetic evidence that the cAMP pathway plays a key role in the decision between budding and filamentous growth morphology in *U. maydis*. This finding may be generally true for smut pathogens because McCluskey et al. (1994) have reported a mutation (*fil1*) that causes filamentous growth in *U. hordei*. Budding growth is restored to strains carrying the *fil1* mutation by addition of cAMP to culture medium. (D. Mills, pers. comm.).

cAMP is implicated in dimorphism

Biochemical evidence for a role for cAMP in morphogenesis has been described for a number of different pathogenic and saprophytic fungi, including many dimorphic species (Maresca et al. 1977; Medoff et al. 1981, 1987; Paris and Garrison 1983; Brunton and Gadd 1989). For example, an increase in intracellular cAMP has been reported during germ-tube formation and subsequent hy-

phal growth in the dimorphic pathogens *H. capsulatum* (Maresca et al. 1977; Medoff et al. 1981), *C. albicans* (Niimi et al. 1980; Sabie and Gadd 1992), and *Blastomyces dermatitidis* (Paris and Garrison 1983). However, contradictory reports have appeared for *C. albicans* suggesting that factors (e.g., carbon source) other than cAMP control germ-tube formation (Cho et al. 1992) and even that an increase in cAMP levels promotes budding (Paveto et al. 1991). In *M. rouxii*, cAMP levels reportedly drop during the emergence of hyphal germ tubes and the level of cAMP remains low during hyphal growth compared with budding growth (for review, see Orlowski 1991). The situation for *U. maydis* appears to be similar to that described for *M. rouxii* in that low cAMP levels (e.g., as a result of a defect in adenylate cyclase) promote hyphal extension at the expense of budding growth. It should be noted that cAMP plays other roles in fungal morphogenesis in addition to influencing dimorphism. For example, a role for cAMP has been described recently for the regulation of infection structure formation in the rice blast fungus *Magnaporthe grisea* (Lee and Dean 1993).

Overall, biochemical studies have provided suggestive evidence for a role for cAMP in dimorphism, although a clear description of the mechanism of cAMP control of morphogenesis has not emerged. In addition, the ambiguous or contradictory results for *C. albicans*, which may be the result of different cultural and environmental conditions employed by various investigators, cause uncertainty about whether changes in cAMP levels are a secondary effect or a cause of the observed morphological changes. Our genetic evidence strongly supports a role for cAMP and, by implication, cAMP-dependent protein kinase, in determining growth morphology in *U. maydis*. It would be interesting to know whether defects in adenylate cyclase influence morphogenesis in other dimorphic fungi.

A role for PKA in morphogenesis

We have found that the filamentous phenotype resulting from a defect in adenylate cyclase could be suppressed by a mutation in the *ubc1* gene encoding the regulatory subunit of cAMP-dependent protein kinase. This result was not surprising considering the well-established role of the regulatory subunit as the major receptor for cAMP in eukaryotes (Taylor et al. 1990). In addition, mutations in the regulatory subunit gene (*BCY1*) of *S. cerevisiae* suppress the growth defect of an adenylate cyclase mutant. Yeast strains with defects in *BCY1* show a variety of additional phenotypes (Cannon and Tatchell 1987; Toda et al. 1987; Cannon et al. 1990). *U. maydis* strains carrying *ubc1-1* or *ubc1::hyg* mutations also have other phenotypes besides suppression of the filamentous growth that results from mutation in *uac1*; these include inhibition of cytokinesis, altered bud site selection, and attenuation of filamentous growth in response to air and mating interactions. These results suggest that phosphorylation events mediated by the catalytic subunit of PKA are involved in these processes.

Given the finding that a mutation in the gene for adenylate cyclase results in filamentous growth in *U. maydis*, we would predict that a defect in the gene encoding the catalytic subunit of PKA would result in a similar phenotype (i.e., mimic conditions of low cAMP). Although we have not yet tested this prediction for *U. maydis*, we note that a mutation in the *cot-1* gene of *N. crassa*, which encodes a polypeptide with sequence similarity to the catalytic subunit of PKA, results in altered cell morphology (Yarden et al. 1992). In this case, *cot-1* mutants have a defect in hyphal extension and show extensive branching.

In fungi, morphogenesis is determined by the deposition of new, vesicle-transported, cell wall material. A mathematical model has been proposed that provides for a hypothetical point for vesicle release, the vesicle supply center (vsc; Bartnicki-Garcia et al. 1989). Changes in the position and/or movement of the vsc yield entirely different shapes. This vsc model can account for most of the morphologies exhibited by fungi and includes shapes typical of *U. maydis* hyphal and yeast cells. An extension of the model is that factors influencing the position or movement of the vsc would have profound effects on cell shape; it is possible that phosphorylation by PKA may be such a factor in *U. maydis*.

Protein phosphorylation and cytokinesis

Mutants defective in *ubc1*, and wild-type cells grown in cAMP, display the interesting phenotypes of multiple budding and frequent lateral budding, presumably as a result of activation of the catalytic subunit of PKA and phosphorylation of as yet unidentified proteins. The multiple-budding phenotype in *ubc1* mutants appears to result from a defect in the separation (cytokinesis) of mother and daughter cells. Daughter cells remain attached and continue to initiate buds at apical or lateral positions. Multiple-budding phenotypes have been described for other fungi. For example, *C. albicans* reportedly displays a multiple-budding phenotype under conditions otherwise conducive to germ-tube formation, that is, upon stimulation of adenylate cyclase activity by treatment with glucagon (Paveto et al. 1990). A multiple-budding phenotype is also found for mutants of *S. cerevisiae* defective in the *TPD1*, *TPD3*, *CDC55*, or *YCK2* genes (Healy et al. 1991; van Zyl et al. 1992; Robinson et al. 1993, 1994). These genes encode polypeptides with high homology to protein phosphorylase 2C, the A and B regulatory subunits of mammalian protein phosphatase 2A, and casein kinase I, respectively. The results in *S. cerevisiae* suggest that phosphorylation and dephosphorylation events control the function of a substrate involved in cytokinesis. Our results indicate that PKA may at least partially fulfill the phosphorylation role in *U. maydis*.

Persistent attachment between mother and daughter cells also occurs during pseudohyphal growth in response to nitrogen starvation in *S. cerevisiae*. The constitutive activation of the RAS–adenylate cyclase path-

way enhances pseudohyphal growth, thus implicating cAMP in the process (Gimeno et al. 1992). Pseudohyphal growth requires the bipolar budding pattern exhibited by a/α diploid cells; this pattern is established from the default random pattern by the actions of the *BUD1*, *BUD2*, and *BUD5* genes (for review, see Drubin 1991; Nelson 1992). Other genes, for example, *ELM1*, *ELM2*, and *ELM3* (elongated morphology), also play a role in morphogenesis in *S. cerevisiae* (Blacketer et al. 1993). Mutations in these genes cause pseudohyphal growth; the *ELM1* gene encodes a novel protein kinase that appears to interact with the product of the *CDC55* gene (Blacketer et al. 1993). Genes encoding type 1-related protein phosphatases have also been found to play a role in pseudohyphal growth in *S. cerevisiae* (Lee et al. 1993) and a casein kinase II homolog recently has been found to play a role in polarized growth in *S. pombe* (Snell and Nurse 1994). As with cytokinesis in *S. cerevisiae*, these studies point to protein phosphorylation as a regulatory function for cell elongation and pseudohyphal growth.

Budding, cAMP, and mating

The finding that a defect in adenylate cyclase results in filamentous growth in *U. maydis* indicates that activation of PKA promotes budding growth. In addition, PKA appears to play a role in determining the position of the bud (lateral vs. apical). Lateral budding in *ubc1-1* mutants and in wild-type cells exposed to cAMP must result from a defect in the position of the bud site at initiation. Mating interactions between haploid cells of *U. maydis*, governed by the *a* and *b* loci, also influence morphogenesis by causing the cessation of budding growth and the establishment of the filamentous, infectious dikaryon. A connection between PKA and the mating pathway is indicated by the fact that mating reactions between strains carrying mutations in the *ubc1* gene fail to form the vigorous filaments indicative of the infectious dikaryon. In addition, a connection is suggested by the fact that compatible strains carrying the *ubc1-1* mutation are attenuated for pathogenicity, a function that requires mating and the establishment of the filamentous dikaryon (S. Gold, unpubl.). Fusion events are not reduced in wild-type strains in the presence of 25 mM cAMP, a condition that simulates the phenotype of the *ubc1-1* mutant (C. Laity, unpubl.). Therefore, the *ubc1-1* defect probably interferes with the establishment or maintenance of filamentous growth because the presence of defective regulatory subunits leads to constitutive activation of PKA, a situation that promotes budding growth. That is, in matings between *ubc1-1* mutants, there may be a competition between the regulatory influence of heterozygosity at the *b* mating-type locus and the activity of the catalytic subunit of PKA. It should be kept in mind, however, that PKA in *U. maydis* undoubtedly has other roles in addition to the regulation of cytokinesis and bud position.

The connection between morphogenesis, cAMP, and mating in *U. maydis* is interesting when considered in

the context of bud site selection in *S. cerevisiae*. The analysis of budding in *S. cerevisiae* has identified a number of genes involved in budding pattern (e.g., *BUD3* and *BUD4*), bud site assembly (e.g., *BEM1*), and cytoskeleton assembly (e.g., *CDC10*) (Chant and Herskowitz 1991; Chenevert et al. 1992; for review, see Drubin 1991). Presumably, similar functions are utilized by *U. maydis* to establish budding growth. To switch to filamentous growth, these functions might be directly or indirectly repressed by *b*-locus-encoded regulatory proteins. In *S. cerevisiae*, mating or cell type regulation of budding pattern may exist; that is, it has been proposed that the *BUD3* and *BUD4* genes are negatively regulated by homeo domain-containing polypeptides encoded by the *MATa/MAT α* loci (Chant and Herskowitz 1991). The *BUD3* and *BUD4* genes are required for establishing the axial budding pattern of haploid cells; these genes may be inactive in diploid cells that encode the $a1/\alpha2$ repressor and that display the polar budding pattern required for pseudohyphal growth. The *b* mating-type locus of *U. maydis* also encodes polypeptides with homeo domain-like motifs; dimers between polypeptides encoded by different *b* alleles are believed to regulate the switch from budding to filamentous growth (Gillissen et al. 1992). Therefore, when cells have mated, it is possible that *U. maydis* employs *b*-encoded regulatory factors to turn off the expression of genes whose products are required for budding. This scenario would be similar to the proposed $a1/\alpha2$ regulation of the *BUD3* and *BUD4* genes, except the *b* proteins in *U. maydis* would repress budding and promote filamentous growth. Our data suggest that PKA phosphorylates factors required for budding; the absence of these factors, for example, in cells heterozygous for *b*, presumably results in filamentous growth by default. The dual regulation of filamentous growth in *U. maydis* by mating and environmental signals is summarized in Figure 6.

Summary

In summary, we have isolated and characterized the genes encoding adenylate cyclase and the regulatory subunit of PKA from *U. maydis*. The analysis of mutants with defects in these genes demonstrates that the cAMP pathway plays a key role in the decision to grow by budding versus hyphal extension (dimorphism). In addition, the phenotypes of mutants defective in the regulatory subunit of PKA indicate that phosphorylation events mediated by PKA control the ability of cells to bud, the separation of mother and daughter cells, and the position of bud formation. Mutations in the *ubc1* gene encoding the regulatory subunit of PKA also attenuate the filamentous growth and the pathogenicity that normally result from mating interactions. Overall, these findings provide important insights into the regulation of dimorphic growth in a fungal pathogen. The information obtained for *U. maydis* promises to have broad applicability, given the probable role of cAMP in dimorphic growth in other pathogenic fungi.

Figure 6. Diagram of environmental and mating control of dimorphism in *U. maydis*. Two pathways are shown to control the decision of *U. maydis* to grow with a budding or a filamentous morphology. That is, two different sets of factors (mating and environmental) trigger filamentous growth; budding growth occurs in nonmated cultures under conditions that result in high cAMP levels. Note that the *a* locus of *U. maydis*, which encodes pheromones and pheromone receptors (Bölker et al. 1992), also influences filamentous growth, presumably through a pathway linked to the *b*-encoded regulatory function (Spellig et al. 1994). Free exchange of air during growth is one environmental signal known to trigger filamentous growth in *U. maydis* (Fig. 5B). Other environmental signals may include nutrient availability and mating pheromones (Kernkamp 1939; Snetselaar and Mims 1992; Spellig et al. 1994).

Materials and methods

Fungal and bacterial strains, growth conditions, and transformation

The *U. maydis* strains employed in this study are listed in Table 2. The *uac1* disruption strain C002P was constructed by insertion of a phleomycin resistance marker (*ble*; Gold et al. 1994) at a *Hind*III site at codon 899 (Fig. 2). This strain was the recipient of the pFuz60 cosmid that confers hygromycin B resistance and carries the *uac1* gene (Barrett et al., 1993). The strain C002a (*uac1::ble uac1-1*) was the recipient of the pUBC1 cosmid carrying the hygromycin resistance marker and the *ubc1* gene (Fig. 1). The *ubc1* disruption strain 521d was constructed by insertion of a 2.7-kb *Bam*HI–*Bgl*III fragment specifying hygromycin resistance into a unique *Bgl*III site at codon 360. Strains C221, 111, and 221 were obtained as progeny from a cross between strains C002a and 518. The *Escherichia coli* strain DH5 α (Bethesda Research Laboratories) was utilized for standard DNA manipulations (Sambrook et al. 1989), and strain DH10B (Bethesda Research Laboratories) was used for electroporation.

Table 2. *U. maydis* strains

Strain	Genotype	Reference
521	<i>a1 b1</i>	Kronstad and Leong (1989)
518	<i>a2 b2</i>	Kronstad and Leong (1989)
C002P	<i>a1 b1 uac1::ble</i>	this work
C002a	<i>a1 b1 uac1::ble uac1-1</i>	this work
C221	<i>a2 b2 uac1::ble uac1-1</i>	this work
111	<i>a1 b1 ubc1-1</i>	this work
221	<i>a2 b2 ubc1-1</i>	this work
C221	<i>a2 b2 uac1::ble uac1-1</i>	this work
521d	<i>a1 b1 ubc1::hyg</i>	this work

Growth conditions for *U. maydis* and mutagenesis with ultraviolet light were as described previously (Barrett et al. 1993). AMP, dAMP, and cGMP (Sigma Chemical Company) were added to solid and liquid potato dextrose medium (PDA, PDB; Difco) at a concentration of 25 mM. *U. maydis* transformations were performed by established methods (Tsukuda et al. 1988).

DNA manipulations

Total *U. maydis* nucleic acid was isolated by disruption of cells with glass beads and extraction with phenol (Kronstad and Leong 1989). Nucleotide sequence determination was performed with the T7 sequencing kit (Pharmacia). Fragments for sequencing were subcloned in the vector pUC128 (Keen et al. 1988) or pBluescript KS+ (Stratagene), and nested deletions were produced with the Erase-a-Base deletion kit (Promega Biotech). Restriction and modifying enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, Promega Biotech, or Pharmacia. A cDNA library was constructed in the phage vector Uni ZAP XR (Stratagene) from polyadenylated RNA purified from a mating mixture of strains 518 and 521 grown on double complete medium containing 1% charcoal (Holliday 1974). The library was screened by plaque hybridization (Sambrook et al. 1989) using the 4.7-kb *Bam*HI fragment (C fragment; Barrett et al. 1993) of *uac1* labeled with 32 P [Oligolabeling kit, Pharmacia]. Southern hybridization analysis, to confirm the presence of disruption mutations in strains C002P (*uac1::ble*) and 521d (*ubc1::hyg*), was carried out using Zetabind membranes (AMF Cuno) according to the manufacturer's instructions, except that dextran sulfate was omitted. The 4.7-kb *Bam*HI fragment of *uac1* and a 2.6-kb *Sac*I fragment of *ubc1* were used as hybridization probes. For each gene, insertion of the antibiotic resistance marker resulted in loss of a restriction fragment of wild-type size and the appearance of a novel fragment of the size expected from the insertion construct. RNA blot analysis was carried out as described previously (Barrett et al. 1993) using the 2.6-kb *Sac*I fragment of *ubc1* as a hybridization probe. Reverse transcriptase–polymerase chain reaction (RT–PCR) was employed to determine whether an intron was present in the *ubc1* gene. This analysis was carried out as described (Kawasaki 1990) using primers IN1 (AAGATCTCAATCGTGATG; codons 12–18) and IN3 (CTGAGCGCCTTGTTTCGATCACAG; codons 239–246).

Microscopy

Colony morphology on PDA was photographed using a Wild M-5 dissecting microscope. Specimens for scanning electron and fluorescence microscopy were grown to mid- to late logarithmic phase in PDB with hygromycin (50 μ g/ml) or cAMP (25 mM). Scanning electron microscopy was carried out using a Hitachi S-4100 microscope. For fluorescence microscopy, cells were mounted in 0.7% low melting point agarose containing 5 μ g/ml of Calcofluor white and 1 μ g/ml of DAPI (Sigma Chemical Co.) and photographed with a Zeiss Axiophot (Carl Zeiss, D-7082 Oberkochen) microscope using UV excitation. Bright-field light microscopy was also performed with a Zeiss Axiophot microscope.

Analysis of multiple budding

Cells for the analysis of budding were grown on PDA medium for 2 days at 30°C and used to inoculate 5 ml of PDB medium with or without 25 mM cAMP. These cultures were grown to saturation (48 hr) at 30°C and 200 rpm and diluted 1:100 into the same medium. Samples (50 μ l) were removed and characterized

for cell density; a minimum of 100 cells were observed for each sample, and the number of cell clusters and lateral buds were determined. Lateral buds were defined as those produced at right angles from the axis of the mother cell. The maximum number of cells per cluster was determined for the largest cluster observed in each sample: Average cells/cluster = total cells/clusters.

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Note

The accession numbers for the sequences reported in this article are L33918 (*uac1*) and L33917 (*ubc1*).

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S Gold, G Duncan, K Barrett, et al.

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