Protein kinase A is involved in the control of morphology and branching during aerobic growth of *Mucor circinelloides*

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INTRODUCTION

The regulation of fungal morphology is an area of growing interest in basic and applied research (Wendland, 2001). The control of fungal morphology would improve the growth properties of filamentous fungi in a fermenter and contribute to increased biomass levels and product yields. Efforts to modulate hyphal branching, improve secretion and reduce the problems associated with high viscosity, oxygen and mass transfer are being pursued (McIntyre et al., 2001).

Dimorphism in fungi is also relevant in the field of fungal virulence (Lengeler et al., 2000). Studies in different fungi, including ascomycetes and basidiomycetes, have converged to define two broadly conserved signal transduction cascades that regulate fungal development and virulence. One is a mitogen-activated protein (MAP) kinase cascade that mediates responses to pheromone. The second is a nutrient-sensing receptor-mediated cAMP protein kinase A (PKA) cascade. These pathways function co-ordinately to regulate mating, filamentation and virulence. Particular attention has been given to the cAMP signal transduction pathway and fungal morphology (Kronstad et al., 1998; D’Souza & Heitman, 2001). This second messenger regulates the activity of PKA by binding to the regulatory subunit (PKAR) and releasing the catalytic subunits (PKAC) from the tetrameric inactive holoenzyme to initiate a phosphorylation cascade (Taylor et al., 1992).
*Mucor circinelloides* is a dimorphic zygomycete that displays multipolar yeast or filamentous morphology in response to a number of environmental conditions, including the gas atmosphere and the level of nutrients (Orlowski, 1991; McIntyre et al., 2002; Wolff et al., 2002). Typically, the yeast morphology is favoured during anaerobic growth in the presence of a fermentable carbon source, while the filamentous form is triggered in the presence of oxygen or upon nutritional challenge. The participation of cAMP in the morphogenetic process of *Mucor* has been shown for both *Mucor rouxii* and *Mucor racemosus* (Orlowski, 1991) and analysed further particularly for *M. rouxii* (Pereyra et al., 1992, 2000). However, no molecular genetic approach had been attempted until recently with this species in order to analyse the role of PKA in *Mucor* morphology.

The role of PKA in the control of morphology and hyphal branching has been shown recently for *Aspergillus niger* (Saudohar et al., 2002) using genetic approaches. While a null mutation in the *A. niger* pkaR gene led to a small colony phenotype and lack of conidiation, an increase in pkaR expression did not result in any measurable phentypic difference (Saudohar et al., 2002). The pkaR and pkaC genes of *M. circinelloides* have been cloned recently. Expression of pkaR was observed during anaerobic yeast growth and during the shift from yeast to filamentous growth. In addition, overexpression of pkaR resulted in an increase in pkaC expression during anaerobic growth. A constructed strain, KFA121, overexpressing the pkaR gene from the promoter of the gpd1 gene (gpd1P) showed a multibranching colony phenotype during growth on plates (Wolff et al., 2002). Here, we confirm that the multibranching phenotype in strain KFA121 is a consequence of the overexpression of PKAR and present a characterization of the biochemical and morphological properties associated with this phenotype. A role for PKA in the control of branching in *M. circinelloides* is presented.

**METHODS**

**Strains and media.** *M. circinelloides* KFA121 was constructed by transforming strain R7B (ATCC 90680, a leuA mutant strain derived from ATCC 1216b) with plasmid pEUKA4, containing the pkaR gene under the control of gpd1P (Wolff et al., 2002). This promoter is inducible by glucose and galactose (Wolff & Arnau, 2002). *M. circinelloides* ATCC 1216b and strain KFA89 (a vector control derivative of R7B; McIntyre et al., 2002) served as reference strains. ATCC 1216b was used as a reference for the morphological experiments and KFA89 was used for the enzymic experiments. We have verified that the two controls are equivalent, since KFA89 is a ‘reconstituted’ ATCC 1216b, i.e. it includes a wild-type leuA gene on a plasmid that contains no other *M. circinelloides* genes (Wolff et al., 2002). The transformation host strain R7B has a point mutation in the coding region of leuA resulting in a single amino acid change (K. F. Appel, A. M. Wolff & J. Arnau, unpublished data). Furthermore, the multibranching phenotype of KFA121 was qualitatively shown by comparison to KFA89 (Wolff et al., 2002), providing added evidence for the similarities between ATCC 1216b and KFA89.

During the morphological analysis in the flow-through cell, all strains were cultivated in liquid YNB medium [1·5 g (NH₄)₂SO₄ l⁻¹, 1·5 g glutamic acid l⁻¹, 0·5 g yeast nitrogen base l⁻¹] adjusted to pH 4·5 with 1 M H₂SO₄ and supplemented with 1 mg thiamine chloride l⁻¹ and 1 mg niacin amide l⁻¹. The nature and concentration of the carbon source were varied as indicated. Cultures were grown at 28 °C in either YNB or rich medium (YPG; yeast, peptone, glucose) as indicated for PKA measurements. Glucose concentration was variable as indicated.

**Flow-through cell experiments and image analysis.** Spores were immobilized in a temperature-controlled flow-through cell mounted on a motorized stage with continuous medium flow, to assess fungal growth and branching in a quantitative way. The construction and set-up of the flow-through cell has been described in detail previously (Sophr et al., 1998). The cell consisted of two microscope slides (26 × 76 mm) separated by a Parafilm M spacer clamped into a steel frame equipped with tubes for feed addition and waste withdrawal. To sterilize both the cell and the tubing, 70 % ethanol was injected through a filter (0·45 μm); this was followed by addition of distilled water after 20 min to remove the ethanol. One millilitre of 0·1 % poly-D-lysine (Sigma) was then filtered into the cell to mediate spore fixation. The cell was inoculated with spores to gain a final number of approximately 20 spores. Medium was continuously added and removed from the cell at a flow rate of 3 ml h⁻¹.

All experiments were carried out at 28 °C. Images of the hyphal elements were obtained at time intervals of 15 min on a Nikon Optiphot 2 microscope equipped with a CCD camera (Bischke CCD-5230P) connected to an image analysis system (QUANTIMET 600S; Leica Cambridge). Automatic image analysis was applied in the detection of hyphal elements and measurements of hyphal length (Sophr et al., 1998). The hyphal tips were counted manually. These values were used to calculate the hyphal growth unit length (HGUL) (total hyphal length of a mycelium divided by its tip number, μm tips⁻¹) (Caldwell & Trinci, 1973).

**Crude extract preparation for PKA studies.** The cells were harvested by centrifugation at 7000 g during 15 min and processed immediately. Crude extracts were prepared by vortexing the cells four times for 1 min at 4 °C with glass beads (460–600 μm diameter) and buffer A (25 mM Tris pH 8, 5 mM EDTA, 3 mM EGTA, 10 mM 2-mercaptoethanol, supplemented with the indicated concentration of the Roche complete EDTA-free protease inhibitor cocktail). Protein concentration was determined by the method of Bradford (1976).

**cAMP-binding assay.** Aliquots from crude extracts, containing 5–10 μg protein, were incubated for 30 min at 30 °C or overnight at 4 °C in a final volume of 70 μl with 0·3 μM [1H]cAMP (62 000 d.p.m. pmol⁻¹) in 0·5 M NaCl in buffer A, filtered through nitrocellulose filters, washed with 30 ml water, dried and counted in liquid scintillation mixture.

**PKA assay.** Aliquots from crude extracts, containing 0·05–0·5 μg protein, were incubated for 10 min at 30 °C in 70 μl of the following incubation mixture: 50 mM Tris/HCl pH 7·2, 15 mM MgCl₂, 200 μM kemptide, 0·1 mM [γ-32P]ATP (specific activity 1300 c.p.m. pmol⁻¹), and 10 μM cAMP, when added. Samples (50 μl) were loaded onto phosphocellulose paper squares and processed according to Roskoski (1983). As opposed to the cAMP-binding assay, which is linear to the protein concentration over a large range of concentrations and can be used directly with crude extracts, linearity is often compromised when measuring PKA activity (Sorol et al., 2001). LOW amounts of protein have to be used to enable quantification of PKA levels. In each case, conditions to attain a linear response of activity versus crude extract protein concentration have to be settled.
One unit of protein kinase activity is defined as picomoles of phosphate incorporated into kemptide per 10 minutes under the standard assay conditions.

Sucrose gradient centrifugation. Crude extracts containing 3 mg protein were loaded onto 4-5 ml 5–20% sucrose gradients in buffer A, and centrifuged for 15 h at 35 000 r.p.m. in a SW 55 Ti rotor of a Beckman ultracentrifuge. Fractions were collected from bottom to top, and in each fraction cAMP-binding and PKA activities were assayed by the standard assays indicated above. Catalase (11-3 S) and horseradish peroxidase (3-5 S) were used as sedimentation markers.

Western blot analysis. Samples of crude extracts were analysed by 10% SDS-PAGE and blotted onto nitrocellulose membranes using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol buffer in a Transphor apparatus. Blots were blocked with 5% non-fat milk, 0-05% Tween 20 in Tris-buffered saline. Membranes were incubated overnight at 4°C with a 1:20 000 dilution of an antibody developed in rabbit against M. rouxi PKAR purified via cAMP-agarose affinity chromatography (Rossi et al., 1992; V. González Polo, S. Rossi & S. Moreno, unpublished data). Secondary anti-rabbit IgG peroxidase-conjugated antibody was used at 1:10 000. After three washes the blots were developed with Chemiluminescence Luminol reagent, and immunoreactive bands were visualized by digital imaging. A duplicate gel was stained with Coomassie brilliant blue to estimate protein loading.

Kemptide and anti-rabbit IgG peroxidase-conjugated antibody were from Sigma Chemical. [γ-32P]ATP and [3H]cAMP were from Perkin Elmer Life Sciences. Phosphocellulose paper was from Whatman. Nitrocellulose membrane ECL Western Blotting Detection Reagent and Roche complete EDTA-free protease inhibitor cocktail tablets were from Amersham Biosciences.

RESULTS

Morphological characterization of the pkaR-overexpressing strain KFA121

After the initial observation that M. circinelloides KFA121, the transformant strain containing the pkaR gene under the control of gpd1P, displayed a hyperbranching phenotype during growth on solid medium, as reported recently (Wolff et al., 2002), a series of flow-through cell experiments was conducted to assess its branching behaviour quantitatively. The gpd1P-driven expression of pkaR in M. circinelloides is induced by glucose or galactose and a correlation between the expression level of its mRNA and the sugar concentration in the medium has been described (Wolff & Arnau, 2002; Larsen et al., 2004). Therefore, cultivations were performed at different concentrations of an inducing carbon source (glucose) and with a non-inducing sugar (xylose) to study the effects on morphology. As a comparison, all growth conditions were also tested with strain ATCC 1216b as a reference strain.

For each hyphal element, the total hyphal length (the length of the skeletonized mycelium after image processing) and the number of branches were determined (Fig. 1). Dividing the total hyphal length of a mycelium by its tip number defines HGUL. HGUL constitutes a useful parameter for comparing branching characteristics between different strains or for a particular strain cultivated under varying conditions. It attains a fairly constant and thus characteristic value during exponential growth, when new branches are initiated at a constant rate. The specific growth rate (µ) was obtained from a semi-logarithmic plot of the hyphal length versus time, where the slope of the resulting curve is equivalent to µ. Values of HGUL and µ for KFA121 and ATCC 1216b for the different media tested are presented in Fig. 2. Each data point constitutes a means result for 9–15 individual hyphal elements. For the reference strain ATCC 1216b, HGUL decreased with increasing glucose concentration, i.e. more branches were formed, reaching 70 and 43% of the maximum HGUL (98 µm tips−1) in medium with a high glucose concentration (1 and 10 g l−1, respectively, Fig. 2a). This is also coherent with the way fungi would grow in nature. In a nutrient-rich environment, energy is easily available so new branches can be initiated rapidly. However, if the nutritional state of the immediate surroundings is poor, growth without formation of excess branches is advantageous, as this would allow the maximum possible spatial extension, towards a potential new nutrient source. Comparing the branching frequencies of M. circinelloides ATCC 1216b during growth with different carbon sources provided at the same concentration showed that mycelia were more densely branched during growth with glucose than with xylose. The transformant strain KFA121, on the other hand, displayed a branching pattern different from that of ATCC 1216b. At elevated glucose concentrations (1 and 10 g l−1), the relative reduction in HGUL compared to the values at low glucose concentration (130 µm tips−1) was about fivefold (Fig. 2a). This means that KFA121 formed an increased number of branches in high glucose where the gpd1P is induced, confirming the previous observations during growth on plates (Wolff et al., 2002). During growth in the presence of xylose, KFA121 formed significantly fewer branches compared to growth with...
glucose at the same concentration, reaching similar values to growth on low glucose (Fig. 2a). No significant differences were observed in $\mu$ for ATCC 1216b and KFA121 in each growth condition (Fig. 2b). The highest values were obtained in $0.2$ g glucose l$^{-1}$ (0.5 $\mu$) and were similar during growth in 1 and 10 g glucose l$^{-1}$ (around 0.4 $\mu$). These results established that the differences in HGUL were not due to differences in $\mu$ (Fig. 2).

Although there was also a difference in branching between growth on glucose and on xylose for the reference strain ATCC 1216b, the observed effect was more pronounced for KFA121. The different branching patterns for both strains during growth with glucose are illustrated by the image series (Fig. 3). The images clearly show the tendency of KFA121 to form very branched mycelia with rather short individual branches, whereas branches formed by ATCC 1216b were comparatively longer. A more accurate way to express this is by calculating the hyphal extension rate ($\mu$ per tip h$^{-1}$) that is obtained by multiplying HGUL by $\mu$ (Fig. 2c). KFA121 displayed a significantly higher hyphal extension rate in low glucose or in xylose, compared to ATCC 1216b (Fig. 2c). In inducing conditions, i.e. at high glucose concentrations, the hyphal extension rate is generally reduced, but for KFA121 the rates were significantly lower than for ATCC 1216b (9 vs 29 in 1 g glucose l$^{-1}$ and 10 vs 16 in 10 g glucose l$^{-1}$, respectively; Fig. 2c), quantitatively demonstrating the gpd1P-dependent hyperbranching phenotype of strain KFA121.

**PKA levels in strain KFA121**

PKAR homologues display two well-conserved cAMP-binding domains (Taylor et al., 1992). Measurement of the level of the PKAR subunit was therefore performed by the $[^{3}H]$cAMP-binding assay in crude extracts of two strains of *M. circinelloides*, KFA89 (vector control) and KFA121. The strains were grown in defined medium, with the addition of increasing concentrations of glucose, or with 10 g xylose l$^{-1}$ as sugar source. The extracts were prepared shortly after germ-tube emission (4–5 h of growth). Under these conditions, the glucose concentration was not significantly altered. The results are shown in Fig. 4. For both strains, an increase in cAMP-binding activity with the increase in glucose was observed. This was expected since a correlation between nutrient level and specific activity of PKAR and PKAC is typically observed (Pereyra et al., 2000). Moreover, at least in *M. rouxii*, more germ tubes are emitted per mother cell in richer medium at the moment of germ-tube emission (Pereyra et al., 2000). Importantly, a net increase in PKAR activity was observed in strain KFA121 at 10 g glucose l$^{-1}$. The mean increase, taken from five independent experiments, was in the range 2.5- to threefold. The results in Fig. 4(a) show a representative

![Fig. 2.](image)
experiment but not a mean, as the absolute levels of PKAR in each experiment depend on the exact physiological stage at which the cells were harvested; at the stage of germ-tube emission the levels of PKAR and PKAC are increasing steadily (Rossi & Moreno, 1994). The overall levels of PKAR were consistently higher in KFA121 compared to KFA89 even at low glucose concentration (0.1 g l⁻¹) or in xylose. These observations were anticipated since there are two reasons for expecting that spores from KFA121 accumulate higher levels of PKAR than spores from KFA89: first, gpd1P-driven expression is strongly induced during sporulation in M. circinelloides (Larsen et al., 2004) and, second, cells from stationary phase accumulate high levels of PKAR which are then incorporated in the spores derived from them (Rossi & Moreno, 1994). These differences should still be observed during the early stages of germination. However, the highest levels of PKAR were obtained for KFA121 grown in high glucose (10 g l⁻¹; Fig. 4a). Thus, these results demonstrate that glucose-dependent expression of PKAR occurs in strain KFA121.

The same crude extracts were used to determine PKA activity using kemptide as a substrate. An overall increase of about twofold was obtained for strain KFA121 compared to strain KFA89 in three independent measurements performed with extracts of cells growing in defined medium containing 10 g glucose l⁻¹ (Fig. 4b). This result is in accordance with previous evidence showing that, at least
at the level of mRNA, overexpression of the pkaR gene in strain KFA121 resulted in a significant increase of pkaC gene expression (Wolff et al., 2002).

To address whether the observed phenotype in strain KFA121 resulted from an up-regulation or a down-regulation of PKA, it was important to establish the ratio between PKAR and PKAC subunits and also if there was free PKAR in excess of the PKAR associated with PKAC as a holoenzyme. The measurement of activities in crude extracts does not provide an accurate measurement since both subunits seem to increase in the KFA121 strain, and reproducibly the increase in PKAR in each independent experiment is around 1.5-fold greater than the increase in PKAC. To have an independent measure, crude extracts from strains KFA89 and KFA121 grown for 7-5 h were ultracentrifuged on 5–20 % sucrose gradients to separate the species according to their sedimentation coefficients. The phosphorylating activity of PKAC and the cAMP-binding activity of PKAR were measured in fractions derived from the gradient (Fig. 5). The results showed unambiguously that, in strain KFA121, there was an excess of free PKAR at a lower sedimentation coefficient than the holoenzyme, i.e. not associated with PKAC activity. A relative increase in PKAR and PKAC activities could be deduced from the areas under the gradient peaks, indicating an overall increase of almost threefold in cAMP-binding activity, when comparing KFA121 (Fig. 5b) to KFA89 (Fig. 5a) extracts. For PKAC activity, the estimated increase was only around 1.5-fold. The PKAR-to-PKAC ratio within the holoenzyme peak in both gradients was maintained, and the excess of PKAR activity in KFA121 extracts was detected in fractions free from PKAC activity and with a much lower sedimentation coefficient than the holoenzyme. The observed increase in PKAC in strain KFA121 was consistent with the previously reported increase in pkaC expression in this strain (Wolff et al., 2002).

Characterization and measurement of PKAR protein levels during germination in strain KFA121

The M. circinelloides pkaR gene encodes a 427 aa protein with a predicted molecular mass of 48.6 kDa (GenBank accession no. AJ400723). Western blot analysis was carried out to investigate the expression of PKAR in strains KFA121 and KFA89 at the protein level (Fig. 6), using a specific anti-PKAR antibody. Two major bands were detected in both preparations, a large band with an estimated molecular mass of around 72 kDa and a smaller band of 60 kDa. Previous studies in M. rouxii using the same antibodies identified a full-length PKAR and a truncated PKAR that results from specific degradation, especially during spore germination (Rossi et al., 1992; Rossi & Moreno, 1994). Thus, the larger band might represent the M. circinelloides full-length PKAR while the presence of the smaller 60 kDa band suggests that a similar mechanism for PKAR degradation is present in this species. The reason for the discrepancy between the theoretical and estimated size for PKAR can be attributed to post-transcriptional modifications but also to abnormal migration observed for PKAR during electrophoresis (Marques Mdo & Gomes, 1992). A minor 83 kDa band was detected in both samples (Fig. 6, lanes 1 and 2). This band may correspond to

![Fig. 5. Sucrose gradient sedimentation of crude extracts (3 mg) from strains KFA89 (a) and KFA121 (b) grown in defined medium with 10 g glucose l⁻¹ for 7-5 h. ▲, PKAC activity measured in the presence of 10 μM cAMP; ○, cAMP-binding activity. Sedimentation markers: Cat, catalase (11.3 S); Per, peroxidase (3.5 S).](image1)

![Fig. 6. Measurement of PKAR by Western blot. Crude extracts (4 μg protein) of strain KFA89 (lane 2) and KFA121 (lane 1) growing in YNB for 5 h were used together with a polyclonal antibody raised against the M. rouxii PKAR. M, molecular mass marker (in kDa).](image2)
PKAR subunits tightly associated to A-kinase-anchoring proteins (AKAPs) responsible for the tethering of PKA holoenzyme to different cellular locations via PKAR (Carr et al., 1999) or covalently modified by an as-yet-unknown post-translational modification. Preliminary results indicate that the high-molecular-mass PKAR species is still observed in denaturing gels even after the addition of 5 M urea to the denaturing cracking mixture (data not shown). Further characterization of this PKAR species is currently under way.

A low level of the full-length PKAR was detected in the control strain, while for strain KFA121 a high level of the 72 kDa band was observed (Fig. 6). Also, a similar level of the 60 kDa band was observed in both strains. These results indicate that PKAR is actively degraded during germination in M. circinelloides and that, in strain KFA121, an excess of full-length PKAR is maintained (Fig. 6). The M. circinelloides pkA gene has also been expressed in Saccharomyces cerevisiae resulting in a unique 72 kDa band detected by Western blot analysis using the same antibody (data not shown). Taken together, the above results establish that the 72 kDa band corresponds to the full-length PKAR in M. circinelloides.

DISCUSSION
The involvement of the M. circinelloides PKAR in branching was quantified and a correlation between increased PKAR levels and enhanced branching was demonstrated in strain KFA121 (Figs 2, 3 and 4). The elevated levels of PKAR in strain KFA121 were also present during the early stages of germination when a decision about germ-tube emission occurs. Concomitant with the increase in PKAR levels via induction of the gpd1P-driven promoter, there was an increase in PKAC levels as determined by its catalytic activity (this work, Fig. 5) and mRNA expression (Wolff et al., 2002). However, the increase in the PKAR level was consistently greater than the increase in the PKAC level. As a consequence, there is an excess of free PKAR not bound to PKAC in the holoenzyme (Fig. 5). An excess of PKAR shifts the following equilibrium to the left:

\[ R_2C_2 + 4\text{ cAMP} \rightleftharpoons R_2cAMP_4 + 2\text{ C} \]

inactive \hspace{1cm} active

This shift to the non-dissociated state has two consequences: first, the existence of more inactive holoenzyme in strain KFA121 than in the control strain and, second, the holoenzyme is more refractive to activation by cAMP and higher concentrations are needed (Houge et al., 1990). We propose that this lower level of PKA activity is responsible for the increased branching displayed in this strain during growth in media containing glucose. This interpretation agrees with the results observed in M. rouxii, where the addition of a cAMP analogue to the growth medium, via a sustained activation of PKA, has as a consequence an impairment of polarized growth, which is replaced by isodiametric growth (Pereyra et al., 1992, 2000). The results presented here provide the basis for the regulation of morphology and branching in M. circinelloides. Genetic control of PKA activity will enable the development of a process for the production of heterologous proteins composed of initial biomass phase as a yeast followed by polar growth and enhanced branching to aid protein secretion. In addition, a more detailed characterization of the PKA subunits and their interaction in M. circinelloides will add to our understanding of this central element of the cellular signal transduction machinery.

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