

Cyclic AMP-dependent protein kinase is involved in morphogenesis of *Aspergillus niger*

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The cAMP signal transduction pathway controls many processes in fungi. The *pkaR* gene, encoding the regulatory subunit (PKA-R) of cAMP-dependent protein kinase (PKA), was cloned from the industrially important filamentous fungus *Aspergillus niger*. To investigate the involvement of PKA in morphology of *A. niger*, a set of transformants which overexpressed *pkaR* or *pkaC* (encoding the catalytic subunit of PKA) either individually or simultaneously was prepared as well as mutants in which *pkaR* and/or *pkaC* were disrupted. Strains overexpressing *pkaR* or both *pkaC* and *pkaR* could not be distinguished from the wild-type, suggesting that regulation of PKA activity is normal in these strains. Absence of PKA activity resulted in a two- to threefold reduction in colony diameter on plates. The most severe phenotype was observed in the absence of PKA-R, i.e., very small colonies on plates, absence of sporulation and complete loss of growth polarity during submerged growth. Suppressor mutations easily developed in the $\Delta pkaR$ mutant and one of these mutants appeared to lack PKA-C activity. These data suggest that cAMP-dependent protein phosphorylation in *A. niger* regulates growth polarity and formation of conidiospores.

Keywords: cAMP-dependent protein kinase, regulatory subunit, signal transduction, morphology

INTRODUCTION

Cyclic AMP is a ubiquitous secondary messenger that controls the activity of the cAMP-dependent protein kinase (PKA). The system is widespread in eukaryotes (Gancedo *et al.*, 1985) and is involved in, for example, regulating metabolic and developmental processes (Daniel *et al.*, 1998).

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Abbreviations: PKA, cAMP-dependent protein kinase; PKA-R, regulatory subunit of PKA; PKA-C, catalytic subunit of PKA.

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cAMP signalling controls a number of developmental events such as growth polarity in the filamentous fungus *Neurospora crassa* (Bruno *et al.*, 1996), cell development of *Blastocladiella emersonii* (de Oliveira *et al.*, 1997) and sexual development of *Schizosaccharomyces pombe* (Maeda *et al.*, 1994). The influence of cAMP via PKA on dimorphic transition was demonstrated for several dimorphic fungi, e.g. *Candida albicans* (Niimi, 1996) and *Mucor rouxii* (Orlowski, 1991). For the plant-pathogenic fungi *Ustilago maydis* (Gold *et al.*, 1994) and *Magnaporthe grisea* (Mitchell & Dean, 1995) and for the human pathogen *Cryptococcus neoformans* (Kronstad *et al.*, 1998), cAMP signalling is directly connected to fungal virulence (reviewed by Borges-Walmsley & Walmsley, 2000). PKA is also involved in metabolic regulation, activating glycolysis (Goncalves *et al.*, 1997) and diauxic transition in *Saccharomyces cerevisiae* (Boy-Marcotte *et al.*, 1998). The inactivation of *Sac. cerevisiae* fructose-1,6-bisphosphatase (Jiang *et al.*, 1998), the stability of *Sac. cerevisiae* neutral trehalase (Zahringer *et al.*, 1998) and the activation of 6-phosphofructo-1-kinase from *Mytilus galloprovincialis*

(Fernandez *et al.*, 1998) are results of PKA phosphorylation. In *Aspergillus nidulans* conidia PKA is proposed to be involved in mobilization of trehalose by phosphorylation of trehalase (d'Enfert *et al.*, 1999).

In most cases the inactive form of PKA is a tetrameric protein composed of two regulatory and two catalytic subunits. Upon binding of cAMP, inactive PKA dissociates into two active catalytic subunits and a dimer of regulatory subunits. Two types of PKA regulatory subunits (PKA-R), type I and type II, have been isolated and four different genes encoding PKA-R ($I\alpha$, $I\beta$, $II\alpha$, $II\beta$) have been identified in higher eukaryotes (Taylor *et al.*, 1992). Fungi usually possess a single gene for PKA-R, which encodes a protein similar to either the mammalian type I or type II regulatory subunits. In *N. crassa*, *Sch. pombe*, *Mag. grisea*, *Sac. cerevisiae*, *U. maydis* and *B. emersonii* type II regulatory subunits have been described (Bruno *et al.*, 1996; de Voti *et al.*, 1991; Adachi & Hamer, 1998; Kunisawa *et al.*, 1987; Gold *et al.*, 1994; Marques & Gomes, 1992). PKA from *Dictyostelium discoideum* (Mutzel *et al.*, 1987) and *Paramecium tetraurelia* (Carlson & Nelson, 1996) have a different structure. The regulatory subunit of these enzymes does not contain dimerization domains – they are heterodimeric proteins composed of only one catalytic and one regulatory subunit.

In the filamentous fungus *Aspergillus niger*, a transient increase in cAMP levels during the early stage of growth in a medium with high initial sucrose concentration has been observed (Legiša *et al.*, 1981; Gradišnik-Grapulin & Legiša, 1997). Simultaneously with the cAMP peak, a change in morphology from bulbous cells to filamentous hyphae took place (Legiša & Gradišnik-Grapulin, 1995; Gradišnik-Grapulin & Legiša, 1997). Growth tests indicated that even moderate overproduction of PKA-C affected growth and sporulation characteristics of *A. niger* transformants (Benčina *et al.*, 1997). During germination of wild-type *A. niger* the level of expression of *pkaC* and the specific PKA activity steadily decreased (Benčina & Legiša, 2000). These data suggest that PKA is involved in morphogenesis. To analyse the *A. niger* PKA enzyme in more detail and study its role in the regulation of morphological and cellular development, we cloned *A. niger pkaR* and constructed *A. niger* strains with disrupted or overexpressed *pkaC* and/or *pkaR*.

METHODS

Strains and plasmids. *A. niger* strains used in this study are listed in Table 1. *A. niger* N400 (CBS 120.49) was used for preparation of a genomic library. The strains used for transformations were *A. niger* NW219 and NW245. *A. niger* 13mcC with two additional copies of *pkaC* (Benčina *et al.*, 1997) and *A. niger* NW219::pyrA were used as reference strains in some analyses. *A. niger* NW274 was used for mitotic recombination experiments.

Escherichia coli LE392 [$\Delta(lacIZY)6$ *galK2 galT22 metB1 trpR55* λ^-] (Promega), was used for phage amplification and purification, *E. coli* DH5 α [$F^-/endA1 hsdR17$ (r_k^- m_k^-) *supE44*

thi-1 recA1 gyrA (Nal^r) *relA1* $\Delta(lacIZIA-argF)$ U169 *deoR* ($\phi 80 \delta lac \Delta(lacZ)$ M15)] (New England Biolabs) and *E. coli* XL-1 Blue MRF' [$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F^- proAB lacI^N \Delta M15::Tn10 (Tet^r)]$] (Stratagene) were used for plasmid DNA propagation and for *in vivo* excision, respectively.

Plasmid vectors pBluescript KS (Stratagene) and pEMBL19 (Roche) were used for subcloning. Plasmid pGW635, containing *A. niger pyrA* (Goosen *et al.*, 1987), and plasmid pIM650, containing *A. nidulans argB* (Johnstone *et al.*, 1985), were used for construction of *pkaR* and *pkaC* disruption plasmids. Phage ExAssist (Stratagene) was used as a helper phage for phagemid excision.

The following gene fragments were used to generate probes for screening *A. niger* genomic and cDNA libraries: a 1.51 kbp *Bgl*II/*Bam*HI fragment from *Mag. grisea pkaR* (Adachi & Hamer, 1998) and a 1.45 kbp *Hind*III fragment from *A. nidulans pkaR* [accession no. AF043231 (gi: 3170247)].

Culture media and growth conditions. For the preparation of conidiospores, *A. niger* strains were grown at 30 °C for 3–4 days on complete medium (CM), originally described for *A. nidulans* (Pontecorvo *et al.*, 1953), using 1% (w/v) glucose as a carbon source, with appropriate supplements and solidified with 1.5% (w/v) agar. Since disruption of the *pkaR* gene in *A. niger* resulted in an inability to form conidiospores, we could not maintain a $\Delta pkaR$ mutant in the form of conidiospores. Instead, mycelium of an *A. niger* $\Delta pkaR$ mutant was picked from a plate and stored in 30% (v/v) glycerol at –70 °C. For submerged growth, medium was inoculated with 10^6 conidiospores ml⁻¹. One litre of minimal medium (MM) contained 6 g NaNO₃, 1.5 g KH₂PO₄, 0.5 g KCl, 0.5 g MgSO₄·7H₂O, pH 6.0, and 0.2 ml trace metal solution (Visniac & Santer, 1957). Glucose was used as a carbon source. For the growth of auxotrophic strains, appropriate supplements were added: 10 mg nicotinamide l⁻¹, 200 mg leucine l⁻¹, 1220 mg uridine l⁻¹ and 200 mg arginine l⁻¹. Mycelium was grown at 30 °C in a rotary shaker at 250 r.p.m.

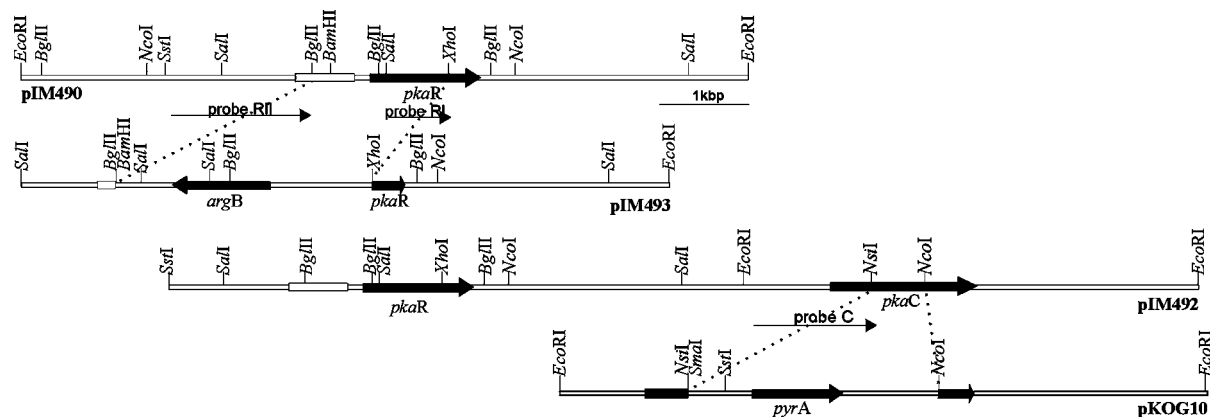
Germination kinetics were analysed by microscopic examination of slides coated with minimal medium containing 2% glucose and 1.5% agar and spot-inoculated with 2×10^4 conidiospores. Spores were incubated at 30 °C. The percentage of germinated spores was followed in time by examining 50–100 spores at 30 min intervals.

DNA manipulation. *A. niger* chromosomal DNA was isolated as described by de Graaff *et al.* (1988). Propagation and isolation of plasmid DNA, Southern blot analysis and other DNA manipulations were essentially done as described by Sambrook *et al.* (1989). [α -³²P]dATP-labelled probes were synthesized using random hexamer primers (Sambrook *et al.*, 1989). Automated sequencing based on the dideoxy chain-termination procedure (Sanger *et al.*, 1977) was done with ALFexpress (Amersham Pharmacia Biotech) using the T7 DNA polymerase sequencing kit (Amersham Pharmacia Biotech). DNA sequence was analysed with PC/GENE and WINSTAR computer program packages. Further analysis of the nucleotide sequence was performed using EDITSEQ and MAPDRAW from the PC/GENE software package, MATINSPECTOR v2.2 (Quandt *et al.*, 1995) and Prosite and InterPro databases.

The *A. niger pkaR* gene and the corresponding cDNA clone were isolated from an *A. niger* N400 genomic library in the λ replacement vector EMBL4 (Promega) and a cDNA library from *A. niger* N400 in λ ZAP II (Stratagene), respectively. Plasmids containing the cloned cDNAs were obtained from

Table 1. *A. niger* strains used in this study

Strain	Genotype (linkage group)
N400 (CBS 120.49)	Wild-type
NW219	<i>cspA1 pyrA6(III) leuA1(IV) nicA1(V)</i>
A395	<i>cspA1 pyrA6(III) leuA1(IV) nicA1(V) pyrA⁺</i>
NW245	$\Delta argB::pyrA(I) cspA1 pyrA6(III) leuA1(IV) nicA1(V)$
NW258 (KOG10/ ΔC)	<i>cspA1 pyrA6(III) leuA1 $\Delta pkaC::pyrA(IV) nicA1(V)$</i>
NW274	<i>funA24 $\Delta argB(I) goxC17(II) cspA1 bioA1(III) prtF28(V)$</i>
NW275 (9 ΔR), 47 ΔR	$\Delta argB::pyrA(I) cspA1 pyrA6(III) leuA1(IV) nicA1 \Delta pkaR::argB(V)$
9 ΔR sp	$\Delta argB::pyrA(I) cspA1 pyrA6(III) leuA1(IV) nicA1 \Delta pkaR::argB(V) pkaC1$
NW276	<i>funA24 $\Delta argB(I) goxC17(II) cspA1 pyrA6(III) leuA1 \Delta pkaC::pyrA(IV) prtF28(V)$</i>
NW277 ($\Delta R\Delta C$)	$\Delta argB::pyrA(I) cspA1 pyrA6(III) leuA1 \Delta pkaC::pyrA(IV) nicA1 \Delta pkaR::argB(V)$
13mcC (Benčina <i>et al.</i> , 1997)	<i>cspA1 pyrA6(III) leuA1(IV) nicA1(V) pkaC⁺ pyrA⁺</i>
38, 10, 39, 42 (mcR)	<i>cspA1 pyrA6(III) leuA1(IV) nicA1(V) pkaR⁺ pyrA⁺</i>
44, 7, 35, 1, 18 (mcRC)	<i>cspA1 pyrA6(III) leuA1(IV) nicA1(V) pkaR⁺ pkaC⁺ pyrA⁺</i>

**Fig. 1.** Partial restriction maps of pIM490 containing *pkaR*, pIM492 containing *pkaC* and *pkaR*, the *pkaR* disruption plasmid, pIM493 and the *pkaC* disruption plasmid, pKOG10. The arrows below the restriction maps indicate the probes used for Southern and Northern analysis. The coding regions and the directions of transcription are presented by filled arrows; open boxes indicate non-coding regions.

the λ ZAP II phages by *in vivo* excision, according to the manufacturer's instructions.

Mitotic recombination was performed as described by Bos *et al.* (1988). Heterokaryon formation between NW276 and NW275 was induced by protoplast fusion (Van Diepingen *et al.*, 1998). Haploidization was induced with benomyl.

To construct a plasmid for simultaneous overexpression of *pkaC* and *pkaR*, pIM492, an 8 kbp *EcoRI* fragment, containing *pkaR*, was inserted into pEMBL19. After removal of one *EcoRI* site, a 5 kbp *EcoRI* *pkaC* fragment from pPKAC1 (Benčina *et al.*, 1997) was inserted into the other *EcoRI* site giving pIM492 (Fig. 1).

To construct pIM493, a plasmid to disrupt *pkaR*, a 1.7 kbp *SalI* fragment of *pkaR* from pIM490 was inserted in pBlue-script, the plasmid obtained was digested with *XhoI*/*BglII* and ligated to the 2.8 kbp *BamHI*/*XhoI* fragment of *argB* from pIM650 (Fig. 1). The 3.3 kbp *XhoI*/*KpnI* fragment of *pkaR* from pIM490 was ligated into this construct to get pIM493. The 7.1 kbp *EcoRI* fragment of this construct was used for transformation of *A. niger* NW245.

To construct the *pkaC* disruption plasmid, pKOG10, a *SmaI*/*NcoI* fragment of *pyrA* was ligated into the *NsiI* (blunt end)/*NcoI* sites of *pkaC* in pPKAC1 (Benčina *et al.*, 1997). A 6.4 kbp *EcoRI* fragment of the resulting plasmid (pKOG10, see Fig. 1) was used for *pkaC* disruption by REMI transformation of *A. niger* strain NW219.

Northern analysis. Mycelium grown in liquid complete medium (CM) for 24 h was transferred for 1 h into MM medium with 1 or 15% glucose, harvested by filtration, washed briefly with ice-cold 0.05 M potassium phosphate buffer pH 7.0, frozen in liquid nitrogen and ground using a micro dismembrator (Braun Biotech). RNA was extracted using the TRIzol reagent (Life Technologies). After electrophoresis, RNA was transferred to nylon membranes (Hybond-N⁺; Amersham Pharmacia Biotech) by capillary blotting in 10 $\times$ SSC. Pre-hybridization and hybridization were performed at 42 °C in formamide hybridization buffer containing 50% (w/v) formamide, 0.75 M NaCl, 50 mM NaH₂PO₄ (pH 7.4), 10 mM EDTA, 2 $\times$ Denhardt's, 0.1% (w/v) SDS and 10% (w/v) dextran sulphate. Northern blots were washed at 65 °C in 4 $\times$ SSC, 0.5% SDS and 2 $\times$ SSC, 0.5% SDS. The

probes used for Northern analysis were as follows: a 0.8 kbp *Bgl*II/*Xho*I fragment from pIM490 for *pkaR* mRNA (probe R, Fig. 1), a 1.2 kbp *Eco*RI/*Kpn*I fragment from pIM492 for *pkaC* mRNA (probe C, Fig. 1) and a 0.7 kbp *Eco*RI fragment of *A. niger* 18S rRNA. The *pkaR* and *pkaC* probes are also depicted in Fig. 1.

Transformation of *A. niger*. Mycelium was obtained by growing *A. niger* for 16–18 h in liquid culture on complete medium with appropriate supplements. Preparation of protoplasts and subsequent transformation of *A. niger* were performed as described by Kusters-van Someren *et al.* (1991). The *A. niger* *pyrA* gene and *A. nidulans* *argB* gene were used as selection markers. For co-transformation of *A. niger* NW219, 1 µg pGW635 DNA and 27 µg of the co-transforming plasmid pIM491 (containing *pkaR*) or pIM492 (containing a copy of both the *pkaR* and the *pkaC* genes) were added to 2×10^7 protoplasts. To disrupt *pkaR*, 5 µg of the 7.1 kbp *Eco*RI *pkaR* fragment from pIM493 was used to transform *A. niger* NW245. To disrupt *pkaC*, 5 µg of the 5 kbp *Eco*RI *pkaC* fragment from pKOG10 was used to transform *A. niger* NW219. The transformants were selected and purified by replating at low spore densities on selective medium without uridine or arginine.

Enzyme assays. Frozen mycelium (0.5 g) obtained as described for RNA isolation, was ground and suspended in 1 ml extraction buffer (350 mM KH_2PO_4 , pH 7.5, 0.1 mM DTT, 10% glycerol). After 15 min extraction, the homogenate was centrifuged at 10000 *g* for 10 min. Supernatant was used for the enzyme assay. Cell extracts (with protein concentration of approximately 2 mg ml⁻¹) were diluted two- to tenfold before measuring enzyme activity. The activity of PKA was detected by the non-radioactive PepTag test method with dye-labelled Kemptide as a substrate, according to the manufacturer's protocol (Promega) or by SpinZyme (Pierce), according to Benčina *et al.* (1997). The incubation time for the enzymic reaction was 30 min at 30 °C. One unit of enzyme activity was defined as the amount of enzyme required to transfer 1 pmol phosphate from ATP to the substrate (Kemptide) per min at 30 °C. The catalytic subunit of bovine heart PKA (Promega) was used as a standard. Protein concentrations in cell-free extracts were determined with the Bicinchoninic-acid protein kit (Sigma), according to the supplier's instructions, and using BSA fraction V (Roche) as a standard.

RT-PCR. RT-PCR was performed according to Benčina & Legiša (1999). Total RNA (2 µg) and primers PKAC14 [5'-AGAAAGCGTGAAACACACAG-3' (Benčina *et al.*, 1997)] and PEPC2 [5'-TATCACGGTGAGAGATACGAGC-3' (Frederick *et al.*, 1993)] (Ransom Hill Bioscience) were used. cDNA product (2 µl) was transferred to 20 µl PCR reaction mixture containing primers PKAC13 [5'-TGGTCATGGAC-TTCGTAGAGGG-3' (Benčina *et al.*, 1997)], PKAC14, PEPC1 [5'-CTATCTGGGTCTCAAGAACACC-3' (Frederick *et al.*, 1993)], PEPC2 (Ransom Hill Bioscience). PCR was performed in a Perkin Elmer thermal cycler GeneAmp PCR System 2400.

Twenty microlitres of the PCR products were separated by 1% (w/v) agarose gel electrophoresis, transferred to Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech) and subsequently the biotin-labelled fragments were detected by the avidin alkaline phosphatase conjugate (Tropix) using CDP-Star (Tropix) as a substrate. Signals were determined by imaging film X-OMATAR (Eastman Kodak). The expected sizes of the PCR products were 230 and 420 bp. The intensity of the signals on the film was measured with an Imaging Densitometer model GS-670 (Bio-Rad) and analysed by Molecular Analyst Software (Bio-Rad).

RESULTS

Cloning and sequence analysis of *A. niger pkaR*

The *A. niger pkaR* gene, encoding PKA-R, was cloned from an *A. niger* N400 genomic library in λEMBL4 using *Mag. grisea pkaR* (Adachi & Hamer, 1998) and *A. nidulans pkaR* [accession no. AF043231 (gi: 3170247)] as probes. Six phages were isolated with the first probe and four phages with the second. Restriction patterns of these phages were similar except for minor differences, indicating that the two different probes hybridized to the same gene. An 8 kbp *Eco*RI fragment from one of the phages originally obtained with the *A. nidulans* probe was cloned in pEMBL19, resulting in plasmid pIM490 (Fig. 1).

The nucleotide sequence of a 3330 bp fragment of pIM490 was determined on both strands. The sequence from position -319 to -326 could not be identified unambiguously due to high GC content. The nucleotide sequence was deposited in the EMBL database under the accession no. AJ296317. In addition, a 2002 bp cDNA fragment of *pkaR* was isolated from an *A. niger* cDNA library using stringent hybridization conditions and a 0.6 kbp *Bam*HI/*Sal*I fragment of pIM490 as a probe.

By comparing the cDNA sequence with the sequence of the genomic clone, a single intron of 700 bp was found from position -112 to -811 with borders 5'-GTAAGG and 3'-CAG. Three putative CCAAT boxes were found upstream from the start codon at positions -361, -426 and -473. Analysis of the promoter region by the MATINSPECTOR v2.2 software package (core and matrix similarity parameters: 1.0 and 0.9) (Quandt *et al.*, 1995) revealed several putative transcription elements, including stress response elements (Stre), a binding site for heat-shock factors (Hsp), a target site for an activator of nitrogen-regulated genes (Nit1), the positive transcriptional regulator Gcr1, the transcriptional activator for control of development, AbaA, and the regulator of asexual reproduction and differentiation of hyphae, StuA. In addition, the promoter region contains several E-boxes (CANNTG) (Murre *et al.*, 1989) and nine C₄T or reverse complementary (AGGGG) sequences (Treger *et al.*, 1998), five of which are inside the intron. No obvious TATA sequence was found in the 5' non-coding region.

The cDNA contained an ORF of 1233 nt encoding a protein of 411 aa with a calculated molecular mass of 44527 Da, which is fairly similar to the apparent molecular mass determined for the purified protein, 48 kDa (Legiša & Benčina, 1994). The coding sequence showed a strong bias for A in the third position of the codon, which was also found in the *A. niger pkaC* gene (Benčina *et al.*, 1997). Three putative structural features were present within PKA-R (Leon *et al.*, 1997; Su *et al.*, 1995). First, a dimerization domain located at the N-terminal third of the protein. Second, a site for interaction between the regulatory and catalytic subunits [¹¹⁷RRTSVSAE] containing an autophosphorylation site (Ser-120). Third, two cAMP-binding sites:

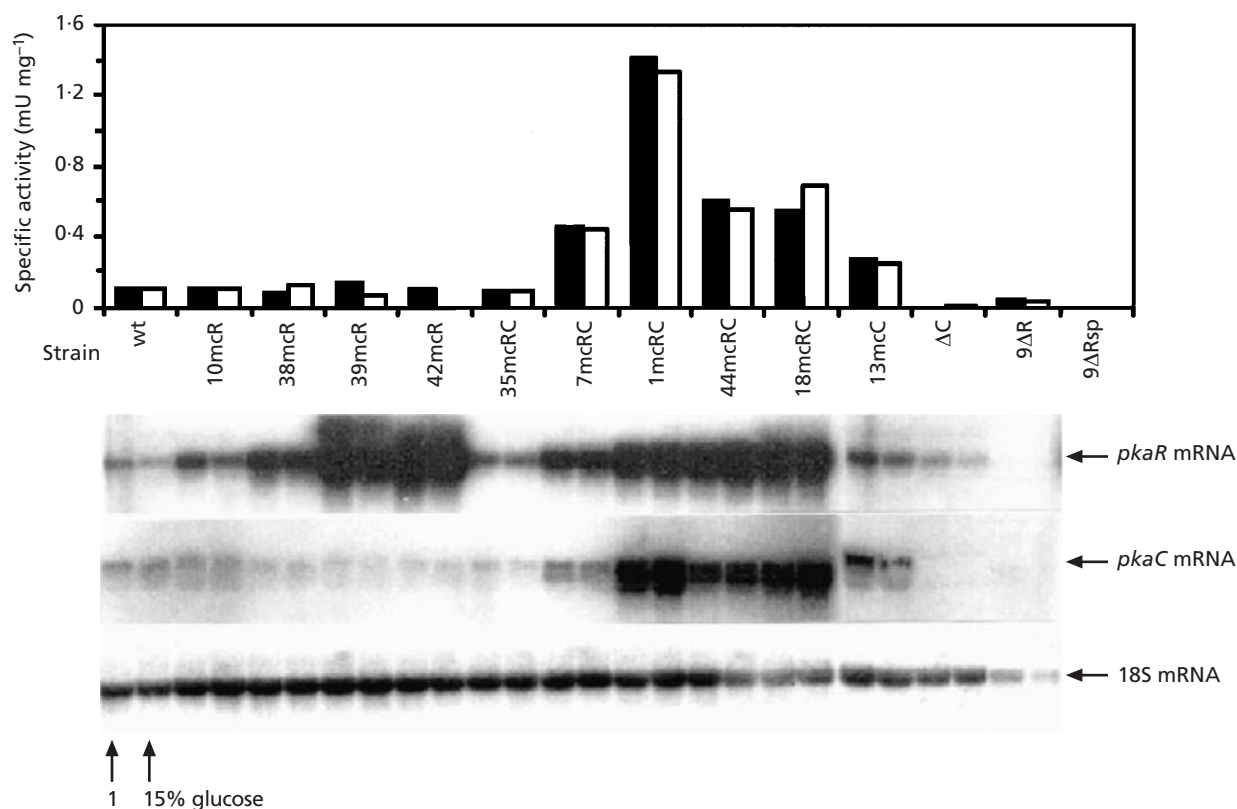


Fig. 2. PKA activities and Northern analysis of selected *A. niger* *pkaR* and *pkaR pkaC* transformants. The following strains were analysed: A395, wild-type (wt); 10mcR, 38mcR, 39mcR, 42mcR, strains containing multiple copies of *pkaR*; 35mcRC, 7mcRC, 1mcRC, 44mcRC, 18mcRC, strains containing multiple copies of both *pkaR* and *pkaC*; 13mcC, multicopy *pkaC*; ΔC, *pkaC* deletion strain NW258; 9ΔR, *pkaR* deletion strain NW275; 9ΔRsp, pseudorevertant of strain 9ΔR capable of conidiation. Cell-free extracts for PKA activity and total RNA were isolated from mycelium that was pre-grown on CM with 1% glucose for 24 h and then transferred to MM with 1% (■) or 15% (□) glucose. After 1 h of incubation, mycelium was harvested and used for the above-mentioned analysis. PKA activities shown in the graph are means from three independent determinations. The probes used for Northern analysis are described in Methods and indicated in Fig. 1. For detection of the *pkaR* transcript, probe RI (Fig. 1) was used. 18S was used as a loading control. In the Northern blots are shown for each strain, showing expression of the genes in mycelium incubated with 1 and 15% glucose.

(²²⁷)VgSv^gpGGSF^gELALmYnaPRAATV and (³⁴⁸)Vksy-
kr^gDYFGEIALLddkPRAAsI (conserved amino acid
residues are underlined). The deduced amino acid
sequence suggests that the PKA-R of *A. niger* is most
closely related to type II PKA-R, showing an overall
identity of 75% with *A. nidulans* *pkaR* [accession no.
AF043231 (gi: 3170247)], 69% with *N. crassa* (Bruno *et al.*, 1996) and 66% with *Mag. grisea* (Adachi & Hamer, 1998).

Downstream of the stop codon the mRNA still contains 459 nt. Putative polyadenylation signals, ATAAA, were found at 16 and 366 nt downstream of the stop codon.

Construction of strains modified in *pkaR* and *pkaC*

To understand the role of the PKA-R in cell physiology, transformants containing multiple copies of *pkaR* and/or *pkaC*, and strains in which these genes were disrupted were prepared.

Multicopy *pkaR* strains (mcR) were isolated after co-transforming *A. niger* with pIM491 using *pyrA* as a selection marker. Similarly, transformants with increased copy numbers of both *pkaC* and *pkaR* (mcRC strains) were produced using pIM492.

Several transformants were analysed for copy number of *pkaR* and *pkaR/pkaC* by Southern analysis. Four transformants were found with multiple copies of *pkaR* (strain 10mcR, one extra copy; strain 38mcR, approximately 10 copies; strains 42mcR and 39mcR contained more than 20 copies). Five transformants contained an increased number of copies for both genes (strain 35mcRC, one extra copy for each gene; strain 7mcRC, 4–5 copies; strain 44mcRC, approximately 10 copies; strains 1mcRC and 18mcRC had at least 20 copies). Increased copy numbers of *pkaC* and *pkaR* resulted in overexpression of the two genes (Fig. 2).

Additionally, transformants with disrupted *pkaR* (ΔR) or *pkaC* (ΔC) were constructed. For disruption of the

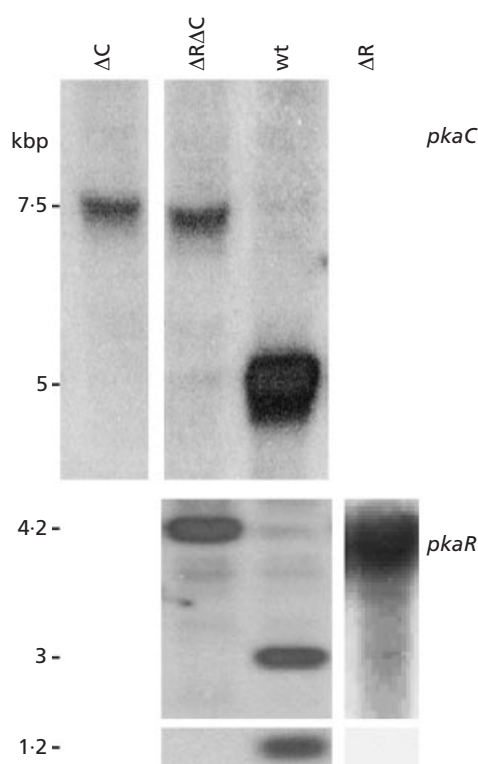


Fig. 3. Disruption of *pkaC* and *pkaR* in *A. niger*. Southern analysis was performed with *EcoRI*-digested genomic DNA of *pkaC* disruption strains (top panels) or *BglIII*-digested genomic DNA of *pkaR* disruption strains (middle and lower panels). The following strains were analysed: wt, wild-type strain N400; ΔR , *pkaR* disruptant NW275; ΔC , *pkaC* disruptant NW258; $\Delta R\Delta C$, *pkaC* *pkaR* double disruptant NW277. Blots were probed with a 1.2 kbp *EcoRI/KpnI* *pkaC* fragment (probe C; Fig. 1) to detect gene replacement for *pkaC* (top panels), a 1.6 kbp *SstI/BglIII* *pkaR* fragment (probe RII; Fig. 1) to detect gene replacement for *pkaR* (middle panels) or a 0.8 kbp *BglIII/XhoI* *pkaR* fragment (probe RI; Fig. 1) to show deletion of part of the *pkaR* gene (lower panels). Molecular size markers are indicated on the left.

pkaR gene, plasmid pIM493 was constructed in which a 5' part of the *pkaR* coding region and part of the promoter was removed and replaced by the *A. nidulans* *argB* gene. To obtain a *pkaC* knock-out strain plasmid

pKOG10 was constructed in which *A. niger* *pyrA* was inserted into *pkaC* after removing a small part of the coding region. Five *pkaR* disruption strains (9 ΔR , 26 ΔR , 29 ΔR , 38 ΔR , 47 ΔR) and one *pkaC* disruption strain (ΔC) were obtained. Gene replacements in strains ΔC and 9 ΔR were verified by Southern analysis (Fig. 3). In strain ΔC the presence of a 7.5 kbp *EcoRI* fragment instead of a 5 kbp fragment indicated proper gene replacement. In ΔR strains replacement of a 3 kbp *BglIII* fragment by a 4.2 kbp fragment established gene replacement. In addition, removal of a 1.2 kbp *BglIII/XhoI* fragment was confirmed in ΔR strains. All five *pkaR* disruptants had very compact colonies and did not sporulate (see below). Strain 9 ΔR was analysed in more detail.

To construct a strain in which both *pkaR* and *pkaC* were disrupted, two consecutive mitotic recombination steps were performed. Linkage analysis on the resulting recombinants allowed chromosomal localization of *pkaC* and *pkaR*. In the first step, strain NW258 (*pyrA6*, *leuA1* Δ *pkaC*::*pyrA* *nicA1*) was recombined with strain NW274 (*fwnA24* Δ *argB*::*pyrA* *goxC17* *bioA1* *prtF28*) (Table 1). From NW258//NW274, 97 descendants were randomly picked and analysed. Considering Δ *pkaC* (ΔC) morphology (see below) and uridine and leucine requirement we found 21 descendants which were Δ *pkaC* *leuA1*, 1 *pyrA6* *leuA1*, 15 *pyrA6* and 60 *pkaC*⁺ *leuA1*⁺ *pyrA*⁺ strains. Since only one *leuA1* *pkaC*⁺ recombinant was found, linkage of *pkaC* with *leuA1* is 99% and *pkaC* is therefore located on chromosome IV. Subsequently, one of the Δ *pkaC*::*pyrA* descendants (NW276) was recombined with NW275 (Δ *argB*::*pyrA* *pyrA6* *leuA1* *nicA1* Δ *pkaR*::*argB*). The diploid of NW275//NW276 showed segregation of all three morphology types (see below) on benomyl plates. Many wild-type recombinants were obtained, some recombinants showing ΔC morphology and a few recombinants with Δ *pkaR* (ΔR) morphology. Since it was rather difficult to isolate and characterize ΔR recombinants, we purified 39 recombinants with ΔC morphology and analysed these in more detail, in particular for the presence of the *pkaR* disruption. In this case the relation between phenotype and genotype was less obvious (Table 2). All recombinants carry the *argB* disruption on chromosome I, but only some have the *pkaR* disruption with the intact *argB* gene. The perfect linkage between *arg*⁺ and *nicA1* proves

Table 2. Phenotype, frequency and genotype of descendants from recombinant NW275 (ΔR)//NW276 (ΔC)

+ indicates the presence of a marker; – indicates the absence. ND, Not detectable. All strains carry the *leuA1* mutation.

Derived genotype		<i>pyrA6</i>	Δ <i>argB</i>	Δ <i>argB</i> :: <i>pyrA</i> <i>fwnA24</i>	Δ <i>pkaC</i> :: <i>pyrA</i>	Δ <i>pkaR</i> :: <i>argB</i> <i>nicA1</i>
Phenotype	Number					
<i>arg</i> [–]	11	+	+	–	ND	–
<i>arg</i> [–] <i>fwn</i>	2	+	–	+	+	–
<i>nic</i> [–]	2	+	+	–	ND	+
<i>nic</i> [–] <i>fwn</i>	24	+	–	+	+	+

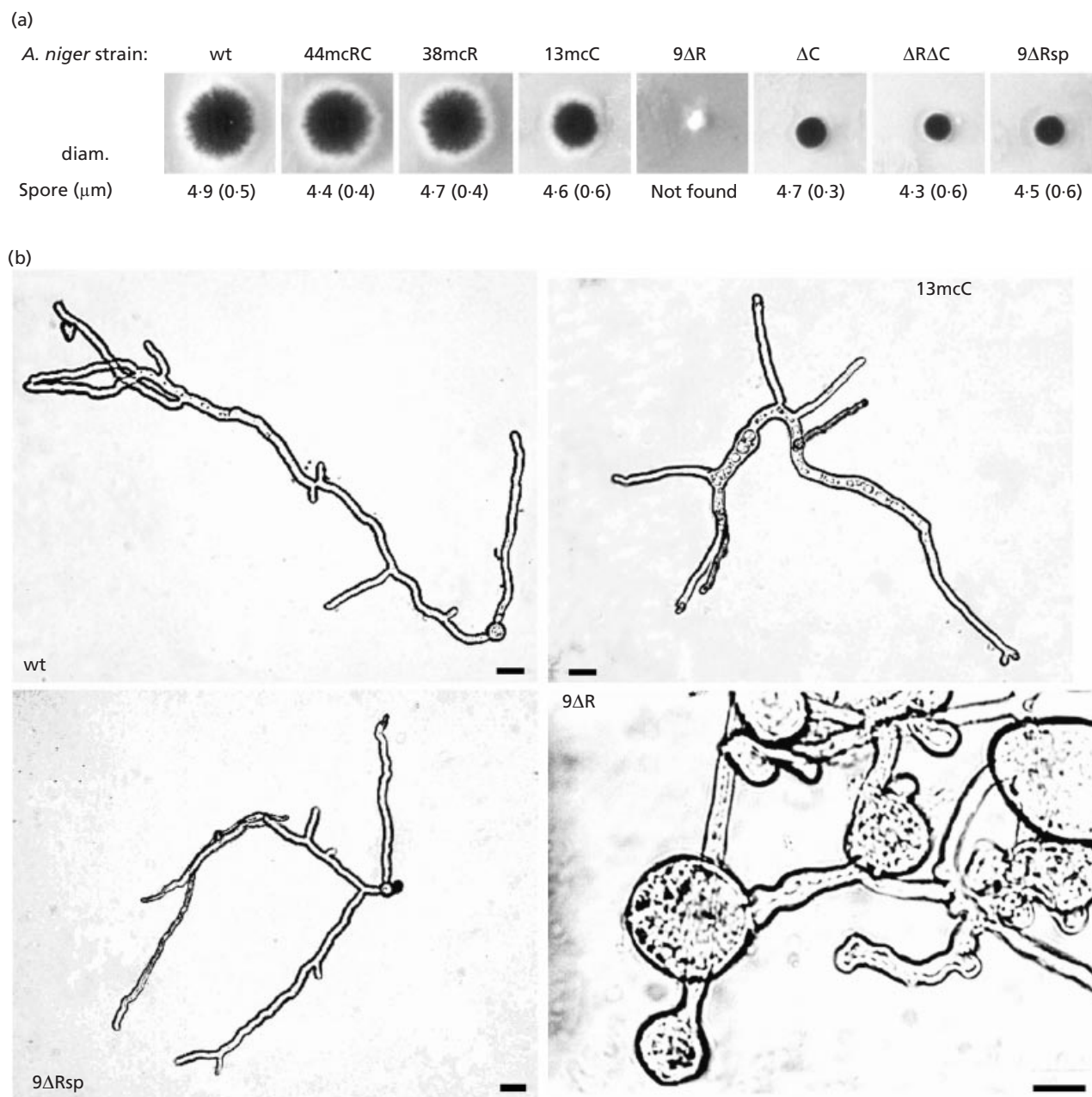


Fig. 4. Morphological characteristics of *A. niger* wild-type and *pka* transformants and mutants. (a) Colony morphology and size of conidiospores. Pictures and spore sizes are from 3-day-old colonies grown on solid minimal medium with 2% glucose at 30 °C. Spore diameter is given with standard deviation in parentheses ($n \geq 10$). (b) Hyphal morphology during submerged growth. Cells were grown for 24 h in minimal medium containing 2% glucose at 30 °C. Bar, 10 μm. Strain designations: wt, wild-type strain A395; 44mcRC, strain with multiple copies of *pkaR* and *pkaC*; 38mcR, multicopy *pkaR* strain; 13mcC, multicopy *pkaC* strain; 9ΔR, *pkaR* disruptant NW275; ΔRΔC, *pkaR pkaC* double disruptant NW277; ΔC, *pkaC* disruptant NW258; 9ΔRsp pseudorevertant of strain 9ΔR.

that *pkaR* is located on chromosome V. All the recombinants with ΔC morphology are expected to contain the *pkaC* disruption. This can be derived from their *pyr*⁺ phenotype. Although all recombinants carry the *pyrA6* mutation, they can be *pyr*⁺ due to either one of the two disruptions carrying *pyrA*; *argB::pyrA* linked to black spore colour and *pkaC::pyrA*. All 26

fawn recombinants were *pyr*⁺, proving the presence of the *pkaC* disruption. Assuming that black recombinants with ΔC morphology also carried the *pkaC* disruption, we selected one black double mutant, NW277, which was used for further experiments. The presence of the *pkaC* and *pkaR* disruptions in NW277 was confirmed by Southern analysis (Fig. 3).

Expression of *pkaC* and *pkaR* and determination of PKA activity in *A. niger* wild-type and various transformants

The level of expression of *pkaR* and *pkaC* in various transformants was determined in mycelium grown on 1 % glucose medium for 24 h and subsequently incubated for 1 h in medium with either 1 or 15 % glucose. The high glucose concentration, 15 %, was used since it has been shown that *pkaC* expression and PKA activity change during growth at high sugar concentration (Benčina & Legiša, 2000). Our results, however, show that the levels of *pkaC*- and *pkaR*-specific mRNA were similar under these two conditions (Fig. 2), indicating that incubation of mycelium at high sugar concentration did not affect the level of expression of *pkaC* and *pkaR*.

Specific PKA activities were measured to correlate it to modifications in the copy number of *pkaC* and *pkaR*. PKA activity was significantly higher in transformants with multiple copies of both *pkaR* and *pkaC* (Fig. 2). The activity correlated qualitatively to copy number and mRNA level (Fig. 2), e.g. transformant 44mcRC had about 10 copies of both genes and 0.6 mU mg⁻¹ (wild-type activity was 0.1 mU mg⁻¹). In transformants with an increased copy number of only *pkaR*, PKA activity remained at the wild-type level (Fig. 2). PKA activity was measured in one *pkaR* disruption strain, 9ΔR, and was found to be similar to wild-type activity (0.175 mU mg⁻¹). Finally, in the *pkaC* disruptant PKA activity was below the detection level (Fig. 2).

Growth and morphology of *A. niger* strains modified in *pkaR* and *pkaC*

The role of PKA activity in morphology and hyphal development was monitored by observing the phenotypic features of individual transformants growing on solid medium and in liquid cultures. By comparing growth of wild-type, various multicopy transformants and the different disruptants on solid medium, three morphological classes were identified on the basis of colony size and the ability to form spores (Fig. 4). Strains in the first group were similar to wild-type and included a *pkaR* multicopy transformant (38mcR), a *pkaC* multicopy transformant (13mcC with a three- to four-fold increased PKA-C level) and a strain with an increased copy number of both genes (44mcCR). The second morphological class included strains with reduced or abolished PKA activity. Transformants in which *pkaC* (ΔC) or both *pkaC* and *pkaR* (ΔRΔC) were disrupted formed colonies with a two- to threefold smaller diameter than the wild-type (Fig. 4). A *pkaR* disruptant (9ΔR) formed a third morphological class and developed colonies of only about half the size of wild-type and no sporulation could be observed after 3 days growth on agar plates.

No major changes in hyphal morphology could be observed for most of the strains after 24 h submerged growth in minimal medium (data not shown). Exceptions were strains with increased *pkaC* copy number or disruption of *pkaR*. Young mycelium of a *pkaC*

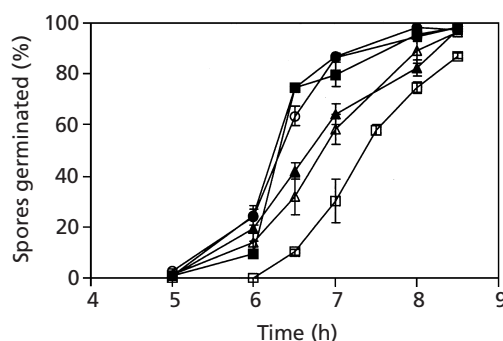


Fig. 5. Germination kinetics of *A. niger* wild-type, *pkaC* and *pkaR* multicopy transformants and $\Delta pkaC$ mutants. Germination was monitored as described in Methods using minimal medium containing 2 % (w/v) glucose at 30 °C. The following strains were analysed: ○, wild-type strain A395; ■, 44mcRC, a strain with multiple copies of *pkaR* and *pkaC*; ●, 38mcR, multicopy *pkaR* strain; □, 13mcC, multicopy *pkaC* strain; △, ΔC, *pkaC* disruptant NW258; ▲, ΔRΔC, *pkaR pkaC* double disruptant NW277. Results are means from three independent determinations; bars represent SD.

multicopy transformant (13mcC) exhibited hyphae with a 30 % increased diameter (Fig. 4), but in a later stage (typically after 28 h growth) newly formed hyphae were similar to the wild-type. To study the morphology of the *pkaR* disruptant (9ΔR) in submerged cultures, peripheral hyphae from a surface-growing colony were taken as an inoculum, since no spores were available. After 24 h incubation remarkable morphological changes were observed. There was a distinct change from polar to apolar growth and enlarged bulbous cells were formed on the tips of filamentous hyphae (Fig. 4).

Germination kinetics were similar for the wild-type strain and strains overexpressing *pkaR* or both *pkaR* and *pkaC* (Fig. 5). Germination of conidiospores from the *pkaC* disruption strain (ΔC) and the *pkaC pkaR* double disruptant (ΔRΔC) was slightly delayed (30 min to 1 h), but conidia from strain 13mcC, overproducing PKA-C, germinated considerably slower than the wild-type strain (1 to 2 h). Like the results obtained for colony morphology, strains ΔC and ΔRΔC behaved similarly in germination experiments, indicating that disruption of *pkaC* was epistatic to deletion of *pkaR*.

Suppression of the *pkaR* mutant phenotype

During propagation of strain 9ΔR on solid medium, sectors appeared in the colonies exhibiting altered morphology and growth rate. One such strain was designated 9ΔRsp (Fig. 4). Interestingly, the morphology of this strain on solid medium resembled that of the strain lacking *pkaC* (ΔC), i.e. compact colonies (Fig. 4). During submerged growth in minimal medium, bulbous cells, typical for strain 9ΔR, were not observed. Instead morphology was very similar to wild-type and the ΔC transformant. The morphological resemblance between the 9ΔRsp and ΔC strains suggested that, due to a suppressor mutation in strain 9ΔR, *pkaC* was inacti-

vated or transcription of *pkaC* was decreased. By RT-PCR, the *pkaC* mRNA level in strain 9ΔR was found to be $103 \pm 24\%$ ($n=3$) of the wild-type level, while in strain 9ΔRsp *pkaC* expression was only $40 \pm 9\%$ ($n=3$). Moreover, the specific activity of PKA in strain 9ΔRsp was below the detection level (Fig. 2). The absence of PKA activity may indicate a mutation in the *pkaC* coding region, resulting in an inactive protein and perhaps reduced mRNA stability, but this has to be analysed further.

DISCUSSION

In this paper we describe cloning of the *Aspergillus niger pkaR* gene, encoding PKA-R. The following data imply that we cloned *pkaR*. First, the deduced PKA-R amino acid sequence is very similar to PKA-R proteins from related fungi, in particular those of *A. nidulans*, *N. crassa* and *Mag. grisea* (Bruno *et al.*, 1996; Adachi & Hamer, 1998). Second, three putative domains involved in PKA-R function are present in the sequence: a dimerization domain, a site for interaction with PKA-C containing an autophosphorylation site (Ser-120) (Kreepipuu *et al.*, 1999) and two cAMP binding sites. Given the similarity to other PKA-R proteins and the structural features of the protein, *A. niger* PKA-R most likely belongs to type II PKA regulatory subunits. The structure of the *A. niger* PKA holoenzyme is most likely tetrameric (Taylor *et al.*, 1992).

The presence of an intron within the 5' non-coding region of *A. niger pkaR* has also been reported for the PKA-R genes of *B. emersonii* (Marques & Gomes, 1992), *N. crassa* (Bruno *et al.*, 1996) and *Mag. grisea* (Adachi & Hamer, 1998). Similar to intron I of *B. emersonii*, the *A. niger pkaR* intron (700 bp) is unusually large compared to the normal size (50–100 bp) of fungal introns (Unkles, 1992). Its role in the promoter region of *pkaR* is not clear, but it might play a role in the expression of the gene, as reported for rat cytochrome *c* (Evans & Scarpulla, 1988). Analysis of the *A. niger pkaR* promoter region revealed no typical GC sites or binding sites for transcription factor Sp1 as could be found in the promoter of *A. niger pkaC* (Benčina *et al.*, 1997) and the *pkaR* promoter of *B. emersonii* (Marques & Gomes, 1992). However, the promoter regions of the *pkaC* and *pkaR* genes of *A. niger* bear some similarities which are indicative of common transcription control mechanisms. Both promoters contain stress response elements (C₄T) and E-motifs. Such elements have been found in the multistress response genes of *Sac. cerevisiae* (Treger *et al.*, 1998).

To analyse the involvement of PKA in morphogenesis, growth and morphology of strains with increased *pkaC* and/or *pkaR* expression as well as strains lacking PKA-C and/or PKA-R were examined on solid and in liquid medium. Strains overexpressing *pkaR* or both *pkaC* and *pkaR* showed higher mRNA levels as expected, but the strains could otherwise not be distinguished from the wild-type, suggesting that regulation of PKA-C activity is normal in these strains. With simultaneous over-

expression of *pkaC* and *pkaR*, one might expect a relatively high PKA-C activity upon binding of cAMP to PKA-R and release of PKA-C, but this does not appear to interfere with regulation of processes which determine morphology. These data suggest that an increased level of PKA-C is not harmful to the cells as long as its activity is properly regulated by PKA-R.

A three- to fourfold increase in the PKA-C level in *pkaC* transformant 13mcC did not affect surface growth, but in submerged cultures, young mycelium of this transformant had thicker hyphae. In a later stage, however, newly formed hyphae were like wild-type. Apparently, overproduction of PKA-C only affects morphology at an early growth stage. This observation agrees with the finding that *pkaC* transcription in *A. niger* significantly decreased between 19 and 28 h after inoculation (Benčina & Legiša, 2000). Thus, if overproduction of PKA-C results in increased hyphal diameter, a decrease in *pkaC* transcription might get the PKA-C/PKA-R balance back to normal, resulting in proper regulation of PKA activity and consequently in a normal hyphal diameter. Alternative mechanisms to control PKA-C level or activity in *pkaC* multicopy transformants may be at the translational level or by inducing the synthesis of PKA-R, but we have no proof that such mechanisms work in *A. niger*.

A *pkaC* disruption strain lacked PKA activity and developed smaller colonies than the reference strain on plates. Germination of ΔC conidiospores was slightly delayed, but hyphal morphology in liquid culture and sporulation was normal. As expected, silencing of *pkaR*, in addition to *pkaC*, did not change the phenotype. Clearly, PKA activity is required for optimal growth and metabolism.

Disruption of *pkaR* gave the most dramatic phenotype, i.e. very small colonies on plates, absence of sporulation and complete loss of growth polarity during submerged growth. Compact colony morphology and inhibition of conidiation was also observed for a wild-type strain grown in the presence of 5 mM cAMP (Benčina *et al.*, 1997). The morphological and developmental effects of both disruption of *pkaR* and continuous presence of cAMP are presumably due to unrestrained PKA activity. We did not, however, provide actual evidence that PKA activity is not regulated at all in the Δ*pkaR* strain. We cannot, for example, exclude the possibility that *A. niger* possesses another PKA-R-type protein that may, at least partially, control PKA activity.

Loss of growth polarity and absence of sporulation has also been described for the *N. crassa mcb* mutant, which is deficient in PKA-R (Bruno *et al.*, 1996). Likewise, a key role for cAMP and PKA is reported for morphogenesis in dimorphic fungi (Borges-Walmsley & Walmsley, 2000). For example, in *U. maydis* disruption of *ubc1*, encoding PKA-R, caused a multiple budding phenotype (Gold *et al.*, 1994). Thus, elevated or unrestrained PKA activity gives a budding growth in dimorphic fungi and loss of growth polarity in filamentous fungi. Filamentous fungi have a short period of

isotropic growth during spore germination and PKA appears to be important for the switch of isotropic to polarized growth (d'Enfert, 1997). Interestingly, the *A. niger* $\Delta pkaR$ strain is able to form hyphae during growth on plate, although to a very limited extent. Surface growth may trigger a signal transduction pathway, also involved in hyphal extension, which is to some extent independent of the PKA pathway.

In summary, disruption of *pkaR* interferes with proper growth and development of *A. niger*. Our data show that cAMP-dependent protein phosphorylation in *A. niger* is involved in developmental processes such as growth polarity in germinating conidia and asexual reproduction, i.e. formation of conidiospores. The availability of a $\Delta pkaR$ mutant will hopefully enable a study aiming to identify the PKA targets directly involved in growth and morphology.

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