Involvement of cAMP and Protein Kinase A in Conidial Differentiation by *Erysiphe graminis* f. sp. *hordei*

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Erysiphe graminis f. sp. *hordei*, the causal agent of barley powdery mildew, is an obligate biotroph. On arrival on the host, a primary germ tube (PGT) emerges from the conidium. An appressorial germ tube (AGT) then appears, forms an appressorium, and effects host penetration. Such developmental precision may be due to multiple, plant-derived signals and to endogenous tactile and chemical signals. The transduction mechanism remains obscure. The isolation of an expressed sequence tag (EST) homologue of the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase A (PKA) enabled the corresponding gene to be characterized and the transcript to be identified in conidia and in PGT and AGT stage spores. cAMP-dependent PKA activity was detected in ungerminated conidia. These data suggest that PKA and cAMP are involved in conidial development. To substantiate this we exploited the responses of developing conidia to various surfaces, including exposure to the host leaf (fully inductive to AGT formation), cellulose membrane (semi-inductive), and glass (non-inductive). Assessment of fungal development, following application of exogenous cAMP or cAMP analogues, revealed that, at different concentrations and on different surfaces, cAMP either promoted or inhibited conidial differentiation. Various PKA inhibitors were tested for their effect on PKA activity and conidial development. A negative correlation was established between PKA inhibition in vitro and fungal development in vivo. Taken collectively, these data suggest that PKA and cAMP play a role in conidial differentiation in this obligate, plant-pathogenic fungus.

Powdery mildews are among the most widespread and damaging of all plant diseases. The powdery mildew fungus *Erysiphe graminis* DC. f. sp. *hordei* Ém. Marchal (syn. *Blumeria graminis*) produces prolific asexual spores, conidia, that infect its host, barley. Conidia penetrate the host plant following germination and differentiation of an appressorium. The developmental program leading to appressorial differen-

tiation (Fig. 1) involves two morphologically distinct germ tubes and is dependent upon prior recognition of the leaf surface (Carver and Ingerson 1987). The leaf recognition process appears to be highly complex. Both physical properties of the leaf surface, such as hydrophobicity, and cuticle-derived chemicals, such as cutin monomers and cellulose, have been shown to promote *E. graminis* differentiation (Francis et al. 1996; Kobayashi et al. 1991). However, none of these factors alone is sufficient to trigger high levels of differentiation on artificial surfaces. This has led to the hypothesis that *E. graminis* conidia recognize multiple leaf-derived signals during the differentiation process (Carver et al. 1996).

Fig. 1. The sequence of events leading from arrival of the *Erysiphe graminis* conidium (28 × 14 mm) on the host leaf surface to the emergence of the primary germ tube and subsequent differentiation of the appressorial germ tube.

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Nucleotide and/or amino acid sequence data can be found at the EMBL data base as accession no. AJ243654.

The cyclic AMP (cAMP) signal transduction pathway is known to influence a variety of phenomena in phytopathogenic fungi, including spore morphogenesis and the establishment of infection within a host plant (Kronstad 1997). For example, cAMP is implicated in formation of the infectious dikaryon in the corn smut pathogen, *Ustilago maydis* (Gold et al. 1994) and is associated with virulence in the chestnut blight fungus, *Cryphonectria parasitica* (Chen et al. 1996). In addition, many plant-pathogenic fungi differentiate a specialized infection structure, the appressorium, in order to directly penetrate their host. cAMP has been found to promote key events associated with appressorial differentiation in the rust fungus, *Uromyces phaseoli* (Hoch and Staples 1984) and has been demonstrated to be a key mediator of both appressorial differentiation and function in the rice blast pathogen *Magnaporthe grisea* (Lee and Dean 1993; Xu et al. 1997). The finding that cAMP is pivotal to pathogenicity in a number of model host-pathogen interactions has led to the proposal that cAMP is a common mediator of virulence and may, therefore, be considered an ideal target for disease intervention strategies (Hamer and Holden 1997).

cAMP-dependent protein kinase A (PKA) is the major downstream effector of cAMP. PKA has been extensively characterized in mammalian systems and in yeast. PKA is a tetrameric protein of two cAMP-binding regulatory subunits (R) and two catalytic subunits (C). Activation of the catalytic subunits results from their dissociation from the regulatory subunits following cAMP binding. In the yeast *Saccharomyces cerevisiae*, three catalytic subunit genes have been identified: *Tpk1*, *Tpk2*, and *Tpk3*. The products of these three genes may show some redundancy (Toda et al. 1987). However, Pan and Heitman (1999) recently demonstrated that the different TPK catalytic subunits play distinct roles during regulation of *S. cerevisiae* pseudohyphal growth, in that *Tpk2* promotes filamentous growth while *Tpk1* and *Tpk3* inhibit filamentous growth. PKA has also been found to be involved in growth and morphological processes in filamentous fungi (Bencina et al. 1997; Bruno et al. 1996). Moreover, PKA has been implicated in fungal pathogenesis in both the rice blast fungus, *M. grisea*, and the smut pathogen, *U. maydis* (Mitchell and Dean 1995; Gold et al. 1997; Xu et al. 1997).

The role of cAMP and PKA in appressorial differentiation has been extensively characterized in *M. grisea*. Here, the addition of exogenous cAMP is sufficient to promote high levels of appressorial differentiation on an otherwise non-inductive surface (Lee and Dean 1993). Ablation of the gene for the catalytic subunit of PKA (*cpkA*) resulted in the delayed formation of nonfunctional appressoria (Xu et al. 1997). Deletion of *MAC1*, the gene encoding adenylate cyclase, has been demonstrated to disrupt appressorial differentiation, although pleiotropic effects were observed on growth and development (Choi and Dean 1997). A mutation in the R subunit of PKA, *sum-99*, was subsequently found to suppress the *mac1* phenotype (Adachi and Hamer 1998). Thus, such mutational analysis supports the involvement of a cAMP-mediated signaling pathway in appressorial differentiation, although the details of this process have not been fully resolved. In *M. grisea*, cAMP acts in conjunction with other signal transduction pathways such as the MAPK homologue, *pmk1*, which has also been found to be central to the differentiation process (Xu and Hamer 1996).

In this paper we have investigated the effect of exogenous cAMP and cAMP analogues on conidial morphogenesis. Furthermore, we have found evidence for both the presence of PKA in *E. graminis* and its importance in the differentiation process.

RESULTS

cAMP-dependent PKA catalytic subunit gene.

A cDNA sequencing project yielded an EST with homology to known fungal PKA catalytic subunits. The amino acid sequence of the *E. graminis* cPKA protein, deduced from the nucleotide sequence data of both the partial cDNA and full-length genomic *cPKA* clones, is shown in Figure 2. Sequence analysis revealed an open reading frame of 1,569 bp, carrying three putative short introns of 50, 49, and 53 bp with consensus 5'GT / 3'AG splice sites (Gurr et al. 1988). The catalytic protein core of cPKA resides between amino acids 160 and 416, in a region that shares 225 of 257 amino acid residues (88%) with the *M. grisea* protein (Mitchell and Dean 1995). The data also reveal other motifs and residues commonly found in catalytic subunit sequences (Taylor et al. 1993; Woodget 1996), including the ATP binding motif, a catalytic loop motif, and a magnesium ion-chelating loop (Fig. 2). A single autophosphorylation site resides at Thr-314. The phosphorylation site, 406-Ser, commonly found within cPKA sequences is absent from the *E. graminis* sequence.

Southern analysis.

Southern analysis of *E. graminis* DNA, restricted with *Bgl*II, *Bam*HI, or *Eco*RV and hybridized with a 1,300-bp *Hind*III fragment of the gene, revealed unique bands of 2.7, 8, and 1.5 kb, respectively (Fig. 3), consistent with the existence of a single copy of the *cPKA* gene in the *E. graminis* genome.

Expression of the cAMP-dependent PKA catalytic subunit transcript over 12 h post inoculation.

Expression of the transcript for the *E. graminis* PKA catalytic subunit was investigated by reverse transcription-polymerase chain reaction (RT-PCR; Fig. 4). Expression was detected in ungerminated conidia and in populations of conidia collected from barley leaves at 4 and 12 h post inoculation. The identity of the 342-bp RT-PCR fragment was confirmed by hybridization to restriction-digested *E. graminis* DNA (not shown).

cAMP-dependent PKA activity detected in protein extracted from ungerminated conidia.

cAMP-dependent phosphorylation of the model PKA substrate kemptide was identified in protein extracted from ungerminated *E. graminis* conidia. Detection of high levels of activity required the addition of either cAMP or the cAMP analogues, 8-bromo-cAMP or cBIMPs (5,6-dichloro-1- β -ribofuranosyl-benzimidazole-3'-5'-monophosphothionate; Sandberg et al. 1991) at a concentration of 1 μ M; AMP and cGMP at 1 μ M had no significant effect (Table 1).

The effect of exogenous cAMP and cAMP analogues on conidial development.

The presence of the *PKA* gene, its transcript, and PKA activity suggested the involvement of cAMP in conidial devel-

<i>Erysiphe</i>	MPTFG-FLKKRKKVSLGHRDLDSFASTLSQPASPITPSTSRGLG-EPIDTTSSKITS---	55
<i>Colletotrichum</i>	MPTLG-FLKK-KRTREGNQD---PQGS--PTSPVPTSSKPFDP-SSISTTLSSLSNNSQ	52
<i>Magnaporthe</i>	MPSLG-FLKK-KRTRDGNND-----NSSQPASPVTPTAAQSFEQAQVLGAPSAINNSHA	52
<i>Aspergillus</i>	MPSLGGLKK-RRTKD-----SQTLS--KELEAGS---AQTQTSFNAAEDHHN	42
<i>Erysiphe</i>	-----PGQK-----LGSNITKDHGRTRTTV-----ED	77
<i>Colletotrichum</i>	ATSQPSGPGQKQEDPASSEAAKNQMYPVTVHPAP---N-----QQHYNQGG--EPQQN	102
<i>Magnaporthe</i>	HT-----QQQSYLVPQPGYSVGTEVQAQQPQMNSISQQQQQAFAPPAHTPSFGTIDPQQS	107
<i>Aspergillus</i>	HN-----HHQHH-----HHLFHHHHQPQPATNSGS-----AANTP-P---QPQDS	78
>		
<i>Erysiphe</i>	LPSMT-----DVS-----HLQQAYR-NLSQGSTASKSNLLTIHNLINPP-----	115
<i>Colletotrichum</i>	LPSISNLINPTQHDASSNGHNYLAHATQTSQPNPSPGTDPTLQQQQAQSMHPPTQPQQ	162
<i>Magnaporthe</i>	LPSISNLMNP-----AVQQNSQPSANFQPQSQSQSQSQSQFPLPPSHGNG	153
<i>Aspergillus</i>	VPQQSN-----RSSGAEKSSDGQVASMQSAVTQAS---PSAHTS	115
<i>Erysiphe</i>	-----QHDGAGQS-----KVMPEK---HMNNKTERP-----VQGK-----AAVAQ	147
<i>Colletotrichum</i>	Q-QQQQQQQQQQQQQQ-----QQQQQQ---QQQTQTQQP-----TAKER---HAATGA	203
<i>Magnaporthe</i>	DQSQQQNFQVQQQIQSQQDAMDIPQSVQDQSHSQQAQPQHQPQHVVQHVNHAHQGSQD	213
<i>Aspergillus</i>	G-LPQPNANAAS-IQN-----IINPSQ-QGAMHSASSG-----HTQSH---HAGRS	156
ATP binding site		
<i>Erysiphe</i>	---VRQTKGKYSLSDFEILRLTGTGSGFRVHLVQSKHNQRFYAVKVLKKQOVVKMKQVEH	203
<i>Colletotrichum</i>	GPAGRQTKGKYTLQDFDILRLTGTGSGFRVHLVQSKHNQRFYAVKVLKKAQVVKMKQVEH	263
<i>Magnaporthe</i>	QQ-TRVTGKGYSLTDFEILRLTGTGSGFRVHLVQSRHNQRFYAVKVLKKAQVVKMKQVEH	272
<i>Aspergillus</i>	---ARTTKGKYSLDDFSLQRTLTGTGSGFRVHLVQSKHNHRYAVKVLKKAQVVKMKQIEH	213
<i>Erysiphe</i>	TNDERSMLQEVKHPFLITLWGTFOQSKNLYMVMDFVEGGELFSLLRKSQDSQIQWPSFTP	263
<i>Colletotrichum</i>	TNDERRMLGEVKHPFLITLWGTFOQSKNLYMVMDFVEGGELFSLLRKSGRFPNPVAKFYA	323
<i>Magnaporthe</i>	TNDERKMLGEVKNPFLITLWGTFOQCRNLYMVMDFVEGGELFSLLRKSGRFPNPVAKFYA	332
<i>Aspergillus</i>	TNDERMLNRVRHPFLITLWGTWQDSRNLYMVMDFVEGGELFSLVRKSQRFPNPVAKFYA	273
Catalytic loop Magnesium ion-chelating loop		
<i>Erysiphe</i>	LKVTLALEYLHKKDIIYRDLKPENLLDRHGHLKITDGFAGAKKVTDITWTLCTGTPDYLA	323
<i>Colletotrichum</i>	AEVTLALEYLHSRDIIYRDLKPEN-LLDRHGHLKITDGFAGKRVDPKWTWTLCTGTPDYLA	382
<i>Magnaporthe</i>	AEVTLALEYLHAKNIIYRDLKPENLLDRHGHLKITDGFAGKRVDPKWTWTLCTGTPDYLA	392
<i>Aspergillus</i>	AEVTLALEYLHTQNIYRDLKPENLLDRHGHLKITDGFAGKEVPDITWTLCTGTPDYLA	333
<i>Erysiphe</i>	EVVSSKGYNKSVDWWSLGILIFEMLCGYTPFWDNGSPMKIYENILKGRVKYPPYIHPDAQ	383
<i>Colletotrichum</i>	EVVSNKGYNKSVDWWSLTIILYEMLCGYTPFWDGSGPLKIYENILKGVKYPAYINPDAQ	442
<i>Magnaporthe</i>	EVVSNKGYNKSVDWWSLGILIFEMLCGYPPFWDGSGPMKIYENILKGVKYPAYINPDAQ	452
<i>Aspergillus</i>	EVVSSKGYNKSVDWWSLGILIFEMLCGFTPFWDGSGSPVKIYENILRGRVKYPPYIHPDAV	393
<i>Erysiphe</i>	DLIQRITADLTKEGLGNLHGGAEGIKSHQWFAEVTWERLAKKDIDAPYVPPVKAGSGDAS	443
<i>Colletotrichum</i>	DLLSKLITADLTKEGLGNLYGGPNVKTTHPWFQEVTDRLARKDIDAPYTPPVKAGAGDAS	502
<i>Magnaporthe</i>	DLLQRLITADLTKEGLGNLYGGSQDVNRNHPWFAEVTWDLARKDIDAPYTPPVKAGAGDAS	512
<i>Aspergillus</i>	DLLSQLITADLTKEGLGNLHGSSDDVKNHPWFAEVTWDLARKDIDAPYVPPIRGGQGDAS	453
• >		
<i>Erysiphe</i>	QFDKYPEETERYGQTGPDEHGSLFENF	470
<i>Colletotrichum</i>	QFDRYPEDPERYGSSGHDEYGHFTDF	529
<i>Magnaporthe</i>	QFDRYPEETERYGQTGHDEYGNLFPGF	539
<i>Aspergillus</i>	QYDRYPEETEYQGMAGEDPHGHLFPDF	480

Fig. 2. Amino acid sequence of the *Erysiphe graminis* cPKA gene (EMBL accession no. AJ243654) aligned with cPKA from *Colletotrichum trifolii* (Yang and Dickman 1997), *Magnaporthe grisea* (Mitchell and Dean 1995), and *Aspergillus nidulans* (Bencina et al. 1997). The putative ATP binding site, catalytic loop, magnesium ion-chelating loop, and site of three short introns (•) are marked, as are residues E, D, and R important in protein folding, and T, a putative auto-phosphorylation site.

opment. Therefore, *E. graminis* development was assayed on barley epidermal strips, hydrated cellulose membranes, 2% water agar, and glass. In each case, the conidia were scored for both the percentage of the total population that germinated and the percentage of the germinated conidia that formed appressoria. On barley epidermal strips, typically 60 to 70% of the population germinated of which 80 to 90% differentiated

an appressorium within 12 h. Inoculation onto cellulose membrane gave lower levels of both germination (50%) and appressorial differentiation (20% of germinated conidia). Conidial development on the cellulose membrane used in this study occurred at a slower rate than observed on the barley epidermis so the conidia were scored at the later time of 24 h post inoculation. Although conidia germinated following inoculation onto 2% water agar and glass, very few, if any, conidia differentiated appressoria. Thus, in terms of promotion of conidial differentiation, these surfaces range from the inductive (barley epidermal strips) through the partially inductive (hydrated cellulose membrane) to the non-inductive (2% water agar and glass).

To assess the effects of cAMP and AMP on conidial development, the epidermal strips or cellulose membranes were laid over solutions of either cAMP or AMP and inoculated, or else spores were inoculated directly onto water agar carrying the

Fig. 3. Southern blot analysis of *Erysiphe graminis* DNA digested with *Bgl*II, *Bam*HI, and *Eco*RV, hybridized to a "high-prime" labeled 1,300-bp *Hind*III fragment (denoted > on Figure 2) and washed under conditions of low stringency.

Fig. 4. Reverse transcription-polymerase chain reaction of the *Erysiphe graminis* (A) PKA catalytic subunit (342 bp amplicon) and (B) β -tubulin (412 bp amplicon) sequences from ungerminated conidia, and conidia collected at 4 and 12 h following inoculation onto barley leaves.

Table 1. Effect of cyclic AMP (cAMP) and related compounds on appressorial differentiation on cellulose membrane and epidermal strips^a

Compound applied	On epidermal strips ^b	On cellulose membrane ^c	Efficiency of PKA activation ^d
Water	81.2 $\pm$ 2.6	14.7 $\pm$ 2.1	13.4
cAMP 1 mM	11.7 $\pm$ 5.3	30.0 $\pm$ 2.2	100
8-bromo-cAMP 1 mM	19.7 $\pm$ 4.5	29.0 $\pm$ 2.7	84.4
cBIMPs 1 mM	26.3 $\pm$ 5.2	27.7 $\pm$ 4.9	43.3
cGMP 1 mM	80.4 $\pm$ 2.1	16.4 $\pm$ 3.7	21.7
AMP 1 mM	79.0 $\pm$ 6.0	13.0 $\pm$ 3.2	8.5

^a In addition, the relative ability of these compounds to promote kemptide phosphorylation is shown in Figure 7. Values shown for kemptide phosphorylation are calculated relative to observed activation by cAMP.

^b Percent appressorial differentiation 12 h post inoculation.

^c Percent appressorial differentiation 24 h post inoculation.

^d Percent phosphorylated peptide in protein kinase A (PKA) assay with protein extract from ungerminated spores.

Fig. 5. The effect of exogenous cyclic AMP on appressorial differentiation on conidia germinated on barley epidermal strips (■) and cellulose membrane (◆). The values shown represent the means of three independent experiments $\pm$ standard error of the mean.

chemicals, and conidial development was monitored. On each surface a range of both cAMP and AMP concentrations were investigated and conidia were scored as above. In addition, cGMP and the cAMP analogues, 8-bromo-cAMP and cBIMPs, were assayed on cellulose membrane and epidermal strips.

Application of 100 μ M and 1 mM cAMP to conidia inoculated onto epidermal strips resulted in a decrease in appressorial differentiation at 12 h post inoculation, as shown in Figures 5 and 6. Application of 1 mM cAMP resulted in decreased germination (data not shown). No effect was observed at lower cAMP concentrations (0.1, 1, and 10 μ M). At 1 mM, application of both 8-bromo-cAMP and cBIMPs reduced the number of hooked appressorial germ tubes but did not affect germination. cGMP and AMP had no effect over the concentration range assayed (1 μ M to 1 mM; Table 1). At 10 mM both AMP and cAMP inhibited differentiation (data not shown).

In contrast to the development on epidermal strips, appressorial differentiation by conidia inoculated onto cellulose membrane was promoted at the lower cAMP concentrations of 1 and 10 μ M (Figs. 5 and 6). Similarly, Table 1 shows that a promotion of appressorial differentiation was observed following application of 8-bromo-cAMP or cBIMPs to cellulose membrane at 1 μ M whereas application of 1 μ M cGMP or AMP had no significant effect.

No induction of appressorial differentiation was observed following application of cAMP to conidia inoculated onto the non-inductive surfaces of 2% water agar or glass.

Effect of staurosporine, H7, H89, and KT5720 on *E. graminis* cAMP-dependent PKA activity and conidial differentiation.

As illustrated in Figure 7, the PKA inhibitors staurosporine, H7, and H89 inhibited appressorial differentiation on epider-

Fig. 6. Conidia ($28 \times 14 \mu$ m) viewed at $\times 40$ magnification, at 12 h post inoculation onto the barley epidermal surface (A) control and (B) treated with 100 mM cyclic AMP (cAMP). Conidia at 24 h post inoculation onto cellulose membrane (C) control and (D) treated with 1 mM cAMP. Conidia at 24 h post inoculation onto 2% water agar (E) control and (F) treated with 1 mM cAMP.

Fig. 7. The effect of the protein kinase inhibitors (A) staurosporine, (B) H7, and (C) H89, on appressorial differentiation by conidia germinated on epidermal strips. In each experiment, 6 × 50 germinated conidia were scored for appressorial differentiation. The values shown the means from three independent experiments, ± standard error of the means.

mal strips. However, KT5720 had no significant effect (data not shown). The inhibitors were also assayed for their ability to inhibit cAMP-dependent phosphorylation of the model PKA substrate, kemptide, by the *E. graminis* conidial protein extract. A range of concentrations between 1 nM and 10 mM were assayed and the IC₅₀ values for inhibition of the *E. graminis* activity were determined as shown in Figure 8. Staurosporine proved to be the most potent inhibitor with an IC₅₀ of 190 nM, followed by H89 with an IC₅₀ of 26 μM, then H7 with an IC₅₀ of 122 μM. KT5720 was found to be a very weak inhibitor of kemptide phosphorylation; maximum inhibition of 20% was observed at 100 μM.

DISCUSSION

The involvement of cAMP signal transduction during the establishment of host-pathogen interactions has been recently investigated in plant-pathogenic fungi such as *U. phaseoli* (Hoch and Staples 1984), *U. maydis* (Gold et al. 1994), and *C. parasitica* (Chen et al. 1996; Kronstad 1997). In particular, the role of cAMP in infection structure differentiation has been characterized in *M. grisea*, largely through the use of mutational analysis (Xu et al. 1997; Choi and Dean 1997; Adachi and Hamer 1998). Powdery mildews are an agronomically important group of plant pathogens; however, due to their obligate, biotrophic lifestyle and genetic intractability, investigation of the molecular events associated with fungal morphogenesis has lagged behind that of other plant pathogens. The barley powdery mildew, *E. graminis* f. sp. *hordei*, like *M. grisea*, is an ascomycete fungus that infects its host via an appressorium. However, conidial differentiation of *E. graminis* is more complex than that of *M. grisea*, in that two distinct germ tubes are found and development of mature appressoria is fully efficient only on host surfaces. Appressorial differentiation by *M. grisea* is more promiscuous and can occur to very high levels on artificial substrata (Gilbert et al. 1996). In this paper we gain some insight into the involvement of cAMP in conidial development in an obligate, biotrophic plant pathogen.

Application of exogenous cAMP at 1 μM stimulated appressorial differentiation on the moderately inductive cellulose membrane, whereas 1 mM cAMP inhibited appressorial differentiation when applied to the highly inductive epidermal strips. On cellulose membrane, higher concentrations (100 μM) inhibited development.

A similar inhibition of differentiation at 1 mM on epidermal strips and promotion of differentiation at 1 μM on cellulose membrane was observed with the cAMP analogues, 8-bromo-cAMP and cBIMPs. No effect was observed with cGMP or AMP on either surface at these concentrations. This reflects the relative potency of these compounds in activation of *E. graminis* PKA.

These results imply that the addition of exogenous cAMP at relatively high concentrations (100 μM and 1 mM) interferes with appressorial differentiation on the inductive barley epidermal surface. In contrast, low levels of cAMP promoted differentiation on cellulose membrane, a surface that is partially inductive to conidial development. However, no such promotion was observed on the non-inductive surfaces of 2% water agar and glass. Thus, application of exogenous cAMP is not sufficient to promote differentiation on a non-inductive sur-

face. Cellulose membrane may provide an additional signal that is lacking on the non-inductive surfaces and that is required for appressorial development. Nevertheless, even on cellulose membrane treated with cAMP, levels of appressorial differentiation did not reach those observed on the barley leaf, indicating either non-maximal stimulation of the cAMP pathway or the requirement for additional signaling pathways.

These observations provide an interesting comparison with both the rice blast fungus, *M. grisea*, and *Colletotrichum trifolii*, the causal agent of alfalfa anthracnose (Lee and Dean 1993; Yang and Dickman 1997). The concentrations of cAMP required to promote development in *E. graminis* are in the μM rather than the mM range. This may reflect a greater permeability of conidia of *E. graminis*, compared with those of *M. grisea*. In addition, in *M. grisea*, application of 50 mM cAMP promotes at least 90% appressorial differentiation on a non-inductive surface, whereas cAMP increases AGT formation by only 15% (Adachi and Hamer 1998).

The principal downstream effector of cAMP is believed to be cAMP-dependent PKA. Characterization of the *E. graminis* PKA catalytic subunit gene revealed that the catalytic protein core shares some 88% identity with the analogous region of the *M. grisea* protein (Mitchell and Dean 1995). Like the *M. grisea* cPKA, the *E. graminis* cPKA gene resides as a single copy within the barley powdery mildew genome. In *S. cerevisiae* there are three different cPKA genes (Toda et al. 1987).

RT-PCR studies revealed that the transcript was present in ungerminated conidia and in populations of conidia collected at the specific time points 4 and 12 h post inoculation, corresponding to the beginning and end points of appressorial differentiation, respectively. Moreover, cAMP-dependent protein kinase activity was detected in ungerminated conidia. This activity was used to assay the inhibitory potency of the serine/threonine protein kinase inhibitors staurosporine, H7, H89, and KT5720. KT5720 was found to only weakly inhibit the *E. graminis* PKA activity, whereas staurosporine, H7, and H89 inhibited the activity with IC_{50} values ranging from the nM to the μM range. H89 is reported to be relatively specific for mammalian PKA, whereas staurosporine and H7 inhibit a range of serine/threonine kinases (Engl et al. 1996). However, although we demonstrate the ability of these compounds to inhibit *E. graminis* PKA activity, we cannot define their relative specificities toward *E. graminis* PKA. Nevertheless, these three compounds are shown to disrupt appressorial differentiation on epidermal strips when applied at concentrations that reflected the IC_{50} values. Thus, taken together these findings not only provide evidence for the presence of cAMP-dependent protein kinase activity in *E. graminis* but also sup-

port the role for protein phosphorylation, possibly modulated by PKA, and its involvement in conidial morphogenesis.

The results presented in this paper demonstrate the importance of cAMP signaling and PKA in appressorial differentiation of *E. graminis* conidia. However, unlike the model systems so far studied, most notably *M. grisea*, the involvement of cAMP in *E. graminis* is not manifest in a simple cAMP-

Fig. 8. The effect of (A) staurosporine, (B) H7, and (C) H89 on inhibition of *Erysiphe graminis* cyclic AMP-dependent protein kinase A (PKA) activity. For each inhibitor, the effect on kemptide phosphorylation in the PKA assay was determined for inhibitor concentrations in the range of 106 to 101 nM. The experiments were repeated three times and the mean $\pm$ standard error is shown. The degree of inhibition at the different concentrations was used to determine the IC_{50} of the inhibitor for the *E. graminis* PKA activity; (A.i.) staurosporine, (B.i.) H7, and (C.i.) H89. Representative assays are shown; (A.ii.) staurosporine, (B.ii.) H7, and (C.ii.) H89. C(-) represents the negative control (no protein added), C(+) the positive control (protein added, but no inhibitor), and CD(+) the solvent control for staurosporine.

mediated trigger of differentiation. This may reflect the high degree of specificity of host recognition required to promote differentiation in *E. graminis* or the greater complexity involved in germinating two types of germ tubes. Not only do multiple external signals appear to be required to promote appressorial differentiation, but these may be transduced by multiple signal transduction pathways. Thus, although signaling via cAMP appears to be necessary for appressorial differentiation in *E. graminis*, it is not sufficient, alone, to trigger the conidial differentiation program in this obligate, plant-pathogenic fungus.

MATERIALS AND METHODS

Fungal and plant material.

E. graminis f. sp. *hordei* race IM82 was maintained in a greenhouse under supplemented light conditions (16-h light period per day) by successive inoculations onto spring barley, *Hordeum vulgare* L. 'Golden Promise', which was grown in a peat-based compost in a greenhouse under natural light conditions and provided the leaf tissue for experimental use. For experiments, inoculations were performed with an inoculation turret, a plastic pipe 16 cm in diameter and 52 cm high with a circular perspex lid, and a 15-cm-diameter plastic tube positioned 70 mm from the top of the turret to form a spout. The surfaces to be inoculated were placed at the base of the turret and conidia were dislodged from infected leaves placed in the spout with a Kenair air duster (Kenro, Swindon, U.K.). The apparatus was left in place for 5 min after the spores had been dislodged to allow them to settle on the surfaces below.

Assay of conidial development on host and artificial substrata following application of cAMP, cAMP analogues, or protein kinase inhibitors.

Barley epidermal strips were prepared from the abaxial surface and were clamped between the jaws of a metal grip. This presented an epidermal cell sheet of approximately 0.5 cm² for inoculation with conidia. Immediately following inoculation, the epidermal strip was placed on top of a 20- μ l droplet of either water, solvent control, or a solution of cAMP, cAMP analogue, or protein kinase inhibitor. The inoculated epidermal strips were incubated at 100% relative humidity (RH), 20°C in the dark.

Cellulose membrane (Medicell International, London) was washed prior to use by boiling three times for 30 min in dH₂O and then placed on glass slides that had been covered with a thin layer (500 μ l) of 2% water agar. The cAMP, cAMP analogue, or protein kinase inhibitor was added to the water agar to give the final concentrations indicated. Following inoculation the membrane was incubated at 100% RH, 20°C in the dark.

Glass slides were covered with a thin layer (500 μ l) of 2% water agar containing the chemical under investigation. Conidia were then directly inoculated onto the solidified water agar layer and incubated at 100% RH, 20°C in the dark.

The percentage of germination was determined by scoring 300 conidia for the presence of at least one germ tube. Differentiation was assessed by scoring 300 germinated conidia for the number of short germ tubes, germ tube elongation, and formation of an appressorium. Each experiment was performed three times and the standard error was determined for mean values from the individual experiments.

Preparation of chemicals.

cAMP, 8-bromo-cAMP, 5,6-dichloro-1- β -ribofuranosylbenzimidazole-3',5'-monophosphate (cBIMPS), cGMP, H7, H89, and KT5720 were purchased from Alexis Biochemicals (Nottingham, U.K.). AMP was purchased from Sigma (Poole, U.K.), and staurosporine from Calbiochem (Nottingham, U.K.). Stock solutions of cAMP, AMP, 8-bromo-cAMP, cBIMPS, cGMP, H7, and H89 were made in dH₂O whereas solutions of staurosporine and KT5720 were made in dimethyl sulfoxide (DMSO).

Collection of stage-specific conidial material.

Conidia were inoculated onto detached barley leaves and harvested at either 4 or 12 h post inoculation by painting the leaf surface with 5% (wt/vol) cellulose acetate dissolved in absolute acetone, stripping the dried inoculated surface, and freezing the cellulose acetate peels directly in liquid nitrogen. Ungerminated conidia were collected by directly shaking the inoculum onto aluminum foil and were immediately frozen in liquid nitrogen.

Cloning of cPKA.

A 652-bp fragment, showing identity to the carboxyl terminus of the catalytic subunit of cAMP-dependent PKA, was identified among sequences derived from an *E. graminis* EST library, prepared from conidia germinated on glass for 6 h (Justesen et al. 1996). A genomic clone isolated from an *E. graminis* λ Bluestar (Novagen, Cambridge, MA) phage library yielded an 8-kb fragment carrying the *cPKA* gene. This was subcloned into pBluescript SK+ and the open reading frame was sequenced on a 377 sequencer (Perkin Elmer, Warrington, U.K.) with 12 "walking" primers specific to *cPKA*. DNA/protein sequence data base searches were performed with BLAST programs (Altschul et al. 1997).

Preparation of genomic *E. graminis* DNA.

E. graminis DNA was prepared from ungerminated conidia according to the CTAB method described for the extraction of plant DNA (Ausubel et al. 1987). A 1,300-bp *Hind*III fragment (denoted by > in Figure 2) was "high-prime" labeled (Boehringer Mannheim, E. Sussex, U.K.) and Southern blot analysis of genomic DNA, restricted with *Bgl*II, *Bam*HI, and *Eco*RV, was carried out under low stringency hybridization and washing conditions as described by Sambrook et al. (1989).

RNA extraction and RT-PCR.

Total RNA was extracted with the Stratagene (Cambridge, U.K.) micro RNA isolation kit. First-strand cDNA was synthesized with a kit from Pharmacia Biotech (St. Albans, U.K.). cDNA (10 ng) was used as a template for PCR. The forward and reverse primers for amplification of PKA were PKAF (GTGGAAGGTGGCGAACTCTT) and PKAR (GGC GTATAGCCACAAAGCAT). The primers TUBF (CATGTT CCGGAGAAAGGCATTT) and TUBR (TGGTCTGTTACTT GACATGCTCT) were used to amplify the *E. graminis* β -tubulin sequence. In both cases, the primers were designed on either side of an intron in the genomic DNA sequence in order to allow distinction between amplification from genomic DNA and cDNA. PCR was performed with SuperTaq polymerase (HT Biotechnology, Cambridge, U.K.). Thermal cy-

cling conditions were as follows: 95°C for 3 min, followed by 28 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, terminated with 72°C for 5 min.

PKA assays.

PKA assays were performed with a non-radioactive PKA assay kit (Promega, Southampton, U.K.) following the manufacturer's protocol. Protein was extracted from 750 mg of conidia collected directly from heavily sporulating colonies with a Hoover attachment to a vacuum pump. The extraction method has been described previously (Yang and Dickman 1997). Protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard, and assays were performed with 2.5 µl of protein at a concentration of 1.5 mg ml⁻¹. The phosphorylated and nonphosphorylated peptides were separated by agarose gel electrophoresis and the relative amount of each determined by densitometry (Flur-S Multimager, Bio-Rad).

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LITERATURE CITED

- Adachi, K., and Hamer, J. E. 1998. Divergent cAMP signaling pathways regulate growth and pathogenesis in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 10:1361-373.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-402.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Strahl, K., eds. 1987. *Current Protocols in Molecular Biology*. John Wiley, New York. p. 2.3.3.
- Bencina, M., Panneman, H., Ruijter, G. J., Legisa, M., and Visser, J. 1997. Characterization and overexpression of the *Aspergillus niger* gene encoding the cAMP-dependent protein kinase catalytic subunit. *Microbiology* 143:1211-1220.
- Bruno, K. S., Aramaya, R., Minke, P. F., Metzner, R. L., and Plamann, M. 1996. Loss of growth polarity and mislocation of septa in a *Neurospora* mutant altered in the regulatory subunit of cAMP-dependent protein kinase. *EMBO J.* 15:5772-5782.
- Carver, T. L. W., and Ingerson, S. M. 1987. Responses of *Erysiphe graminis* germings to contact with artificial and host surfaces. *Physiol. Mol. Plant Pathol.* 30:359-372.
- Carver, T. L. W., Ingerson-Morris, S. M., and Thomas, B. J. 1996. Influences of host surface features on development of *Erysiphe graminis* and *Erysiphe pisi*. Pages 255-266 in: *Plant Cuticles – An Integrated Functional Approach*. G. Kerstiens, ed. Bios Scientific Pub., Oxford.
- Chen, B., Gao, S., Choi, G. H., and Nuss, D. L. 1996. Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. *Proc. Natl. Acad. Sci. USA* 93:7996-8000.
- Choi, W., and Dean, R. A. 1997. The adenylate cyclase gene *MAC1* of *Magnaporthe grisea* controls appressorium formation and other aspects of growth and development. *Plant Cell* 9:1973-1983.
- Engh, R. A., Girod, A., Kinzel, V., Huber, R., and Bossmeyer, D. 1996. Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulphonyl protein kinase inhibitors H7, H8 and H89. *J. Biol. Chem.* 271:26157-26164.
- Francis, S. A., Dewey, F. M., and Gurr, S. J. 1996. The role of cutinase in germling development and infection by *Erysiphe graminis* f. sp. *hordei*. *Physiol. Mol. Plant Pathol.* 49:201-211.
- Gilbert, R. D., Johnson, A. M., and Dean, R. A. 1996. Chemical signals responsible for appressorium differentiation in the rice blast fungus *Magnaporthe grisea*. *Physiol. Mol. Plant Pathol.* 48:335-346.
- Gold, S., Brogdon, S. M., Mayorga, M. E., and Kronstad, J. 1997. The *Ustilago maydis* regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize. *Plant Cell* 9:1585-1594.
- Gold, S., Duncan, G., Barrett, K., and Kronstad, J. 1994. cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. *Genes Dev.* 8:2805-2816.
- Gurr, S. J., Unkles, S., and Kinghorn, J. 1988. The structure and organisation of nuclear genes of filamentous fungi. Pages 93-139 in: *Gene Structure in Eukaryotic Microbes*. Vol. 23. J. R. Kinghorn, ed. Society of General Microbiology Special Publication, IRL Press, Oxford.
- Hamer, J. E., and Holden, D. W. 1997. Linking approaches in the study of fungal pathogenesis: A commentary. *Fungal Genet. Biol.* 21:11-16.
- Hoch, H. C., and Staples, R. C. 1984. Evidence that cAMP initiates nuclear division and infection structure formation in the bean rust fungus *Uromyces phaseoli*. *Exp. Biol.* 8:37-46.
- Justesen, A., Somerville, S., Christiansen, S., and Giese, H. 1996. Isolation and characterization of two novel genes expressed in germinating conidia of the obligate biotroph *Erysiphe graminis* f. sp. *hordei*. *Gene* 170:131-135.
- Kobayashi, I., Tanaka, C., Yamaoka, N., and Kunoh, H. 1991. Morphogenesis of *Erysiphe graminis* conidia on artificial membranes. *Trans. Mycol. Soc. Jpn.* 32:187-198.
- Kronstad, J. W. 1997. Virulence and cAMP in smuts, blasts and blights. *Trends Plant Sci.* 2:193-199.
- Lee, Y.-H., and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* 5:693-700.
- Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* 7:1869-1878.
- Pan, X., and Heitman, J. 1999. Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 19:4874-4887.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sandberg, M., Butt, E., Nolte, C., Fischer, L., Halbruegge, M., Beltman, J., Jahnson, T., Genieser, H.-G., Jastorff, B., and Walter, U. 1991. Characterisation of Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3',5'-monophosphorothioate (Sp-5,6-DCI-cBIMPs) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem. J.* 279:521-527.
- Taylor, S. S., Zheng, J., Radizio-Andzel, E., Knighton, D. R., Eyck, L. F. T., Sowdaski, J. M., Herberg, F. W., and Yonemoto, W. M. 1993. cAMP dependent protein kinase defines a family of enzymes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 340:277-287.
- Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. 1987. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* 50:277-287.
- Woodget, J. R. 1996. *Protein Kinases*. Oxford University Press, Oxford.
- Xu, J.-R., and Hamer, J. E. 1996. MAP kinase and cAMP signalling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* 10:2696-2706.
- Xu, J.-R., Urban, M., Sweigard, J. A., and Hamer, J. E. 1997. The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration. *Mol. Plant-Microbe Interact.* 10:187-194.
- Yang, Z., and Dickman, M. B. 1997. Regulation of cAMP and cAMP dependent protein kinase during conidial germination and appressorium formation in *Colletotrichum trifolii*. *Physiol. Mol. Plant Pathol.* 50:117-127.