Molecular Microbiology (2002) **44**(4), 1001–1016

cAMP and ras signalling independently control spore germination in the filamentous fungus *Aspergillus nidulans*

Sabine Fillinger,[1†] Marie-Kim Chaveroche,[1] Kiminori Shimizu,[2] Nancy Keller[2] and Christophe d'Enfert[1*]

[1]*Unité Microbiologie et Environnement, CNRS URA 2172, Institut Pasteur, 25, rue du Docteur Roux, 75724 Paris Cedex 15, France.*

[2]*University of Wisconsin Madison, Department of Plant Pathology, 1630 Linden Drive, Madison, WI 53706, USA.*

Summary

The role of cAMP signalling during germination of asexual spores (conidia) of the filamentous fungus *Aspergillus nidulans* was investigated. *A. nidulans* strains defective for adenylate cyclase (CyaA) or for the functionally overlapping cAMP-dependent protein kinase (PkaA) and newly characterized SchA protein kinase, homologous to *Saccharomyces cerevisiae* Sch9, show altered trehalose mobilization and kinetics of germ tube outgrowth, in addition to other defects in colony formation. cAMP-dependent trehalose breakdown is triggered by the addition of a carbon source independently of further catabolism, suggesting that cAMP signalling controls early events of conidial germination in response to carbon source sensing. Additional results suggest that cAMP has targets other than PkaA and SchA and that PkaA retains activity in the absence of cAMP. Conversely, PkaA regulates cAMP levels in *A. nidulans* because these are elevated by ≈ 250-fold in a strain that lacks PkaA. Furthermore, analysis of mutant strains impaired in both adenylate cyclase and RasA GTPase previously implicated in the control of *A. nidulans* spore germination suggested that RasA and cAMP signalling proceed independently during germination in *A. nidulans*.

Introduction

Germination of asexual spores (conidia) constitutes the first step in the colonization process of a new environment by filamentous ascomycetes. Although the study of conidial germination has attracted growing interest in recent years, expression studies or mutational analyses have only yielded scarce information on the signal transduction pathways that are involved during this process (d'Enfert, 1997; Harris, 1999; Momany and Taylor, 2000).

Analysis of the *rasA* gene encoding a homologue of the human ras proto-oncogene has revealed a role for ras signalling during conidial germination in *Aspergillus nidulans* (Som and Kolaparthi, 1994). Indeed, *A. nidulans* mutants overproducing a dominant active form of RasA ($RasA^{G17V}$) produce giant swollen spores with multiple nuclei but do not undergo germ tube formation, suggesting that RasA controls the switch from isotropic to polar growth that is normally observed during conidial germination. Recent results from Osherov and May (2000) show that overproduction of $RasA^{G17V}$ can bypass the carbon source requirement that triggers morphological changes at the onset of germination, suggesting that RasA signalling might also mediate carbon source sensing during germination.

In fungi, cAMP signalling has been involved in such diverse cellular processes as stress response, metabolism, development and virulence (reviewed by Thevelein and de Winde, 1999; Lengeler *et al*., 2000). cAMP signalling also controls many of the events that are linked to growth resumption and is therefore likely to regulate fungal spore germination. Involvement of cAMP signalling during the germination of ascospores in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has indeed been demonstrated (Thevelein, 1984a; Herman and Rine, 1997; Hatanaka and Shimoda, 2001). In contrast, conflicting results have been obtained for conidia from filamentous ascomycetes, probably because most of the studies have relied on analogues of cAMP or chemical inhibitors of protein kinase A (PKA) (Ruan *et al*., 1995; Bruno *et al*., 1996; Yang and Dickman, 1997; Hall *et al*., 1999; Osherov and May, 2000). Genetic evidence for involvement of the cAMP pathway in conidial germination has been obtained in *Neurospora crassa*, in which the *mcb* mutation, which affects the regulatory subunit of the cAMP-dependent PKA in an undefined manner, results in an abnormal morphology of germinating spores, thus suggesting that cAMP signalling controls

Accepted 8 February, 2002. *For correspondence. E-mail denfert@pasteur.fr; Tel. (+33) 1 40 61 32 57; Fax (+33) 1 45 68 87 90. †Present address: Unité de Phytopharmacie et Médiateurs Chimiques, INRA Versailles-Grignon, Route de St-Cyr, 78026 Versailles Cedex, France.

the switch from isotropic to polar growth (Bruno *et al*., 1996). Yet, the study of trehalose breakdown in *N. crassa* and *A. nidulans* suggests a possible involvement for cAMP signalling at an earlier stage during the germination process. Breakdown of the trehalose pool in spores is one of the earliest metabolic events observed at the onset of germination (Schmit and Brody, 1976; Thevelein, 1984b; d'Enfert, 1997). In *A. nidulans* and *N. crassa*, trehalose breakdown is catalysed by a neutral trehalase activated in a cAMP-dependent manner, as is also the case in *S. cerevisiae*, *S. pombe* and other yeasts (Kopp *et al*., 1993; Cansado *et al*., 1998; d'Enfert *et al*., 1999). This suggests that cAMP signalling is involved at the onset of conidial germination in filamentous fungi. Consistent with this assumption are the recent results of de Pinho *et al*. (2001), who have shown that trehalose breakdown during germination of *N. crassa* conidia is impaired in the *mcb* mutant and *cr-1* mutant, deficient in adenylate cyclase.

In order to get a better picture of the role that cAMP signalling might play during conidial germination, we have initiated a detailed investigation of the components of cAMP signalling in the model filamentous fungus *A. nidulans*. Among eukaryotes, the components of the cAMP signalling cascade are well conserved (Francis and Corbin, 1994; van Biesen *et al*., 1996; Thevelein and de Winde, 1999). The main route that leads to cAMP signalling is: (i) the sensing of an external signal through a G-protein coupled receptor; (ii) the subsequent activation of the Gα-subunit of a heterotrimeric G-protein; and (iii) the activation of adenylate cyclase, which is responsible for cAMP synthesis. cAMP binding to the regulatory subunit of PKA causes its dissociation from the catalytic subunit (PKAc). Active PKAc controls protein activities via phosphorylation of conserved Ser and Thr residues. In *S. cerevisiae*, multiple PKAc genes have been identified that perform different but overlapping functions (Toda *et al*., 1987; Robertson and Fink, 1998; Pan and Heitman, 1999; Robertson *et al*., 2000). Furthermore, Ser/Thr protein kinases that can suppress defects resulting from PKAc deletion have been characterized in *S. cerevisiae* (Sch9; Toda *et al*., 1988) and *S. pombe* (Sck1/Sck2; Jin *et al*., 1995; Fujita and Yamamoto, 1998). These kinases are members of the AGC family of Ser/Thr protein kinases and are more closely related to mammalian PKB/Akt (Hanks and Hunter, 1995; Fabrizio *et al*., 2001). Yet, the nature of the link between Sch9-like kinases and cAMP signalling is still unclear (Kraakman *et al*., 1999; Lorenz *et al*., 2000; Fabrizio *et al*., 2001). Another signalling interaction has been demonstrated in *S. cerevisiae*, in which adenylate cyclase activity is also under the control of the GTPases Ras1 and Ras2 (Broek *et al*., 1985; Toda *et al*., 1985). However, a similar control of adenylate cyclase by ras homologues has not been demonstrated in other eukaryotes (Thevelein and de Winde, 1999; Lengeler *et al*., 2000). In the slime mould *Dictyostelium discoideum*, RasC was shown to regulate adenylate cyclase activity through regulation of the heterotrimeric G-proteins (Lim *et al*., 2001).

When this study was initiated, only genes encoding a ras homologue (*rasA*; Som and Kolaparthi, 1994) and a PKA catalytic subunit (*pkaA*; Shimizu and Keller, 2001) had been characterized in *A. nidulans*. Although PkaA had been shown to control hyphal growth, asexual development and secondary metabolism, its role during spore germination had not been investigated (Shimizu and Keller, 2001). In this paper, we have studied (i) the role of the adenylate cyclase (CyaA), the cAMP-dependent protein kinase catalytic subunit (PkaA) and a Sch9-like kinase (SchA) during *A. nidulans* conidial germination using strains with null mutations in the corresponding genes; (ii) the relationships between these signalling elements; and (iii) the relationship between cAMP signalling and RasA signalling. Taken together, our results suggest that cAMP and RasA signalling act independently to control *A. nidulans* spore germination in response to carbon source sensing.

Results

Characterization of novel components of the cAMP signalling cascade in A. nidulans

Inspection of *A. nidulans* EST sequences available from the *Aspergillus nidulans* cDNA Sequencing Project (Prade *et al*., 2001) revealed a sequence encoding a polypeptide with homology to the *S. cerevisiae* Sch9 kinase (c6c06; 65.8% identity over 123 amino acids). The corresponding genomic fragment of chromosome VIII was sequenced (GenBank accession no. AY043352; for details, see *Experimental procedures*). Comparison of the amino acid sequence with protein databases revealed strong similarity to the *Botrytis cinerea* Pka2 protein (51% identity, 63% similarity), previously annotated as a PKA catalytic subunit (GenBank CAC03748), to the *S. cerevisiae* Sch9 kinase (Toda *et al*., 1988) and to the *S. pombe* Sck1 protein (Jin *et al*., 1995), suggesting that the cloned *A. nidulans* gene (referred to as *schA*) encodes a genuine homologue of Sch9 and related kinases. A homologue of the SchA protein was also identified in the *N. crassa* genome sequence (contig 1.9, position 115 591, http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). A distinctive feature of the *A. nidulans*, *B. cinerea* and *N. crassa* proteins and of the *S. cerevisiae* Sch9 and *S. pombe* Sck1/Sck2 is the occurrence in their amino-terminal moiety of residues typical of phospholipid-binding domains also found in phospholipases and protein kinases C (Fig. 1A: C2-like domain; Rizo and Südhof, 1998). The carboxy-terminal moiety shows a high

Table 1. Adenylate cyclase domain conservation [percentage of identical (similar) amino acids in the *A. nidulans* CyaA protein and related fungal adenylate cyclases][a].

Species	*M. grisea*[b]	*N. crassa*[b]	*B. cinerea*[b]	*S. cerevisiae*[b]
Domain				
LRR[c]	49 (63)	49 (61)	53 (66)	28 (40)
PP2C[c]	64 (76)	57 (70)	50 (64)	37 (54)
CYCc[c]	69 (78)	65 (76)	65 (72)	57 (66)

a. The five proteins were aligned using the CLUSTALW program, and the functional domains were assigned according to those in the *S. cerevisiae* Cyr1 protein (P08678). These defined regions were aligned again using the CLUSTALW/PILEUP programs.
b. Accession numbers: *M. grisea*, AF006827; *N. crassa*, Q01631; *B. cinerea*, CAB77164; *S. cerevisiae*, P08678.
c. LRR, leucine-rich region; PP2C, protein phosphatase 2C; CYCc, adenylate cyclase catalytic domain. The *A. nidulans* CyaA domains correspond to positions 796–1288 (LRR), 1384–1670 (PP2C) and 1617– 1897 (CYCc).

degree of conservation with the catalytic domain of other Ser/Thr kinases (Fig. 1B: S_TKc; Taylor and Radzio-Andzelm, 1994; Hanks and Hunter, 1995) including PKA catalytic subunits. In contrast, the C-terminal extension of Sch9-like kinases harbouring an extension to the Ser/Thr kinase domain (S_TK_X; Fig. 1B) differs significantly from that of PKA catalytic subunits.

No EST could be identified displaying homology to fungal adenylate cyclases. Oligonucleotides were designed that correspond to highly conserved amino acid sequences in fungal adenylate cyclases and were used to amplify a region of the *A. nidulans* genome leading to the identification of the corresponding gene localized on chromosome II (GenBank accession no. AY043351). The resulting 2132-amino-acid protein shows a modular architecture similar to that of other fungal adenylate cyclases with a leucine-rich repeat domain (LRR, position 796–1288), a protein phosphatase 2C domain (PP2C, position 1384–1670) and an adenylate cyclase catalytic domain (CYCc, position 1617–1897) (Table 1), suggesting that the cloned *A. nidulans* gene (referred to here as *cyaA*) encodes a genuine adenylate cyclase.

Construction of A. nidulans *strains with null mutations at the* schA *and* cyaA *loci*

Null mutations at the *schA* and *cyaA* loci were introduced by gene replacement using the procedure described by Chaveroche *et al*. (2000) (for details, see *Experimental procedures*). Although *A. nidulans schA*Δ strains had a colony morphology similar to that of an isogenic wild-type strain, *A. nidulans cyaA*Δ strains formed small colonies with a dense mycelium and few conidiophores (Fig. 2A), suggesting that CyaA is involved in both vegetative growth and asexual development. Interestingly, the colony morphology of the *A. nidulans cyaA*Δ mutant differs markedly from that of the *A. nidulans pkaA*Δ mutant that lacks PKAc (Shimizu and Keller, 2001). Indeed, the *A. nidulans pkaA*Δ mutant forms small colonies that are densely sporulated (Shimizu and Keller, 2001; Fig. 2A). In addition, *pkaA*Δ mutants show a certain degree of thermosensitivity, which is not observed in the *cyaA*Δ mutant (Fig. 2A). Determination of cAMP levels in mycelium of *A. nidulans* wild-type and *cyaA*Δ strains showed that cAMP synthesis is fully abolished in the mutant strain (Table 2), thus suggesting that *cyaA* encodes the unique adenylate cyclase active during mycelial growth.

cAMP signalling is required for efficient germination of A. nidulans *conidia*

In order to evaluate the role of cAMP signalling during conidial germination, trehalose breakdown and germ tube outgrowth were evaluated for *A. nidulans cyaA*Δ, *pkaA*Δ and *schA*Δ conidia germinated in minimal glucose medium. Results presented in Fig. 2B show that the kinetics of trehalose breakdown are slower in the *schA*Δ and *pkaA*Δ conidia than in wild-type conidia, but that most of the trehalose pool is eventually degraded. In contrast, degradation of trehalose is impaired in the *cyaA*Δ conidia, with the trehalose pool remaining unaffected after 2.5 h of germination. Results presented in Fig. 2C show that the *schA*Δ mutation does not affect the kinetics of germ tube outgrowth, whereas the *pkaA*Δ mutation results in slower kinetics. This reduction is more pronounced in the *cyaA*Δ mutant. Taken together, these results demonstrate that adenylate cyclase and PKAc are important determinants of conidial germination in *A. nidulans* controlling various aspects of this process.

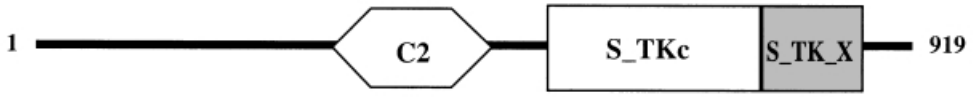
PkaA and SchA have overlapping functions during germination

As Sch9 and Sck1 are multicopy suppressors of defects resulting from PKA deletion in *S. cerevisiae* and *S. pombe*, respectively (Toda *et al*., 1988; Jin *et al*., 1995), we tested whether the *schA* deletion phenotype would be

Table 2. cAMP levels in *A. nidulans* strains defective for adenylate cyclase and/or cAMP-dependent protein kinase (values are the average of three independent assays, and standard deviations are indicated).

Strain	cAMP (pmol mg^{-1} protein)
CEA178 (*wA3 pyroA4*)	163 ± 30
CEA201 (*cyaA*Δ::*pyrG*$^+$)	ND
CEA198 (*wA3 pyroA4 pkaA*Δ::*argB*$^+$)	42 600 ± 3000
CEA192 (*wA3 pyroA4 pkaA*Δ::*argB*$^+$ *cyaA*Δ::*pyrG*$^+$)	ND

ND, not detectable.



A

```
SchA_Neucr  319:KLtIkvGEArGfRRCRDPYVVVVFQRSELiSPGPRRAAENCDD.......AAIAAVQTGGIPIQRQSSdSGrPMAIPmrSrQSSNTSrTDFNTFRRRNS
Pka2_Botci  320:KLtvkINEArGfRRSVDPYVVAVFQRNELySKGPQSEEDDeDDGNSHSPGGIPIGGIPIGGIPITRSGSdSGrSMAIPMKSrQSSNTSlsEYDFK....
SchA_Emeni  350:KLHvkISEgKGLQPgFDPYVVCVEWNEVHS...KSVQDEQQE............SLKRQQKELEQSALdSGrPMAIPMN.LQSSHNNTLESTDHR....
KPC1_Botci  261:QLVlrIQAVKDVDhaTTS....RFTR.....................................................GPeTLIAvK...VEDNVvArtKNSR....
KPC1_Aspng  249:LLtlrIHAVKDVDhaASS....RFSR.....................................................GPeTFIVlK...VEDTIKArtKATR....
KPC1_Neucr  249:TLsIrILAVKDVDhaPLG....RFAR.....................................................SPeTFIAvK...AEDIVvArtKPSR....
PLC1_Maggr  709:LFsIDLISAQQLMRPHNLRDRHTLDPYIEVEAFLADDKRNTNK.....................EYSDAGPS....LTKHKTPiAkDNGFN....
PLC1_Botci  749:NFsIDLISAQQLMRPKGLPSNRSVDPYIEVEVYHADDKSKTKG.....................VIGEGGLDASakGSSGIGAPHKRRTHIVQENGFN....

SchA_Neucr  411:SQSRRSFTNPTWdAEAVFDVVDSDMLvdisVY........GQGPSGEEFLGHVDFQANKAE....PIrGWFPLQGHADTMAE
Pka2_Botci  416:NKGRKMFTPPkWdAEAVFDVVGSDTRVNiTiY........DrAQQAEEFLGHVDLQANLVENVdAPVSGWFQLRGKDMTSNA
SchA_Emeni  430:..GHAPITDPhWNHEAVFDVFGDQSEIdvsVY........DrNDS.EAFLGHVRLcVNLKEd.NSRlDGW.............
KPC1_Botci  305:........TDkWeNEIHNIYVDKANEIelTVY........DkAGQQALPiGLIWvriSDIVe.eLRRKRI.............
KPC1_Aspng  293:........IDrWQDETFNIDiDKANEIelTVY........DkSGDrPTPiGLIWvriSDIAe.eLRRKKI.............
KPC1_Neucr  293:........NDkWeAEFHTFPVDKTNEIeFTVY........DkPAchPVPiaLIWvriSDIVe.eLRRKKI.............
PLC1_Maggr  775:.........PIfdQRYNFNFNTKYPdlIFIRWTVRLS.EGGSQSDrSTILG..TESvNKIRSLGRTVFN.............
PLC1_Botci  873:.........PVENKKFNPAITTKFPEIVFVRWTVRCSSDGHSYNDKTLPLaTYTAKLSSIKQGYRTTPL.............
```

B

```
PkaA_Emeni  140:.................................NTPQVSR.AERTTKGKYtLdDFAIQRTLGTGSFGRVHLVQSKHNhRyYAiKVLKKAQVVkmKQiEHT
PkaC_Aspng  143:.............................SGHTQSHHAGRSDARTTKGKYsLdDFSIQRTLGTGSFGRVHLVQSKHNhRfYAvKVLKKAQVVkmKQiEHT
CpkA_Maggr  202:.............................HVNHGHQGSQDQQTRVTKGKYsLtDFeILRTLGTGSFGRVHLVQSrHNQRfYAvKVLKKAQVVkmKQVEHT
Tpk1_yeast   73:.........................................QARVISGKYsLQDFQILRTLGTGSFGRVHLirSrHNQRyYAMKVLKKAiVVrlKQVEHT
Pka2_Botci  461:DLQANLVENVDAPVSGWFQLRG.KDMTSNAGNVGEVHYEISFQRTEFRhYGPdDFQILkpIGKGtFGQVYQVRKKDTkRIYAMKVLQKkviVQKKeVAHT
SchA_Ncras  456:DFQANKAE....PIRGWFPLQGHADTMAENAPTGELYYEAHYQRAEQKhfGPdDFQILRLIGKGtFGQVYQVRKKDTGRIYAMKVLSKkviVQKKeVAHT
SchA_Emeni  472:RLCVNLKED.NSRLDGWFPLKG..RAAGDSRVSGEIHYEMRFEkTPKQVGPNDFQILkpIGKGtFGQVYQVKKKDTrRIYAMKVLSKkvilQKKeVAHT

PkaA_Emeni  207:NDERMLN...RVRHPFlVnLWGTwQDARnLYMVMDfvEGGELFSLLRKSQRFPNPVAKFYAAEVTLALEYLHSLnIIYRDLKPENlLLDrHGHlKITDF
PkaC_Aspng  215:NDERMLN...RVRHPFliTLWGTwQDSRnLYMVMDfvEGGELFSLvRKSQRFPNPVAKFYAAEVTLALEYLHTQnIIYRDLKPENlLLDrHGHlKITDF
CpkA_Maggr  274:NDERMLG...EVKNPFliTLWGTFQDCRnLYMVMDfvEGGELFSLLRKSGRFPNPVAKFYAAEVTLALEYLHAknIIYRDLKPENlLLDrHGHlKITDF
Tpk1_yeast  133:NDERMLS...IVTHPFIiRmWGTFQDAQqifMiMDYiEGGELFSLLRKSQRFPNPVAKFYAAEVCLALEYLHSkDIIYRDLKPENILLDkNGHIKITDF
Pka2_Botci  560:VGERNILVRTATADSPFIVGLKFSFQTPTDLYlVTDYmSGGELFWHLQKEGRFDeQRAKFYIAElILALEHLHKhDIvYRDLKPENILLDANGHIALCDF
SchA_Ncras  552:VGERNILVRTAMSDSPFIVGLKFSFQTPSDLYlVTDYmSGGELFWHLQKDGkFEeKRAKFYIAElILAiqHLHENDIvYRDLKPENILLDANGHIALCDF
SchA_Emeni  569:VGERNILVRTAMAASPFIVGLKFSFQTPTDLYlVTDYmSGGELFWHLQKEGRFQeFRAKFYIAElAMALqHLHDhDIvYRDLKPENILLDANGHIALCDF

PkaA_Emeni  304:GFAKRVP...DTTwTLCGTPDYLAPEVVASK.GYNKSVDWWSLGILIFEMLCGftPFwDQGSPvKiYQNIIAGrikFP.PyLHPDaVDLLSRLITSDLtk
PkaC_Aspng  312:GFAKRVP...DTTwTLCGTPDYLAPEVVSSK.GYNKSVDWWSLGILIFEMLCGftPFwDSGSPvKiYENIIRGrVkyP.PyLHPDaVDLLSQLITADLtk
CpkA_Maggr  371:GFAKRVP...DXTwTLCGTPDYLAPEVVSNK.GYNKSVDWWSLGILIyEMLCGyPPFwDSGSPmKiYENILKGKVRyP.AyiNPDaQDLLQRLITADLtk
Tpk1_yeast  230:GFAKQVP...DVTyTLCGTPDYiAPEVVSTK.pYNKSiDWWSFGILIyEMLAGytPFYDS.NTmKTYEKILNaPLRFP.PfFNEDVkDLLSRLITRDLsQ
Pka2_Botci  660:GLsKANLTKNATTNTFCGTpeYLAPEVlLDEAGYTKMVDfWSLGvLvFEMCCGwsPFYAE.DTQQMYKNIAFGKVRFPRDTLTTegrnFVKGLlNRnPKh
SchA_Ncras  652:GLsKANLTKNDTTNTFCGTteYLAPEVlLDEAGYTKMVDfWSLGvLvFEMCCGwsPFYAE.DTQQMYKNIAFGKVRFPRDTLSLegrnFVKGLlNRnPKh
SchA_Emeni  669:GLsKANLTQNDTTNTFCGTteYLAPEVlLDEQGYTKMVDfWSLGvLvFEMCCGwsPFYAE.DTQQMYKNIAFGKVRFPRDALSTegrnFVKGLlNRnPKh

PkaA_Emeni  399:RLGNLHGCPdDiKNHPWFAEVTWDrLYRKeIdAPYVPPirGCGDASQyeNYQEESEPYGQAGEDPHGHLFPDF.....................
PkaC_Aspng  407:RLGNLHGCSdDvKNHPWFAEVTWDrLARKdIdAPYVPPirGCGDASQyDRYPEETEqYGMAGEDPHGHLFPDF.....................
CpkA_Maggr  466:RLGNLYGCSQDvrNHPWFAEVTWDrLARKdIdAPYTPPvKACAGDASQFDRYPEETERYGQtGHDEYGNLFPGF.....................
Tpk1_yeast  324:RLGNLQNGTeDvKNHPWFKEVVWekLLSrNIeTPYEPPiQQCQGDTSQFDKYPEEDinYGVQGPDPYADLFRDF.....................
Pka2_Botci  759:RLG.ATDDAeelKRHPfFQdiDWdALYRKEVAPPFKPKIKS.ETDTSnFDPEFTAeTPVDSPDDSALNGSSLNARAAALAAG.MATSTPLSPGMQANFKGFTFVDESSI
SchA_Ncras  751:RLG.ATDDAeelKRHAfFAdiDWdALSKKLITPPFKPQLKS.DTDVSYFDPEFTAeTPVDSPDDSALNGSSLNGRAAALARG.YATSTPLSPSVQANFQGFTFVDESAL
SchA_Emeni  768:RLG.AQNDAKeLMAHPFFHdiDWeALCRKEVIPPFKPKIKS.DTDTSnFDPEFTAeTPVENSNSLNDRAAALANGFMAASTPLSPGMQANFQGFTFVNESSI

PkaA_Emeni      :.....................................................................
PkaC_Aspng      :.....................................................................
CpkA_Maggr      :.....................................................................
Tpk1_yeast      :.....................................................................
Pka2_Botci  856:EENMKDRIKDEYEDRMDQDNNDPER..DWEDTFDIPDKSRMDRMSGVVRNHNEDERMFNGDDFH.
SchA_Ncras  848:EENMHDRHYNRYEDDEMDDVADRRRTDDWEDVHG.ADQRAANRMSGVVKSNTTEDQMFGGTNFDP
SchA_Emeni  866:DHHFKNEVGDRMDEDYEPMHRSHRS..........GNSIDHRMAGVQKTGDAGEIFNVDDNFDM
```

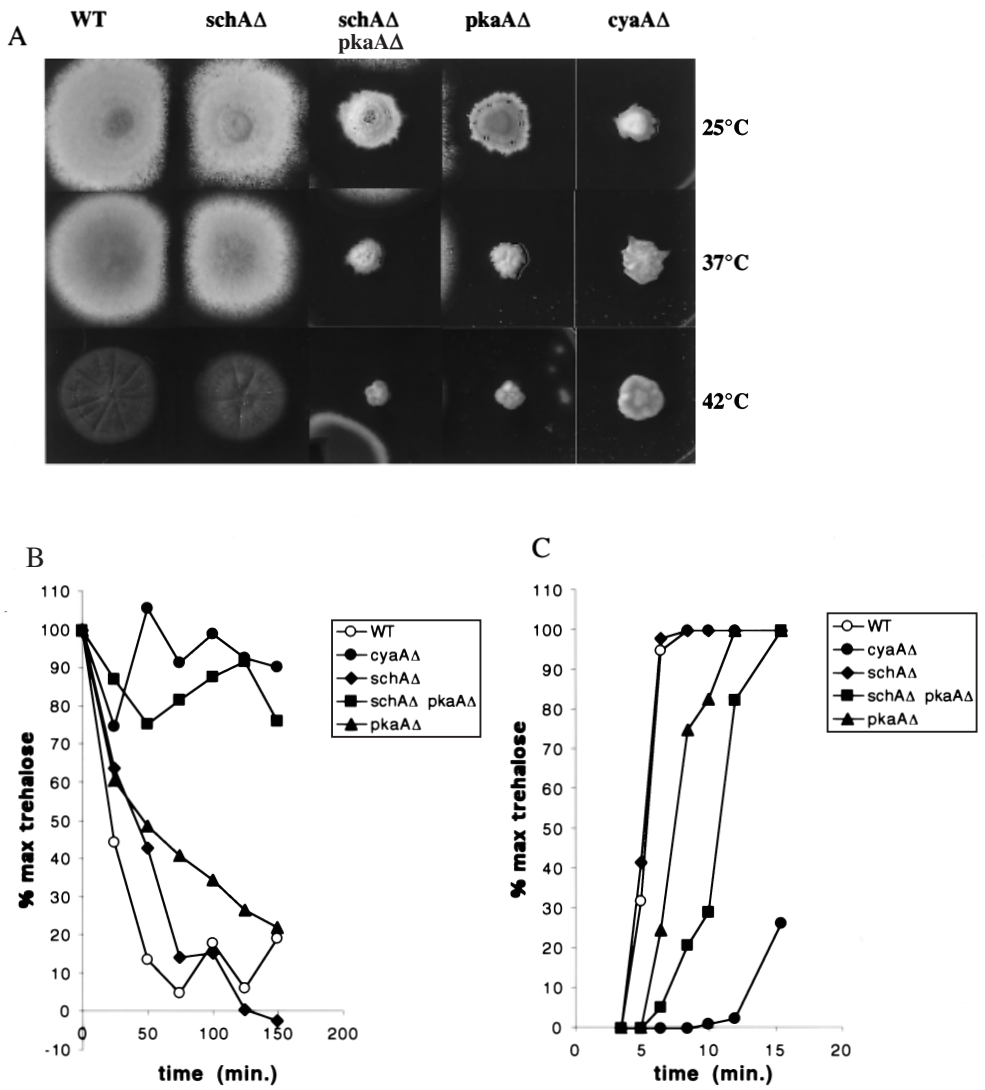


Fig. 2. Mutations in the *cyaA*, *pkaA* and *schA* genes impair colonial morphology, trehalose breakdown and germ tube outgrowth.
A. Colonial morphology of *A. nidulans* strains CEA178 (WT), CEA182 (*schA*Δ), CEA184 (*schA*Δ *pkaA*Δ), CEA198 (*pkaA*Δ) and CEA196 (*cyaA*Δ) on complete glucose medium after 3 days of incubation at 37°C and 42°C or 5 days at 25°C.
B. Kinetics of trehalose breakdown in germinating conidia of *A. nidulans* strains CEA178, CEA182, CEA184, CEA198 and CEA196 inoculated in minimal glucose medium at 37°C. Trehalose levels in each sample were normalized for the trehalose content in resting conidia (100%) of the corresponding strain.
C. Kinetics of germ tube outgrowth in *A. nidulans* strains CEA178, CEA182, CEA184, CEA198 and CEA196 inoculated in minimal glucose medium at 37°C. The number of conidia showing a germ tube was recorded at different times in at least two microscopic fields and is presented as a percentage of the total number of conidia in these fields. Results are representative of two independent experiments.

Fig. 1. Conserved domains in the *A. nidulans* SchA protein.
A. C2-like domains in the amino-terminal region of *A. nidulans* SchA and related Sch9-like protein kinases, in fungal protein kinases C and phospholipases C.
B. Ser/Thr protein kinase domains of fungal cAMP-dependent protein kinases and Sch9-like protein kinases. Alignments were produced using the PILEUP program of the UWGCG package version 9 (Devereux *et al.*, 1984). Conserved residues (identical, upper case letters; similar, lower case letters) have a black background (thresholds are of 30% in A and 50% in B). The regions corresponding to the catalytic domain (S_TKc) and to the extension to Ser/Thr-type protein kinases (S_TK_X; Taylor and Radzio-Andzelm, 1994; Hanks and Hunter, 1995) are indicated by the double line and the grey line respectively. Sch9-like proteins: SchA_Emeni, *A. nidulans*; Pka2_Botci, *B. cinerea* (CAC03748); SchA_Neucr, *N. crassa* (see text). Protein kinases C: KPC1_Aspng, *A. niger* (AAA97433); KPC1_Botci, *B. cinerea* (CAB59301); KPC1_Neucr, *N. crassa* (CAA72731). Phospholipases C: PLC1_Botci, *B. cinerea* (AAB39564); PLC1_Maggr, *M. grisea* (AAC72385). PKAc: PkaA_Emeni, *A. nidulans* (AF262987); PkaC_Aspng, *A. niger* (CAA64172); CpkA_Maggr, *M. grisea* (AAA93199); Tpk1_yeast, *S. cerevisiae* (P06244).

enhanced in a *pkaA*Δ background. A double mutant, *schA*Δ *pkaA*Δ, was constructed by genetic crossing. As shown in Fig. 2A, the resulting strain shows the same colony morphology and the same temperature sensitivity profile as the *pkaA*Δ single mutant. Interestingly, however, inactivation of *schA* in a *pkaA*Δ background almost completely blocks trehalose breakdown (Fig. 2B) and increases the delay in germ tube formation in comparison with the *pkaA*Δ strain (Fig. 2C). These results indicate that the two protein kinases are involved in the germination process and that PkaA or SchA can fulfil part of the functions of SchA or PkaA respectively.

Relative roles of CyaA and PkaA during germination

Because *A. nidulans* strains with a null mutation in the *cyaA* or *pkaA* gene display distinct defects during germination and colony formation (see above), we investigated the relationship between CyaA and PkaA. First, we evaluated whether overproduction of PkaA can bypass the need for cAMP synthesis during germination. An ethanol-inducible and glucose-repressed *alcA(p)–pkaA* allele (Shimizu and Keller, 2001) was introduced into a *cyaA*Δ strain, and spore germination was monitored under inducing conditions. Results presented in Fig. 3 show that elevated levels of PkaA accelerate germination kinetics in a *A. nidulans* strain lacking adenylate cyclase. Interestingly, highly asynchronous germination is observed in the spore population of *cyaA*Δ strains, whatever the expression level of *pkaA*, with some spores that do not appear to produce germ tubes (Fig. 3). However, all the spores of the *cyaA*Δ and *cyaA*Δ *alcA(p)–pkaA* strains will germinate eventually (data not shown).

Secondly, the relationship between PkaA and CyaA was investigated through a comparative analysis of mutant strains in which *cyaA* or *pkaA* or both genes are deleted. Figure 4A shows that the germination delay of conidia from the double mutant in minimal glucose medium is slightly more pronounced than the delay observed for *cyaA*Δ conidia. Vegetative growth is also affected by both mutations in an additive way as shown in Fig. 4B. Therefore, the *cyaA*Δ and *pkaA*Δ mutations have additive effects on germ tube outgrowth and vegetative growth. Measurements of cAMP accumulation in strains CEA178 (WT), CEA201 (*cyaA*Δ), CEA198 (*pkaA*Δ) and CEA192 (*cyaA*Δ *pkaA*Δ) revealed an increase of ≈ 250-fold in a *pkaA*Δ strain in comparison with a wild-type strain (Table 2), showing that PkaA regulates cAMP levels in *A. nidulans*. Yet, the feedback inhibition exerted by PKA on cAMP levels is unlikely to account for the phenotypic differences observed between the *pkaA*Δ and *cyaA*Δ mutants, as cAMP is undetectable in the *pkaA*Δ *cyaA*Δ and *cyaA*Δ mutants, which are phenotypically distinct for both germination and colony morphology (Table 2 and Fig. 4B).

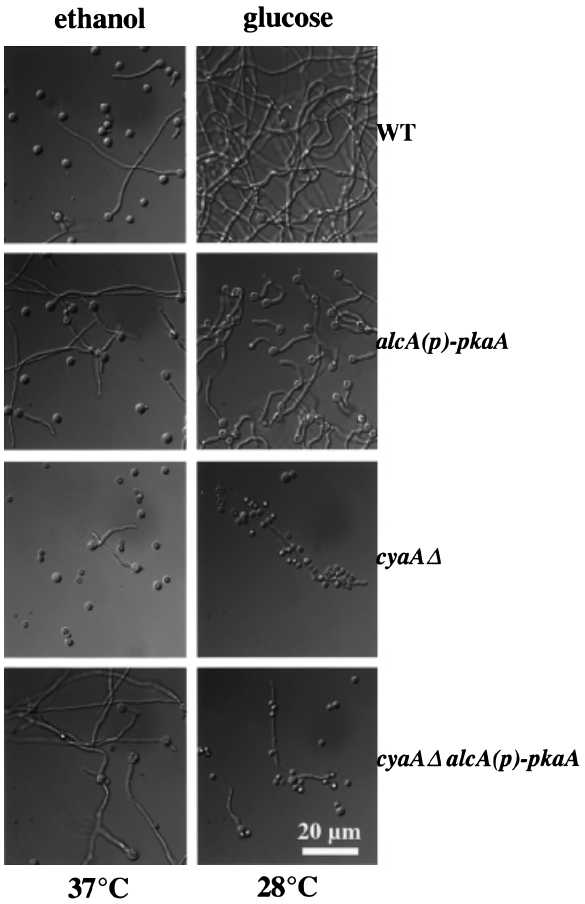


Fig. 3. Overexpression of PkaA partially alleviates the germination defect of an *A. nidulans* strain lacking adenylate cyclase. Conidia of *A. nidulans* strains CEA209 (WT), TKIS20.1 (*alcA*(*p*)*–pkaA*), CEA221 (*cyaA*Δ) and CEA217 (*cyaA*Δ *alcA*(*p*)*–pkaA*) were germinated in minimal medium with ethanol or glucose as carbon source. Pictures were taken after 16 h of growth at 37°C (ethanol) or 28°C (glucose), and representative pictures obtained by light microscopy are presented.

Taken together, these results suggest that (i) PkaA is one of several targets of cAMP synthesis that are required for efficient spore germination and colony formation in *A. nidulans*; (ii) PkaA in *A. nidulans* retains some activity in the absence of cAMP during spore germination; (iii) PkaA regulates cAMP levels in *A. nidulans*; and (iv) additional signalling pathways are involved in the spore germination process.

RasA acts independently of CyaA

In *S. cerevisiae*, the cAMP/PKA pathway is regulated in part by the Ras1/2 GTPases (Broek *et al*., 1985; Toda *et al*., 1985). In *A. nidulans*, overexpression of the dominant-active (*rasA*^{G17V}) or dominant-negative forms (*rasA*^{S22N}) of the homologous *rasA* gene results in alterations in the germination process and in other developmental transitions (Som and Kolaparthi, 1994; Osherov and May,

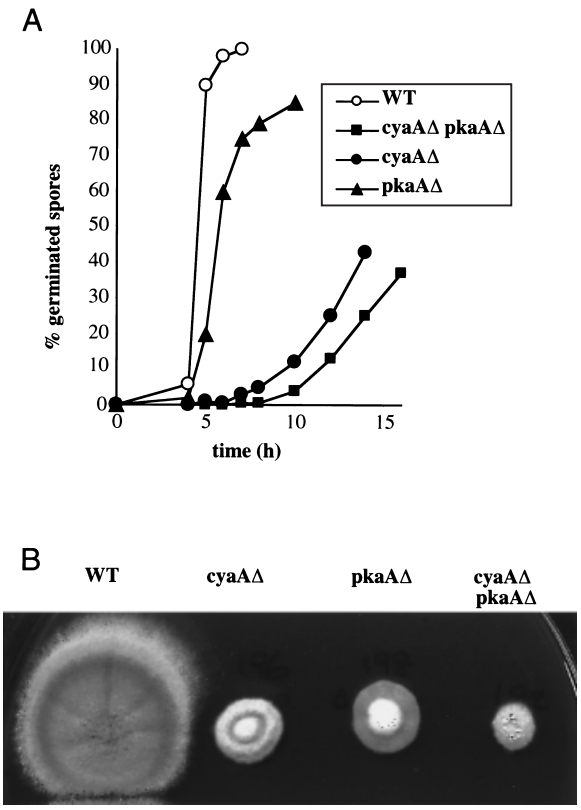


Fig. 4. Simultaneous inactivation of the *A. nidulans cyaA* and *pkaA* genes results in synthetic defects during spore germination and colony formation.
A. Kinetics of germ tube outgrowth in *A. nidulans* strains CEA178 (WT), CEA196 (*cyaA*Δ), CEA198 (*pkaA*Δ) and CEA192 (*cyaA*Δ *pkaA*Δ) inoculated in minimal glucose medium at 37°C. The number of conidia showing a germ tube was recorded at different times in at least two microscopic fields and is presented as a percentage of the total number of conidia in these fields. Results are representative of two independent experiments.
B. Colonial morphology of *A. nidulans* strains CEA178, CEA196, CEA198 and CEA192 on complete medium with glucose as the carbon source after a 3 days incubation at 37°C.

2000). In order to establish whether the function of RasA during spore germination is also mediated through adenylate cyclase in *A. nidulans*, we created *alcA(p)–rasA*^{G17V} *cyaA*Δ and *alcA(p)–rasA*^{S22N} *cyaA*Δ double mutants and observed their germination behaviour. Figure 5A shows that the *alcA(p)–rasA*^{S22N} and *cyaA*Δ mutations have additive effects, resulting in slower germination kinetics than those observed in the single mutant strains. Conversely, the dominant-active *alcA(p)–rasA*^{G17V} allele blocks germ tube development even in the absence of a functional adenylate cyclase (Fig. 5A). Microscopic examinations of germinating *alcA(p)–rasA*^{G17V} and *alcA(p)–rasA*^{G17V} *cyaA*Δ spores show comparable phenotypes with giant multinucleated swollen spores (Fig. 5B). These results suggest that *rasA* regulates germination independently of adenylate cyclase. This hypothesis was corroborated by cAMP measurements in the *alcA(p)–rasA*^{G17V} mutant (Table 3).

The shift from *alcA(p)–rasA*^{G17V} repressing conditions (glucose) to inducing conditions (ethanol) results in a twofold increase in intracellular cAMP in the wild-type and mutant strains, showing that this increase is independent of RasA activation.

Trehalose breakdown results from carbon source sensing

Osherov and May (2000) have proposed that RasA sig-

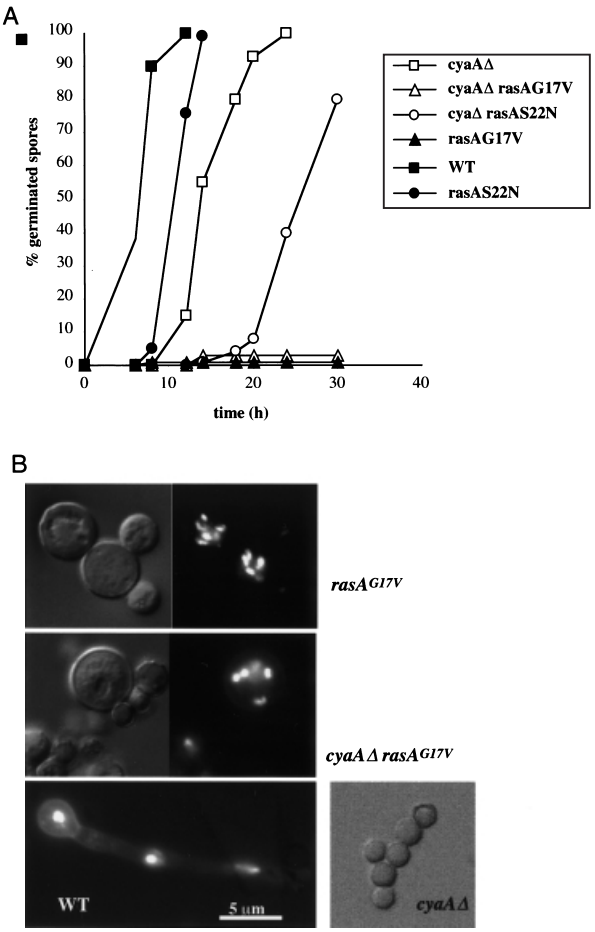


Fig. 5. RasA and cAMP signalling act independently during *A. nidulans* conidial germination.
A. Kinetics of germ tube outgrowth in *A. nidulans* strains CEA204 (WT), CEA201 (*cyaA*Δ), CEA199 (*cyaA*Δ *rasA*^{G17V}), CEA202 (*cyaA*Δ *rasA*^{S22N}), CEA200 (*rasA*^{G17V}) and CEA203 (*rasA*^{S22N}) inoculated in minimal ethanol medium at 37°C. The number of conidia showing a germ tube was recorded at different times in at least two microscopic fields and is presented as a percentage of the total number of conidia in these fields. Results are representative of two independent experiments.
B. Conidia of *A. nidulans* strains CEA204 (WT), CEA201 (*cyaA*Δ), CEA200 (*rasA*^{G17V}) and CEA199 (*cyaA*Δ *rasA*^{G17V}) were germinated for 18 h at 37°C in minimal medium with ethanol as carbon source. Representative pictures obtained by light transmission or fluorescence microscopy after staining with Hoechst 33258 are shown. In the case of CEA201 (*cyaA*Δ), one field of ungerminated conidia is shown to give a point of comparison for conidia diameter.

Table 3. Overexpression of an activated form of RasA does not trigger cAMP synthesis (values are the average of three independent assays, and standard deviations are indicated).

	cAMP (pmol mg^{-1} protein)	
Carbon source	Glucose	Ethanol
Strain		
CEA204 (wild type)	95 ± 40	152 ± 60
CEA200 (*argB*$^{+}$::*alcA(p)–rasA*^{G17V})	69 ± 15	153 ± 90

nalling is required for carbon source sensing at the onset of spore germination. The results presented above suggest that RasA signalling is independent of cAMP synthesis. In order to test whether adenylate cyclase is nevertheless activated in response to carbon source addition, we evaluated the requirements for the activation of trehalase, which is a direct outcome of the activation of the cAMP/PKA pathway during germination. Figure 6A shows that the addition of glucose alone is sufficient to trigger trehalose breakdown and that this process is independent of the addition of a nitrogen source. Furthermore, trehalose breakdown can be activated by the addition of 6-deoxyglucose, a non-metabolizable analogue of glucose that cannot trigger subsequent steps of spore germination (Osherov and May, 2000). In order to confirm that trehalose breakdown can be induced independently of the metabolization of the carbon source, we evaluated the ability of an *A. nidulans* strain devoid of hexokinase and glucokinase, and hence unable to phosphorylate hexoses, to mobilize trehalose in response to the addition of a carbon source. The results presented in Fig. 6B show that the addition of fructose to spores from the *glkA1 frA2* strain triggers trehalose breakdown with kinetics similar to those observed in a wild-type strain. The sole difference that is observed between the wild-type and mutant strains is the accumulation of glucose in the germinating spores of the mutant strain, resulting from its inability to phosphorylate glucose. Similar results were obtained when glucose, ethanol or acetate was used to trigger trehalose breakdown, although the kinetics of trehalose breakdown were specific to each compound (data not shown). Taken together, these data suggest that neutral trehalase activation at the onset of spore germination and, therefore, activation of adenylate cyclase results from carbon source sensing and is independent of hexose phosphorylation.

Discussion

We have reported the first detailed investigation of the role of several signalling elements in the germination of conidia from the filamentous fungus *A. nidulans*. Our results clearly indicate that cAMP signalling plays a critical role in the germination process. Indeed, inactivation of adenylate cyclase results in a severe delay in – but not a complete arrest of – germ tube emergence, suggesting that cAMP synthesis is an important but not essential element for germination. Involvement of the cAMP/PKA signalling pathway during germination is also supported by our analysis of mutant strains that lack the PkaA and/or SchA Ser/Thr protein kinases. These kinases control the kinetics of trehalose breakdown and germ tube emergence probably with overlapping functions, as the combination of null mutations in the *pkaA* and *schA* genes results in synthetic defects during vegetative spore germination. These results are in agreement with those obtained recently by de Pinho *et al*. (2001), who showed that trehalose metabolism is altered during germination of *N. crassa* mutants impaired in the adenylate cyclase or

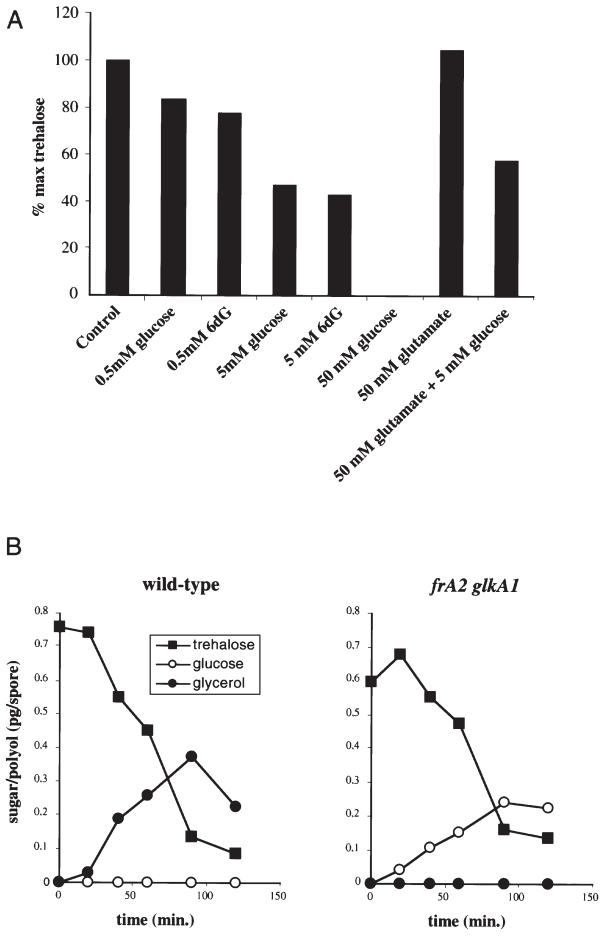


Fig. 6. Trehalose breakdown is induced in response to carbon source sensing.
A. Trehalose content of conidia of *A. nidulans pabaA1* germinated for 1 h in salt solution without carbon and nitrogen source (control) or with glucose (0.5–50 mM), 6-deoxyglucose (6dG, 0.5–5 mM) and/or 50 mM glutamate. Trehalose levels in each sample are expressed as a percentage of the trehalose content in the control.
B. Soluble sugars and polyols in conidia of *A. nidulans* strain CEA53 (WT) and NW194 (*frA2 glkA1*) germinated in minimal fructose medium.

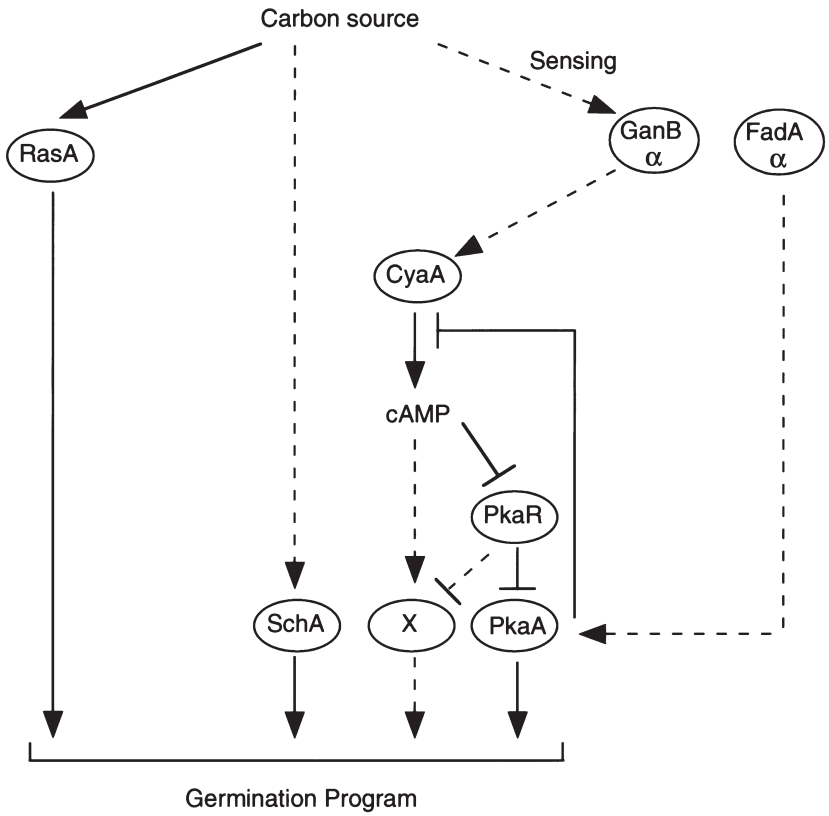


Fig. 7. Proposed model depicting cellular components involved in the activation of the germination programme and in the regulation of the cAMP signalling pathway in *A. nidulans*. Solid lines indicate genetically determined steps and dashed lines hypothesized steps. Sensing of an external carbon source is mediated by the GTPase RasA (Osherov and May, 2000) and probably also by the Gα-subunit GanB (GenBank AAF12813). Activation of GanB results in the activation of adenylate cyclase and the production of cAMP, which relieves the inhibition of PkaA mediated by the PKA regulatory subunit (PkaR; C. d'Enfert, M.-K. Chaveroche and S. Fillinger, unpublished data) and activates additional targets (X). PkaA regulates intracellular levels of cAMP through feedback control, possibly mediated by a low-affinity phosphodiesterase. The SchA kinase is simultaneously activated through an unknown process. PkaA maintains some activity in the absence of cAMP, which might depend on signalling through the Gα-subunit FadA (Shimizu and Keller, 2001). RasA-mediated signalling is independent of cAMP signalling.

regulatory subunit of PKA. Our current model depicting the cellular components involved in the control of *A. nidulans* conidial germination is shown in Fig. 7.

Control of germination by the SchA kinase

In *S. cerevisiae*, SCH9 was originally identified as a multicopy suppressor of cAMP signalling defects (Toda *et al*., 1988). Sch9 seems to be involved in the cAMP-independent activation of PKA targets in response to nitrogen or amino acids when a fermentable carbon source is abundant (Crauwels *et al*., 1997; Kraakman *et al*., 1999; Lorenz *et al*., 2000; Fabrizio *et al*., 2001). Crauwels *et al*. (1997) have shown that inactivation of *SCH9* causes an increase in PKA activity, but the basis for this phenomenon is unknown. The same phenomenon could explain the lack of a germination defect in the *A. nidulans schA*Δ mutant as opposed to the germination defect in a *schA*Δ *pkaA*Δ mutant. Synthetic defects with the *pkaA*Δ mutation during germination are actually the sole detectable phenotypes linked to *schA* inactivation, suggesting that one role for SchA and related kinases of filamentous fungi is in germination. Whether these kinases also have a role in ageing, as has been proposed for Sch9 and PKB/Akt, remains to be evaluated (Fabrizio *et al*., 2001 and references therein). The nature of the signal that Sch proteins transduce during germination remains to be characterized. Sch proteins have a carboxy-terminal domain highly homologous to the AGC family of Ser/Thr protein kinases (Fabrizio *et al*., 2001) and a long amino-terminal extension harbouring a C2-like domain found in protein kinases C (type α, β, γ) and phospholipases A and C in particular (for a review, see Rizo and Südhof, 1998). C2 domains have been shown to bind phospholipids, and it is therefore tempting to speculate that the C2-like domain of Sch proteins might have similar properties that could contribute to its function during germination.

Targets of cyclic AMP

The current knowledge of cAMP signalling in fungi would suggest comparable phenotypes for adenylate cyclase and PKAc mutants, resulting in both cases in a strong reduction in PKA activity (D'Souza and Heitman, 2001). Our results show that this is not the case in *A. nidulans*, as *cyaA*Δ and *pkaA*Δ mutants have very distinct germination and colony morphology defects. Furthermore, overexpression of *pkaA* only partially suppresses the germination defect of a *cyaA*Δ mutation. Taken together, these data clearly indicate the occurrence in *A. nidulans* of targets depending on cAMP other than PkaA (Fig. 7). A similar conclusion has been drawn by Adachi and Hamer (1998), who postulated the occurrence in the plant

pathogen *Magnaporthe grisea* of divergent cAMP signalling pathways regulating growth and pathogenesis and not relying on multiple PKA catalytic subunits. The PKA-related kinase SchA could be one of the targets of cAMP in *A. nidulans*. However, simultaneous inactivation of SchA and PkaA does not lead to the same phenotype as adenylate cyclase inactivation, suggesting that other targets of cAMP exist, whatever the regulation exerted by cAMP on SchA may be. The occurrence of a second PKAc in *A. nidulans* was suggested by Shimizu and Keller (2001). Three or two PKACs with partially overlapping cellular functions have been described for *S. cerevisiae* (Toda *et al*., 1987; Robertson and Fink, 1998; Pan and Heitman, 1999; Robertson *et al*., 2000) and for the basiodiomycete *Ustilago maydis* (Duerrenberger *et al*., 1998) respectively. The situation in filamentous ascomycetes is still unclear. Examination of the *N. crassa* genome (10× coverage; http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) suggests the occurrence of two kinases closely related to PKAc other than *Nc*SchA in this species, whereas only one PKAc can be identified in the *A. fumigatus* genome (6× coverage; http://www.tigr.org/).

Regulation of the PkaA cAMP-dependent protein kinase catalytic subunit

An unexpected result of this study is the difference between *cyaA*Δ *pkaA*Δ and *cyaA*Δ mutants, which cannot simply be explained by multiple targets of cAMP and feedback inhibition of PkaA (see below) on cAMP accumulation, but suggests that PkaA maintains some activity in the absence of cAMP in *A. nidulans*. Partial inhibition of PkaA by its regulatory subunit in the absence of cAMP might explain our observation. In higher eukaryotes, complex regulation patterns of PKA activity are known to be regulated in a spatiotemporal fashion involving compartmentation of the regulatory subunit of PKA at different subcellular locations through interaction with A-kinase anchoring proteins (for a review, see Edwards and Scott, 2000), but similar proteins are not known in fungi. Alternatively, regulation of PkaA by a cAMP-independent component could be postulated. Evidence has been obtained for supplementary PKA-regulating elements in *S. cerevisiae*. Durnez *et al*. (1996) observed that nitrogen-induced activation of trehalase by PKA in glucose-repressed yeasts is not mediated by cAMP. Also, the glucose-induced upshift in ribosomal protein gene expression depending on PKA was shown to be independent of cAMP increase (Neumann-Silberberg *et al*., 1995). Both phenomena were shown to depend on a functional Sch9 protein (Crauwels *et al*., 1997). A similar regulation of PkaA in response to nitrogen and the involvement of SchA in this regulation remain to be explored in *A. nidulans*. In this species, Shimizu and Keller (2001) have shown that the FadA Gα-subunit (Yu *et al*., 1996) positively regulates PkaA to prevent conidiogenesis, hence the hypersporulating phenotype of a *pkaA*Δ mutant. Because *cyaA*Δ mutants do not show this hypersporulating phenotype, it seems likely that the effect of FadA on PkaA is independent of cAMP production. In this regard, FadA could be one of the potential components responsible for cAMP-independent regulation of PkaA (Fig. 7).

Regulation of adenylate cyclase and cAMP levels

Modulation of adenylate cyclase activity in *S. cerevisiae* was thought for a long time to be dependent only on the GTPases Ras1 and Ras2. Recent results have shown that the G-protein α-subunit Gpa2 regulates growth and pseudohyphal development via the cAMP/PKA cascade in response to glucose (Kübler *et al*., 1997; Lorenz and Heitman, 1997; Colombo *et al*., 1998). In *A. nidulans*, three α-subunits of heterotrimeric G-proteins have been identified, namely FadA, GanA and GanB (Yu *et al*., 1996; GenBank accession nos AAD34893 and AAF12813 respectively), but their role in the control of adenylate cyclase is still unknown. Results obtained by Som and Kolaparthi (1994) and Osherov and May (2000) have shown that RasA is involved in the control of developmental transitions in *A. nidulans*, including germination. However, our results indicate that RasA does not regulate adenylate cyclase activity in *A. nidulans* (Fig. 7). Indeed, the decrease in germination kinetics resulting from the expression of a dominant-negative RasA protein is maintained in the absence of a functional adenylate cyclase. Furthermore, the development of giant and multinucleated spores as a result of the expression of a dominant-active RasA protein during germination is only delayed when adenylate cyclase is not functional. Finally, there is no increase in cAMP levels consecutive to the overexpression of the dominant-active RasA protein. RasA might regulate *A. nidulans* development via regulation of a mitogen-activated protein kinase pathway, as has been observed in several other fungi (for a review, see Lengeler *et al*., 2000).

Osherov and May (2000) proposed that RasA is involved in carbon source sensing, as a strain expressing the dominant-active RasA protein initiates the morphological transitions that precede germ tube outgrowth independently of a carbon source. Despite repeated attempts, we were not able to reproduce these results and, therefore, to evaluate the contribution of adenylate cyclase to this phenomenon (data not shown). In contrast, we have shown that trehalose breakdown can be induced by a non-metabolizable analogue of glucose or in a strain that is unable to metabolize hexoses, suggesting that carbon source sensing in germinating spores is sufficient to

induce trehalose breakdown. This contrasts with the results obtained in *S. cerevisiae*, in which glucose-induced cAMP signalling requires both sensing of extracellular glucose by a G-protein coupled receptor and hexose phosphorylation (Beullens *et al*., 1988; Rolland *et al*., 2000). As adenylate cyclase activity is necessary for trehalose breakdown in response to carbon source addition, we can postulate that carbon source sensing during germination is mediated through adenylate cyclase whatever the contribution of RasA to this process might be (Fig. 7). Which of the three Gα-subunits, FadA, GanA or GanB, is involved in carbon source sensing and activation of adenylate cyclase remains to be investigated. Because of its highest similarity to the Gpa2 and Gpa1 nutrient sensors of *S. cerevisiae* and *Cryptococcus neoformans* (Alspaugh *et al*., 1997; Kübler *et al*., 1997; Lorenz and Heitman, 1997; Colombo *et al*., 1998) and to GNA-3 in *N. crassa* previously shown to be involved in the positive control of adenylate cyclase (Kays *et al*., 2000), GanB would appear as the most likely candidate for the control of adenylate cyclase during germination in response to carbon source availability (Fig. 7).

In addition to the regulation of adenylate cyclase by upstream regulatory components, our results show that feedback inhibition by PkaA is also involved in controlling cAMP levels in *A. nidulans* (Fig. 7). A similar phenomenon has been described in *S. cerevisiae* and appears to act at the level of both cAMP synthesis and the Pde1 phosphodiesterase (Nikawa *et al*., 1987; Mbonyi *et al*., 1990; Ma *et al*., 1999). Several PKA phosphorylation sites could be identified in CyaA (data not shown), and feedback regulation might be exerted directly on adenylate cyclase in *A. nidulans*. Although homologues of *PDE1* could not be identified in the currently available genomic data for *A. nidulans*, such homologues are present in the genome of *N. crassa* and *A. fumigatus*, indicating that Pde1-mediated feedback control of cAMP could also occur in filamentous fungi. Further experiments are nevertheless required to identify the targets of PkaA that are necessary for feedback control of cAMP in *A. nidulans* and to define the role that this control plays in the biology of the fungus.

Conclusion

In summary, our results show that cAMP/PKA signalling is a critical but not essential component of asexual spore germination in the filamentous fungus *A. nidulans*. Signalling through the RasA GTPase, which proceeds independently of cAMP synthesis in *A. nidulans*, is also important for the germination process. The involvement of two different pathways in germination might explain why *A. nidulans* strains specifically deficient for conidial germination have never been obtained through classical mutagenesis. Identification of the targets of the cAMP/PKA and RasA signalling pathways and of their connections will be a major challenge for future studies. We have hypothesized the occurrence in *A. nidulans* of more than one direct target of cAMP and of additional regulatory pathways for the PKA catalytic subunit. In this regard, *A. nidulans* provides a genetically tractable model to expand our knowledge of asexual spore germination in filamentous fungi and, more broadly, of cAMP signalling in lower eukaryotes.

Experimental procedures

Aspergillus nidulans *strains and growth conditions*

Aspergillus nidulans and strains used in this study are listed in Table 4. Growth conditions for *A. nidulans* strains have been described previously (d'Enfert and Fontaine, 1997). Cultures of *A. nidulans* strains for trehalose measurements were grown at 37°C in minimal medium supplemented with glucose (50 mM or lower concentrations when indicated) and inoculated at 2×10^7 conidia ml^{-1}. 6-Deoxyglucose (Sigma) was used at 5 mM or lower. Culture conditions for cAMP measurements were as follows: CEA178, CEA192, CEA198 and CEA201 were grown in supplemented liquid minimal medium with 50 mM glucose as carbon source for 18 h at 37°C, or for 16 h at 30°C plus 2 h at 37°C in the case of CEA178, in order to adjust vegetative growth between the strains. CEA200 and CEA204 were grown in liquid minimal medium (50 mM glucose) for 16 h at 30°C. After harvest, half the mycelium was frozen immediately, and the other half was transferred into fresh minimal medium with 170 mM ethanol as carbon source and grown at 37°C for another 3 h.

Soluble sugars and polyols in germinating conidia were measured using previously described procedures (d'Enfert and Fontaine, 1997). Conidiospore germination was monitored on agar-coated slides or on coverslips incubated in liquid medium as described previously (Fillinger *et al*., 2001a,b). Nuclear staining was realized with Hoechst 33258 (2 μg ml^{-1} in water) and examined in a Leitz DMBR fluorescence microscope.

DNA manipulations and characterization of the A. nidulans schA *and* cyaA *genes*

Oligonucleotides used in this study are listed in Table 5. Probes for genes potentially encoding a ScSCH9 homologue or an adenylate cyclase were obtained by amplification. Oligonucleotides SCH9F and SCH9R were deduced from the sequence of EST c6c06 available from the *A. nidulans* EST sequencing program (Prade *et al*., 2001), and genomic DNA from *A. nidulans* FGSC773 prepared according to the method of Girardin *et al*. (1993) was used. Oligonucleotides CYAAF and CYAAR were based on amino acid sequences (EGDAFMV and MDYYGPM respectively) conserved in fungal adenylate cyclases (Kore-eda *et al*., 1991; Loubradou *et al*., 1996; Adachi and Hamer, 1998). The polymerase chain reaction (PCR)-generated fragments were purified and labelled with [α-^{32}P]-dCTP using the Rediprime kit

Table 4. *A. nidulans* strains used in this study.

Strain	Genotype[a]	Origin
FGSC773	*wA3 pyroA4 pyrG89 veA1*	FGSC[b]
AST27[c]	*biA1; argB*$^{+}$*::alcA(p)–rasA*^{G17V} *veA1*	Som and Kolaparthi (1994)
AST29[c]	*biA1; argB*$^{+}$*::alcA(p)–rasA*^{S22N} *veA1*	Som and Kolaparthi (1994)
AST33[c]	*biA1 veA1*	Som and Kolaparthi (1994)
TKIS18-11	*pabaA1 yA2 ΔargB::trpCDB veA1 trpC801 pkaAΔ::argB*$^{+}$	Shimizu and Keller (2001)
TKIS20.1	*yA2 pabaA1 trpC*$^{+}$*::alcA(p)–pkaA veA1*	Shimizu and Keller (2001)
NW194[d]	*wA3 pyroA4 pyrG89 treA::neo frA2 glkA1 veA1*	P. van der Vondervoort and J. Visser
CEA53	*wA3 pyroA4 pyrG89 treA::(neo-AgpyrG-neo) veA1*	d'Enfert and Fontaine (1997)
CEA166	*wA3 pyroA4 pkaAΔ::argB*$^{+}$ *pyrG89 veA1*	This study
CEA178	*wA3 pyroA4 veA1*	This study
CEA179	*wA3 pyroA4 pyrG89 cyaAΔ::pyrG*$^{+}$ *veA1*	This study
CEA182	*wA3 pyroA4 pyrG89 schAΔ::pyrG*$^{+}$ *veA1*	This study
CEA184	*wA3 pyroA4 pyrG89 schAΔ::pyrG*$^{+}$ *pkaAΔ::argB*$^{+}$ *veA1*	This study
CEA192	*wA3 pyroA4 pkaAΔ::argB*$^{+}$ *cyaAΔ::pyrG*$^{+}$ *veA1*	This study
CEA196	*wA3 pyroA4 cyaAΔ::pyrG*$^{+}$ *veA1*	This study
CEA198	*wA3 pyroA4 pkaAΔ::argB*$^{+}$ *veA1*	This study
CEA199	*cyaAΔ::pyrG*$^{+}$ *argB*$^{+}$*::alcA(p)–rasA*^{G17V} *veA1*	This study
CEA200	*argB*$^{+}$*::alcA(p)–rasA*^{G17V} *veA1*	This study
CEA201	*cyaAΔ::pyrG*$^{+}$ *veA1*	This study
CEA202	*cyaAΔ::pyrG*$^{+}$ *argB*$^{+}$*::alcA(p)–rasA*^{S22N} *veA1*	This study
CEA203	*argB*$^{+}$*::alcA(p)–rasA*^{S22N} *veA1*	This study
CEA204	*veA1*	This study
CEA209	*yA2 pabaA1 veA1*	This study
CEA217	*yA2 pabaA1 cyaAΔ::pyrG*$^{+}$ *trpC*$^{+}$*::alcA(p)–pkaA veA1*	This study
CEA221	*yA2 cyaAΔ veA1*	This study

a. Genotypes at the *yA*, *argB* and *pyrG* loci were not tested in *wA3*, *arg*$^{+}$ or *pyr*$^{+}$ strains obtained from genetic crosses.
b. Fungal Genetic Stock Center, Kansas City, KA, USA.
c. Kindly provided by T. Som, Thomas Jefferson University, Philadelphia, USA.
d. Kindly provided by J. Visser, Wageningen University, The Netherlands.

(Amersham) and used to screen the chromosome-specific libraries of *A. nidulans* genomic DNA (Brody *et al*., 1991), which were obtained from the Fungal Genetic Stock Center (Kansas City, KA, USA). Cosmid W17A03 from chromosome VIII was identified using the 380 bp probe corresponding to the *A. nidulans* ScSCH9 homologue, whereas three cosmids from chromosome II were identified, namely W09D06, L05E09 and W02E08, using the 250 bp probe corresponding to *A. nidulans* adenylate cyclase. DNA sequencing was performed on double-stranded cosmids W17A03 and W09D06. The sequence of a 3792 bp fragment of cosmid W17A03 is deposited at the GenBank nucleotide sequence data library

Table 5. Oligonucleotides used in this study.

Oligonucleotide	Sequence
SCH9F	5′-AAGGTCCGGTTCCCTCG-3′
SCH9R	5′-TGGTGATCGATCGAACTC-3′
schF1zeo	5′-TTCTTTCTTGCATTCGGCATGCCAGCATCACGCTGCATTCCGA CACTCTTTCACGGGAATTCTCAGTCCTGCTCC-3′
schBpyr	5′-AAGACAAGATATATGCAGACATCACAACTCCACTAGCATGC ATCGAAAGCCATAAGAATTCGCCTCAAACAATGC-3′
SCH9R3	5′-CGACTAAGCAAAGTGATTCC-3′
sch1	5′-GAAAACACGGAGTTGTCG-3′
CYAAF	5′-GARGGNGAYGCNTTYATGGT-3′
CYAAR	5′-CATNGGNCCRTARTARTCCAT-3′
cyaF1zeo	5′-ACCACCTTCTCTAGGCGCCCCGAATGACCTATACACCTCCAT GTTCTCGTGGGGCGGAATTCTCAGTCCTGCTCC-3′
cyaBpyr	5′-GACGGGTAGGCAGGATAGATGTCATGGGGTATGATGAGAGT AAAAGAGCGGGTATGAATTCGCCTCAAACAATGC-3′
CYAR1	5′-CCGCCGTCAGCAACGGCCG-3′
CYAF-1	5′-CAGGTCTTTGGGAGACGGCC-3′
cya1	5′-CTCTGGGAACAACAGTGG-3′
CYAR.9	5′-CAGATGGTCGCCGGTGATC-3′
pkaCF	5′-GGCATCTCAAGATTACCG-3′
pkaCR	5′-AACCGCTTGGTAAGATCC-3′
pkaC1	5′-GCCACGGTCTTGTTTTAGG-3′
pkaC2	5′-GGTGGTTATGGTGGTTATGG-3′
pkaRP	5′-GCATAGAATTTCGCGACAGG-3′
alcAF2	5′-AGAGACGGAGCACTTTCTGG-3′

under accession number AY043352. It encodes a 2757 bp ORF interrupted by two introns of 394 bp and 73 bp, respectively, leading to a protein of 919 amino acids. The sequence of a 7775 bp region in cosmid W09D06 is deposited in the GenBank nucleotide sequence data library under accession number AY043351. It encodes a 6396 bp ORF interrupted by three introns of 97 bp, 45 bp and 47 bp respectively. The location of introns in both genes was confirmed by analysing reverse transcriptase (RT)–PCR products obtained from RNA samples prepared from vegetatively growing mycelium as described previously (d'Enfert et al., 1999) and using oligonucleotides appropriately spaced alongside the determined nucleotide sequences.

Construction of A. nidulans *mutant strains by gene replacement and genomic analysis of* A. nidulans *strains*

Null mutations at the *schA* and *cyaA* loci were introduced by gene replacement using the procedure described by Chaveroche *et al*. (2000). The bifunctional *Escherichia coli/A. nidulans zeo*/*pyrG* transformation marker from plasmid pCDA21 was amplified using oligonucleotides schF1zeo and schBpyr or cyaF1zeo and cyaBpyr. The resulting PCR products were used to transform *E. coli* strain KS272 carrying corresponding target cosmids and plasmid pKOBEG, which encodes the red$\alpha\beta\gamma$ functions of phage lambda. Using this approach, derivatives of cosmids W17A03 or W09D06 with part of the coding sequence of *schA* (amino acids 22–919) or *cyaA* (amino acids 83–2132) replaced by the *zeo/pyrG* cassette were obtained and used to transform *A. nidulans* strain FGSC773 as described previously (Osmani *et al*., 1987). Transformants with a deletion at the target locus were first identified by PCR using oligonucleotides SCH9F and SCH9R or CYAR1 and CYAF-1, which yield 383 bp or 400 bp products only if the wild-type *schA* or *cyaA* genes are present respectively. Deletion of *schA* or *cyaA* was confirmed by Southern analysis. A probe for *schA* was generated using oligonucleotides SCH9R3 and sch1. Hybridization of *Eco*RI-digested genomic DNA with this probe yielded a 2.7 kb fragment when the wild-type *schA* allele was present, whereas no fragment was observed in a strain with the *schA*Δ allele. A probe for *cyaA* was generated using oligonucleotides cya1 and CYAR.9. Hybridization of *Eco*RI-digested genomic DNA with this probe yielded a 10 kb fragment in a wild-type strain, whereas a 4.5 kb fragment was observed in a strain with the *cyaA*Δ allele. Similar procedures were used to confirm the nature of the allele at the *schA* and *cyaA* loci when scoring progenies resulting from genetic crosses. In addition, the following strategies were used to evaluate the nature of the allele at the *pkaA* locus or the occurrence in progenies of the *trpC*::*alcA(p)–pkaA*, *argB*::*alcA(p)–rasA*, *argB*::*alcA(p)–rasA*^{G17V} and *argB*::*alcA(p)–rasA*^{S22N} *pkaA*: amplification with oligonucleotides pkaCF and pkaCR, which yield a PCR product of 348 bp only when the wild-type *pkaA* allele is present; or Southern hybridization of *Eco*RI-digested genomic DNA with a PCR probe obtained using oligonucleotides pkaC1 and pkaC2, resulting in a 1.4 kb fragment in a wild-type strain and a 3.3 kb fragment in a *pkaA*Δ strain; *trpC*::*alcA(p)–pkaA*, amplification with oligonucleotides alcAF2 and pkaRP, which yield a PCR product of 1060 bp only when the fusion allele is present; *argB*::*alcA(p)–rasA*, Southern hybridization of *Eco*RI-digested genomic DNA with a plasmid pAST33 (Som and Kolaparthi, 1994) as a probe, resulting in a 5 kb fragment in a wild-type strain and a 1.5 kb fragment in a *argB*::*alcA(p)–rasA* strain.

cAMP assay

Aspergillus nidulans crude extracts were obtained from small portions of frozen mycelia that were ground under liquid nitrogen, then resuspended in 0.5 ml of extraction buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5). An aliquot of 0.1 ml of this suspension was used for protein assay after centrifugation using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard. The rest was boiled for 5 min, then centrifuged at 13 000 r.p.m. for 5 min. cAMP concentration was determined in the supernatant using the cAMP (^{3}H) assay system (TRK432/Amersham) according to the supplier's instructions. Assays were made at least in triplicate on two independent cultures. cAMP concentrations are expressed in pmol mg^{-1} protein.

Acknowledgements

Thanks are due to Tapan Som, Peter van de Vondervoort and Jaap Visser for providing strains, and to Maxime Schwartz for his constant interest and support in the course of this study. S.F. was the recipient of a postdoctoral fellowship in the framework of the EU Biotechnology Programme (BIO4-CT98-0268).

References

Adachi, K., and Hamer, J. (1998) Divergent cAMP signaling pathways regulate growth and pathogenesis in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **10:** 1361–1373.

Alspaugh, J.A., Perfect, J.R., and Heitman, J. (1997) Cryptococcus neoformans mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. *Genes Dev* **11:** 3206–3217.

Beullens, M., Mbonyi, K., Geerts, L., Gladines, D., Detremerie, K., Jans, A.W., and Thevelein, J.M. (1988) Studies on the mechanism of the glucose-induced cAMP signal in glycolysis and glucose repression mutants of the yeast *Saccharomyces cerevisiae*. *Eur J Biochem* **172:** 227–231.

van Biesen, T., Luttrell, L.M., Hawes, B.E., and Lefkowitz, R.J. (1996) Mitogenic signaling via G protein-coupled receptors. *Endocrinol Rev* **17:** 698–714.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Brody, H., Griffith, J., Cuticchia, A.J., Arnold, J., and Timberlake, W.E. (1991) Chromosome-specific recombinant DNA library from the fungus *Aspergillus nidulans*. *Nucleic Acids Res* **19:** 3105–3109.

Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J., and Wigler, M. (1985) Differential activation of

yeast adenylate cyclase by wild-type and mutant RAS proteins. *Cell* **41:** 763–769.

Bruno, K.S., Aramayo, R., Minke, P.F., Metzenberg, R.L., and Plamann, M. (1996) Loss of growth polarity and mislocalization of septa in a *Neurospora* mutant altered in the regulatory subunit of cAMP-dependent protein kinase. *EMBO J* **15:** 5772–5782.

Cansado, J., Soto, T., Fernandez, J., Vicente-Soler, J., and Gacto, M. (1998) Characterization of mutants devoid of neutral trehalase activity in the fission yeast *Schizosaccharomyces pombe*: partial protection from heat-shock and high-salt stress. *J Bacteriol* **180:** 1342–1345.

Chaveroche, M.K., Ghigo, J.M., and d'Enfert, C. (2000) A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* **28:** E97.

Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., *et al.* (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J* **12:** 3326–3341.

Crauwels, M., Donaton, M.C.V., Pernambuco, M.B., Winderickx, J., de Winde, J.H., and Thevelein, J.M. (1997) The Sch9 protein kinase in the yeast *Saccharomyces cerevisiae* controls cAPK activity and is required for nitrogen activation of the fermentable-growth-medium-induced (FGM) pathway. *Microbiology* **143:** 2627–2637.

D'Souza, C., and Heitman, J. (2001) Conserved cAMP signaling cascades regulate fungal development and virulence. *FEMS Microbiol Rev* **25:** 349–364.

Devereux, J., Haerberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12:** 387–395.

Duerrenberger, F., Wong, K., and Kronstad, J. (1998) Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. *Proc Natl Acad Sci USA* **95:** 5684–5689.

Durnez, P., Pernambuco, M.B., Oris, E., Arguelles, J.C., Mergelsberg, H., and Thevelein, J.M. (1996) Activation of trehalase during growth induction by nitrogen sources in the yeast *Saccharomyces cerevisiae* depends on the free catalytic subunits of cAMP-dependent protein kinase, but not on functional Ras proteins. *Yeast* **10:** 1049–1064.

Edwards, A.S., and Scott, J.D. (2000) A-kinase anchoring proteins: protein kinase A and beyond. *Curr Opin Cell Biol* **12:** 217–221.

d'Enfert, C. (1997) Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans and Neurospora crassa*. *Fungal Genet Biol* **21:** 163–172.

d'Enfert, C., and Fontaine, T. (1997) Molecular characterization of the *Aspergillus nidulans treA* gene encoding an acid trehalase required for growth on trehalose. *Mol Microbiol* **24:** 203–216.

d'Enfert, C., Bonini, B.M., Zapella, P.D.A., Fontaine, T., da Silva, A.M., and Terenzi, H.F. (1999) Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Mol Microbiol* **32:** 471–484.

Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M., and Longo, V.D. (2001) Regulation of longevity and stress resistance by Sch9 in Yeast. *Science* **292:** 288–291.

Fillinger, S., Chaveroche, M.-K., van Dijck, P., de Vries, R., Ruijter, G., Thevelein, J., and d'Enfert, C. (2001a) Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147:** 1851–1862.

Fillinger, S., Ruijter, G., Tamas, M.J., Visser, J., Thevelein, J.M., and d'Enfert, C. (2001b) Molecular and physiological characterization of the NAD-dependent glycerol 3-phosphate dehydrogenase in the filamentous fungus *Aspergillus nidulans*. *Mol Microbiol* **39:** 145–157.

Francis, S.H., and Corbin, J.D. (1994) Structure and function of cyclic nucleotide-dependent protein kinases. *Annu Rev Physiol* **56:** 237–272.

Fujita, M., and Yamamoto, M. (1998) *S. pombe sck2*$^{+}$, a second homologue of *S. cerevisiae SCH9* in fission yeast, encodes a putative protein kinase closely related to PKA function. *Curr Genet* **33:** 248–254.

Girardin, H., Latgé, J.-P., Skirantha, T., Morrow, B., and Soll, D. (1993) Development of DNA probes for fingerprinting *Aspergillus fumigatus*. *J Clin Microbiol* **31:** 1547–1554.

Hall, A.A., Bindslev, L., Rouster, J., Rasmussen, S.W., Oliver, R.P., and Gurr, S.J. (1999) Involvement of cAMP and protein kinase A in conidial differentiation by *Erysiphe graminis* F. sp. *hordei*. *Mol Plant–Microbe Interact* **12:** 960–968.

Hanks, S.K., and Hunter, T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9:** 576–596.

Harris, S.D. (1999) Morphogenesis is coordinated with nuclear division in germinating *Aspergillus nidulans* conidiospores. *Microbiology* **145:** 2747–2756.

Hatanaka, M., and Shimoda, C. (2001) The cyclic AMP/PKA signal pathway is required for initiation of spore germination in *Schizosaccharomyces pombe*. *Yeast* **18:** 207–217.

Herman, P.K., and Rine, J. (1997) Yeast spore germination: a requirement for Ras protein activity during re-entry into the cell cycle. *EMBO J* **16:** 6171–6181.

Jin, M., Fujita, M., Culley, B.M., Apolinario, E., Yamamoto, M., Maundrell, K., and Hoffman, C.S. (1995) sck1, a high copy number suppressor of defects in the cAMP-dependent protein kinase pathway in fission yeast, encodes a protein homologous to the *Saccharomyces cerevisiae* SCH9 kinase. *Genetics* **140:** 457–467.

Kays, A.M., Rowley, P.S., Baasiri, R.A., and Borkovich, K.A. (2000) Regulation of conidiation and adenylyl cyclase levels by the Galpha protein GNA-3 in *Neurospora crassa*. *Mol Cell Biol* **20:** 7693–7705.

Kopp, M., Müller, H., and Holzer, H. (1993) Molecular analysis of the neutral trehalase gene from *Saccharomyces cerevisiae*. *J Biol Chem* **268:** 4766–4774.

Kore-eda, S., Murayama, T., and Uno, I. (1991) Isolation and characterization of the adenylate cyclase structural gene of *Neurospora crassa*. *Jpn J Genet* **66:** 317–334.

Kraakman, L., Lemaire, K., Ma, P., Teunissen, A.W.R.H., Donaton, M.C.V., van Dijck, P., *et al.* (1999) A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP

pathway during the transition to growth on glucose. *Mol Microbiol* **32:** 1002–1012.

Kübler, E., Mösch, H.-U., Rupp, S., and Lisanti, M.P. (1997) Gpa2p, a G-protein α-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. *J Biol Chem* **272:** 20321–20323.

Lengeler, K.B., Davidson, R.C., D'Souza, C., Harashima, T., Shen, W.-C., Wang, P., *et al.* (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol Mol Biol Rev* **64:** 746–785.

Lim, C.A., Spiegelman, G.B., and Weeks, G. (2001) RasC is required for optimal activation of adenylyl cyclase and Akt/PKB during aggregation. *EMBO J* **20:** 4490–4499.

Lorenz, M.C., and Heitman, J. (1997) Yeast pseudohyphal growth is regulated by GPA2, a G protein α homolog. *EMBO J* **16:** 7008–7018.

Lorenz, M.C., Pan, X., Harashima, T., Cardenas, M.E., Xue, Y., Hirsch, J.P., and Heitman, J. (2000) The G protein-coupled receptor Gpr1 Is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Genetics* **154:** 609–622.

Loubradou, G., Begueret, J., and Turcq, B. (1996) An additional copy of the adenylate cyclase-encoding gene relieves developmental defects produced by a mutation in a vegetative incompatibility-controlling gene in *Podospora anserina*. *Gene* **170:** 119–123.

Ma, P., Wera, S., Van Dijck, P., and Thevelein, J.M. (1999) The PDE1-encoded low-affinity phosphodiesterase in the yeast *Saccharomyces cerevisiae* has a specific function in controlling agonist- induced cAMP signaling. *Mol Biol Cell* **10:** 91–104.

Mbonyi, K., van Aelst, L., Arguelles, J.C., Jans, A.W., and Thevelein, J.M. (1990) Glucose-induced hyperaccumulation of cyclic AMP and defective glucose repression in yeast strains with reduced activity of cyclic AMP- dependent protein kinase. *Mol Cell Biol* **10:** 4518–4523.

Momany, M., and Taylor, I. (2000) Landmarks in the early duplication cycles of *Aspergillus fumigatus* and *Aspergillus nidulans*: polarity, germ tube emergence and septation. *Microbiology* **146:** 3279.

Neumann-Silberberg, F.S., Bhattacharya, S., and Broach, J.R. (1995) Nutrient availability and the RAS/cyclic AMP pathway both induce expression of ribosomal protein genes in *Saccharomyces cerevisiae. Mol Cell Biol* **15:** 3187–3196.

Nikawa, J., Cameron, S., Toda, T., Ferguson, K.M., and Wigler, M. (1987) Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. *Genes Dev* **1:** 931–937.

Osherov, N., and May, G. (2000) Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. *Genetics* **155:** 647–656.

Osmani, S.A., May, G.S., and Morris, R.N. (1987) Regulation of the mRNA levels of *nimA*, a gene required for the G2-M transition in *Aspergillus nidulans*. *J Cell Biol* **104:** 1495–1504.

Pan, X., and Heitman, J. (1999) Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19:** 4874–4887.

de Pinho, C.A., de Lourdes, M., Polizeli, T.M., Jorge, J.A., and Terenzi, H.F. (2001) Mobilisation of trehalose in mutants of the cyclic AMP signalling pathway, *cr-1* (CRISP-1) and *mcb* (microcycle conidiation), of *Neurospora crassa*. *FEMS Microbiol Lett* **199:** 85–89.

Prade, R.A., Ayoubi, P., Krishnan, S., Macwana, S., and Russell, H. (2001) Accumulation of stress and inducer-dependent plant-cell-wall-degrading enzymes during asexual development in *Aspergillus nidulans*. *Genetics* **157:** 957–967.

Rizo, J., and Südhof, T.C. (1998) C_2-domains, structure and function of a universal Ca^{2+}-binding domain. *J Biol Chem* **273:** 15879–15882.

Robertson, L.S., and Fink, G.R. (1998) The three yeast A kinases have specific signaling functions in pseudohyphal growth. *Proc Natl Acad Sci USA* **95:** 13783–13787.

Robertson, L.S., Causton, H.C., Young, R.A., and Fink, G.R. (2000) The yeast A kinases differentially regulate iron uptake and respiratory function. *Proc Natl Acad Sci USA* **97:** 5984–5988.

Rolland, F., de Winde, J.H., Lemaire, K., Boles, E., Thevelein, J.M., and Winderickx, J. (2000) Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. *Mol Microbiol* **38:** 348–358.

Ruan, Y.J., Kotraiah, V., and Straney, D.C. (1995) Flavonoids stimulate spore germination in *Fusarium solani* pathogenic on legumes in a manner sensitive to inhibitors of cAMP-dependent protein kinase. *Mol Plant-Microbe Interact* **8:** 929–938.

Schmit, J.C., and Brody, S. (1976) Biochemical genetics of *Neurospora crassa* conidial germination. *Bacteriol Rev* **40:** 1–41.

Shimizu, K., and Keller, N.P. (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* **157:** 591–600.

Som, T., and Kolaparthi, V.S.R. (1994) Developmental decisions in *Aspergillus nidulans* are modulated by ras activity. *Mol Cell Biol* **14:** 5333–5348.

Taylor, S.S., and Radzio-Andzelm, E. (1994) Three protein kinase structures define a common motif. *Structure* **2:** 345–355.

Thevelein, J.M. (1984a) Cyclic-AMP content and trehalase activation in vegetative cells and ascospores of yeast. *Arch Microbiol* **138:** 64–67.

Thevelein, J.M. (1984b) Regulation of trehalose mobilization in fungi. *Microbiol Rev* **48:** 42–59.

Thevelein, J.M., and de Winde, J.H. (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* **33:** 904–918.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., *et al.* (1985) In yeast, *RAS* proteins are controlling elements of adenylate cyclase. *Cell* **40:** 27–36.

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.

Toda, T., Cameron, S., Sass, P., and Wigler, M. (1988) *SCH9*,

a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes Dev* **2:** 517–527.

Yang, Z., and Dickman, M.B. (1997) Regulation of cAMP and cAMP dependent protein kinase during conidial germination and appressorium formation in *Colletotrichum trifolii*. *Phys Mol Plant Pathol* **50:** 117–127.

Yu, J.-H., Wieser, J., and Adams, T.H. (1996) The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. *EMBO J* **15:** 5184–5190.