A Subunit of Protein Kinase A Regulates Growth and Differentiation in the Fungus *Mucor circinelloides*

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The cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling pathway plays a role in regulating development, growth, and virulence in a number of fungi. To determine whether PKA plays a similar function in zygomycete fungi, a mutant of *Mucor circinelloides* was generated that lacks *pkaR1*, one of the regulatory subunits of PKA. The mutant showed a reduction in growth and alterations in germination rates, cell volume, germ tube length, and assexual sporulation. The lack of *pkaR1* gene resulted in a highly decreased, but not null, cAMP binding activity and in a protein kinase activity that was still dependent on cAMP, although with a higher −/+ cAMP activity ratio, suggesting the existence of other cAMP binding activities. Consequently, three proteins analogous to *pkaR1* were predicted from the recently sequenced genome of *M. circinelloides* and were named *pkaR2*, *pkaR3*, and *pkaR4*. Two of the proteins, *pkaR2* and *pkaR3*, with cAMP binding activity were isolated from the wild-type strain and identified by mass spectrometry. The expression of all genes was detected at the mRNA level by semiquantitative reverse transcription-PCR, and they showed a differential expression at different developmental stages. This is the first time that a fungus is reported to have more than one gene encoding the regulatory subunit of PKA.

Filamentous fungi are defined by their ability to form highly polarized hyphae, which is a prerequisite for efficient colonization of growth niches and substrate utilization. During vegetative growth, the establishment of polarity is an important initiation step for primary germ tube emergence from the spore/conidium on the one hand, and branch emergence from existing hyphae on the other. Once established, polarity must be maintained during hyphal extension. Structural components of the cytoskeleton (especially actin) play a pivotal role in establishing and maintaining fungal polar growth (10).

One of the key regulators of polarity in fungi, as well as of other processes such as development, mating, and virulence is the cyclic AMP (cAMP)-dependent protein kinase A (PKA) (5, 14). This enzyme in its inactive form is a tetramer composed of two regulatory subunits (R) bound to two catalytic subunits (C). In response to signals that increase intracellular cAMP levels, cAMP binds to the regulatory subunit and triggers conformational changes that release the active catalytic subunit. In mammalian systems there are four genes coding for the R subunit—R1α, R1β, R1α, and R1β—and three genes coding for the C subunit—Ca, Cβ, and Cy.

In several Ascomycota (*Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, *Aspergillus nidulans*, and *Aspergillus fumigatus*) and two Basidiomycota (*Ustilago maydis* and *Cryptococcus neoformans*) PKA C subunits are coded by two or three genes, whereas only one gene codes for the R subunit. Genetic evidences have shown the participation of PKA in cell polarity in some of these models. Thus, in *S. cerevisiae* pseudohyphal differentiation was altered by deletion of the gene coding for the R subunit, and each of the three C subunits has been shown to play different roles in this process (26, 32). In *C. albicans*, hyphal morphogenesis is necessary to its pathogenesis. Deletion of the gene coding for the R subunit impairs filamentous growth. Both catalytic isoforms, Tpk1 and Tpk2, act positively in the morphogenetic process, although Tpk2 is specifically required for invasiveness on solid medium, a characteristic of infective hyphal growth (4, 7, 41). In the plant pathogen *U. maydis*, the filamentous form of the fungus is the pathogenic one; knockout mutants for the gene coding for the regulatory subunit are unable to form dikaryotic mycelium and have a defect in bud-site selection and cytokinesis (13). In several *Aspergillus* species, the deletion of genes coding for R subunits impairs polar growth, whereas the deletion of one or more genes for C subunits has shown that PKA is involved in other processes such as growth and tolerance to oxidative stress (2, 17, 22, 42, 46). In the mammalian pathogen *C. neoformans* PKA strongly regulates its virulence (11).

We are interested in studying the involvement of cAMP pathway, via PKA, in the growth and morphology of *Zygomycetes*, in particular those of the genus *Mucor*. Both *Mucor circinelloides* and *M. rouxii* are dimorphic fungi from the new subphylum *Mucoromycota* that display multipolar yeast or filamentous morphology in response to a number of environmental conditions, including the gas atmosphere and the level of nutrients (25). We have demonstrated that spores of *M. rouxii* grown in the presence of a cAMP analog show an isodiametric growth, with total impairment of polarity, and that PKA is involved in this process (28, 29). We have also studied PKA by biochemical approaches and shown that in both fungi the holoenzymes are tetrameric and that they resemble their mammalian counterparts. However, the affinity in
the interaction between R and C subunits is higher for the fungal enzymes (24, 27).

No molecular genetic approaches had been attempted until recently in order to analyze the role of PKA in Mucor morphology. The pkaR gene, renamed pkaR1 here, and pkaC gene of M. circinelloides have been cloned recently (47). A high level of expression of pkaR1 was observed during anaerobic yeast growth and during the shift from yeast to filamentous growth. In addition, overexpression of pkaR1 resulted in an increase in pkaC expression during anaerobic growth, multibranching of colonies growing on plates, and reduced hyphal extension rate (19, 47).

To analyze the M. circinelloides PKA enzyme in more detail and to study its role in the regulation of morphological and cellular development, we generated a M. circinelloides mutant with a disrupted pkaR1 gene. Analysis of the morphological features associated with this mutation suggests that this gene is involved in several developmental processes in this fungus. Moreover, we identified three genes with sequence homology to pkaR1 in the M. circinelloides genome sequence. The R-subunit genes displayed a differential expression during aerobic and anaerobic development. It is the first time that a fungus is reported to have more than one gene coding for the regulatory subunit of PKA.

Materials and Methods

Strains, growth, and transformation conditions. Strain MU402 (23), a uracil and leucine auxotroph derived from RTB (33), was used as the recipient strain in transformation experiments to knock out the pkaR1 gene. The leucine auxotroph RTB (wild type) (33) is a leuA mutant strain derived from M. circinelloides formosanus CBS277.49 (40). This strain was used as a control in all experiments because it has the same auxotrophy as the pkaR1 mutant. Cultures were grown at 26°C in YNB, YPG, or MMC medium (23), which were supplemented with uridine (200 µg/ml) or leucine (20 mg/ml) when required. The pH was adjusted to 4.5 and 3.2 for mycelial and colonial growth, respectively. Transformation was carried out as described previously (31). Complemented strain was grown in YNB medium without leucine.

Nucleic acid isolation and analysis. General procedures for plasmid DNA purification, cloning transformation of Escherichia coli, and standard manipulations for hybridization analyses were performed as described in standard manuals (37). DNA from M. circinelloides was prepared as described previously (36). For Southern blot analysis, restriction-digested chromosomal DNA (1 µg) was blotted onto positively charged nylon filters (Hybond-N+; GE Healthcare Life Sciences) and hybridized at 42°C to radioactively labeled probes in hybridization solution with 0.1 g of dextran sulfate/ml. For Northern blot hybridizations 15 to 25 µg of total RNA from each sample was electrophoresed in 1.2% agarose formaldehyde gels using 1× morpholinepropanesulfonic acid, blotted onto positively charged nylon filters (Hybond-N+) and hybridized in 0.9 M NaCl-0.5% sodium dodecyl sulfate (SDS)-0.1 g of dextran sulfate/ml. Probes were labeled with [α-32P]dCTP using the Ready-to-Go DNA labeling beads (GE Healthcare Life Sciences).

Plasmid pUC18R harboring the pkaR1 gene from position –430 to position 1982 (GenBank accession no. A4400723) was generated by molecular subcloning into the pUC18 vector of a 2.413-bp EcoRI DNA fragment, which was obtained by PCR using genomic DNA as a template and specific primers, including the EcoRI restriction sites.

Plasmid pRgrY, which contains the M. circinelloides pkaR gene (30) flanked by pkaR1 sequences, was constructed to disrupt pkaR1. To generate this plasmid, the pUC18R was PCR amplified by using the primers pkaR-p1 (5′-CCACAGAGTCCTTTGGAGGACCCAGG-3′) and pkaR-p2 (5′-ATTTTAGCTCGAAGGAGGTGAAGGC-3′), both of which include BglII restriction sites (in italics). These primers amplify outwardly from the pkaR1 gene toward the vector sequence, producing a deletion of 0.564 kb of the pkaR1 coding region, which the PCR product digested with BglII was ligated with a pRgrY 3.2-kb BamHI fragment from pEPm1 (3) to produce pRgrY plasmid. The plasmid was linearized with EcoRI digestion and introduced into MU402 protoplasts by transformation. The pkaR2 plasmid was proposed by transformation of pkaR2 plasmid with a plasmid overexpressing pkaR1, grown in minimal medium with or without leucine depending on the strain, were incubated for 30 min at 30°C in a final volume of 70 µl with 0.3 µM [3H]cAMP (62,000,000 pmol µCi-1) and 0.5 M NaCl in buffer 10 mM Tris-HCl (pH 8). At this concentration the cAMP was saturated. An aliquot was spotted on nitrocellulose membrane filters under vacuum and washed with 20 mM Tris-HCl buffer (pH 7.5) (12). R activity was estimated by calculating the amount of cAMP binding in picomoles. Standard PKA assay. The PKA C-subunit activity was determined by assay of its phosphotransferase activity with kemptide as a substrate. The phosphorylation of kemptide was performed by adding an aliquot of PKA, semipurified by DEAE-cellulose, to a standard incubation mixture containing 15 mM MgCl2, 0.1 mM [γ-32P]ATP (700,000 cpm/µl), 200 µM kemptide, and 10 µM cAMP. After 15 min at 30°C, aliquots were processed according to the phosphocellulose paper method (34). PKA activity was expressed in units defined as picomoles of phosphate incorporated into substrate/min at 30°C.

M. circinelloides R-subunit purification. Wild-type strain RTB and strain ΔR (106 spores/ml) were cultivated in liquid YPG medium (pH 4.5). Cultures were grown for 3 to 4 h until germ tube emission. The R subunit was isolated by using N′-cAMP-agarose from BioLog (35). Briefly, crude extracts from both strains were loaded onto the resin; the resin was washed exhaustively with 0.5 M NaCl. Finally, SDS-polyacrylamide gel electrophoresis (PAGE) cracking buffer was added to the resin to elute proteins bound to the resin. M. circinelloides PKA holoenzyme purification. Wild-type and ΔR strains (106 spores/ml) were cultivated in liquid medium YPG. Cultures were grown for 4 to 5 h until the emission of germ tube. PKA holoenzyme was prepared as described previously (27) using buffer A (25 mM EDTA (5 mM Tris-HCl (pH 8), 5 µM MgCl2, 10 mM EGTA, 10 mM 2-mercaptoethanol, and complete EDTA-free protease inhibitor cocktail from Roche) through DEAE-cellulose eluted with 0.35 M NaCl.

Western blotting. Samples of purified R preparations were analyzed by SDS-PAGE and blotted onto nitrocellulose membranes using 25 mM Tris–192 mM glycine–20% (vol/vol) methanol buffer. Blots were blocked with 5% nonfat milk and 0.05% Tween 20 in Tris-buffered saline. Primary antibodies were prepared against R-subunit (27) using buffer A (25 mM EDTA (5 mM Tris-HCl (pH 8), 5 µM MgCl2, 10 mM EGTA, 10 mM 2-mercaptoethanol, and complete EDTA-free protease inhibitor cocktail from Roche) through DEAE-cellulose eluted with 0.35 M NaCl.

Microscopic analysis. For microscopic assays, the cells were fixed with 8% glutaraldehyde and analyzed by using a Nikon E-600 microscope. Pictures were taken with a Nikon Cool Pix-5000 camera. The cell volume and hyphal length were calculated by analyzing 500 cells from each strain and using Science Lab98, Image Gauge version 3.12. The values are expressed in arbitrary units, and each value represents the mean ± the standard error of the mean (SEM) for three independent experiments.

R-sequence prediction and MS analysis. Access to the information of M. circinelloides genome in its 4× and 8× assemblies was generously facilitated by the DOE Joint Genome Institute and the Mucor Genome project. Putative additional R genes were predicted by blasting the database with the already-described pkaR1 sequence from M. circinelloides. cDNA and protein sequences were derived after intron predictions and sequence homology search. Mass spectrometry (MS) data from tryptic digestion of SDS-PAGE gel bands were obtained by using a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometer, Ultraflex II (Bruker), in the MS facility CEQUIBIEM in Argentina. For the identification of some proteins, manual spectrum interpretation was carried out by comparison of the experimental MS and tandem MS (MS/MS) data with the in silico digestion of the predicted R coding genes.

Semiquantitative RT-PCR. RT-PCR was performed by samples of wild-type strain cells from M. circinelloides grown up to different stages using standard procedures. Semiquantitative reverse transcription-PCR (RT-PCR) of each pkaR gene was performed using the elongation factor EF-1α gene (tef-1) as an internal standard. The tef-1 gene has been demonstrated to be constitutively expressed throughout germination in M. circinelloides (45). The RT-PCR ampli...
fication reaction, using Superscript II transcriptase reverse from Invitrogen, was calibrated in order to determine the optimal number of cycles that would allow detection of the appropriate mRNA transcripts while still keeping amplification on solid medium and a reduced hyphal extension rate (19, 47).

RESULTS

Construction of a knockout strain for the pkaR gene. It has been reported that a M. circinelloides transformant overexpressing the pkaR gene under the control of gpd1 promoter (KFA121) displays a hyperbranching phenotype during growth on solid medium and a reduced hyphal extension rate (19, 47). After obtaining these results, we decided to delete the pkaR gene to determine in a direct way its role on the morphology adopted by spores upon germination. One null mutant for the pkaR gene was generated by gene replacement using a knockout vector that contained the pyrG gene, used as a selective marker, flanked by sequences of the pkaR gene and adjacent regions. A restriction fragment from this plasmid containing the pyrG gene and sufficient sequences of the pkaR gene to allow homologous recombination (21, 31) was used to transform strain MU402, which is wild-type for PKA but auxotrophic for uracil and leucine. Several initial transformants were grown in selective medium for several vegetative cycles to allow homologous recombination (21, 31) was used to transform strain MU402, which is wild-type for PKA but auxotrophic for uracil and leucine. Several initial transformants were grown in selective medium for several vegetative cycles to finally obtain one homokaryotic transformant named MU419 (ΔR).

The disruption of the pkaR1 gene was confirmed by Southern blot analysis and PCR (Fig. 1A and B). DNA from the

FIG. 1. Disruption of pkaR1 gene. (A) Genomic DNA from the wild-type strain, MU402, and the pkaR1 knockout mutant (ΔR) was digested with EcoRI and hybridized with the pkaR1 probe (left panel) and the pkaR1 internal probe that only hybridized with the fragment replaced in the disruption (right panel). The positions and sizes of the DNA fragments are indicated in the middle of both Southern blots. (B) PCR performed with primers (p1 and p2) that hybridized inside pkaR1 and pyrG sequences. (C) Northern blot developed with pkaR1 probe; in the bottom panel is shown the total RNA loaded in each lane stained with ethidium bromide. (D) Genomic structure of the wild-type (wt) locus for pkaR1 and after homologous recombination with the replacement fragment. The primers and probes used are indicated. E, EcoRI; B, BglII (wild-type strain) and BglII/BamHI (mutant strain).
The transformant and the parental strain was digested with EcoRI and hybridized with a pkaR1 probe that hybridizes with the wild-type and the disrupted pkaR1 alleles (Fig. 1A, left panel). The transformant showed an expected fragment with an increase of 2.6 kb in its size compared to the fragment of the wild-type strain, indicating that the pkaR1 wild-type allele had been replaced. The shift in the mobility of the fragments came from the replacement of a 0.6-kb DNA fragment from the wild-type allele for a 3.2-kb DNA fragment containing the pyrG sequence (Fig. 1D). The gene replacement was confirmed by hybridization with an internal pkaR1 probe, which hybridized only with DNA from the wild-type strain (Fig. 1A, right panel). Moreover, the absence of wild-type DNA fragments in the transformant in both hybridizations confirmed that it is homokaryotic for the deletion of pkaR1 gene.

The disruption of pkaR1 gene was also confirmed by PCR using a primer that hybridized in a pkaR1 genomic region outside of the replacement fragment and another that hybridized inside the pyrG sequence. An expected 1.7-kb DNA fragment was only obtained in PCKs using DNA from the mutant, confirming the insertion of pyrG sequence in the pkaR1 gene (Fig. 1B).

The expression of pkaR1 has been described to be higher after the shift from anaerobic yeast growth to aerobic filamentous growth than during anaerobic yeast growth (47). Therefore, a Northern blot analysis was performed with RNA from cells shifted from anaerobic to aerobic growth. A band corresponding to the pkaR1 mRNA was observed only in the wild-type strain, confirming that the mutant strain lacks a functional pkaR1 gene (Fig. 1C).

Biochemical characterization of the knockout mutant for pkaR1. Deletion of the regulatory subunit of PKA was expected to result in loss of the cAMP binding activity and, consequently, in loss of regulation of the PKA activity by cAMP. In order to test this prediction, these activities were measured in the wild-type strain and the null pkaR1 mutant. Since the expression of pkaR1 was found to be increased after the shift from anaerobic to aerobic filamentous growth (47), the level of the cAMP binding activity was analyzed in this developmental transition. A [3H]cAMP binding assay was performed in extracts from the wild-type (R7B) and mutant strains grown until the emission of the germ tube (4 to 5 h of growth) after the shift from anaerobiosis to aerobiosis. In the presence of saturating amounts of cAMP, the mutant strain, ΔR, showed a decrease of threefold in cAMP binding activity compared to the wild-type strain, but the activity was still significant (Fig. 2A). This decrease in binding activity was due to the absence of the pkaR1 gene because it was reversed by the introduction of a self-replicative plasmid (Fig. 2A) containing a wild-type allele of pkaR1 under the control of the potent gpd1 promoter (47). In fact, the presence of this plasmid provoked an increase in cAMP binding activity of ~1.5-fold in the
corresponding transformant (ΔR+R) compared to the wild-type strain, which can be due to an overexpression of pkaR1 gene. The total amount of cAMP binding activity attained was, as expected, lower than the one observed when the same plasmid was introduced in a wild-type strain (19). The overexpression of pkaR1 using a heterologous promoter was due to the fact that no expression was observed when a plasmid containing the wild-type allele plus 0.5-kb of upstream sequence was introduced in the mutant strain (data not shown).

Semipurified samples from strains subjected to an anaerobic-aerobic shift as described above were used to determine the pkaC activity using kemptide as a substrate in the presence or absence of cAMP. Even though the wild-type and mutant strains showed a PKA activity regulated by cAMP, the cAMP activity ratio was higher in the mutant strain (0.6) than in the wild-type strain (0.23), indicating a lower cAMP dependence (Fig. 2B). In addition, the total PKA activity, measured in the presence of cAMP, was 2.5-fold higher in the wild-type strain than in the mutant strain (Fig. 2B). These results indicate that the lack of the regulatory subunit encoded by pkaR1 gene modified the kinase activity but, surprisingly, this activity was still dependent on cAMP, although it was less regulated. The presence of cAMP binding activity and dependence on cAMP in the mutant strain suggested the existence of more than one regulatory subunit. Although it was possible that other proteins different from the R subunit of PKA could be responsible for the remaining cAMP binding activity, we considered the existence of other gene(s) encoding R subunit(s) in M. circinelloides to be highly probable. This idea was reinforced when semipurified holoenzyme preparations from the wild-type strain, the ΔR mutant, and the complemented transformant were analyzed by Western blotting with a polyclonal antibody against the R subunit from M. rouxii. The sample from the wild-type strain and the ΔR+R strain presented a protein band that was absent in the mutant strain (Fig. 2C), suggesting that it corresponds to the product of pkaR1 gene.

Moreover, two additional bands were revealed in all strains (Fig. 2C), including the mutant strain, suggesting that they corresponded to products of other pkaR genes (see below).

These results indicate that the mutant pkaR1 null, which has a decreased cAMP binding activity and a consequent PKA activity with a decreased cAMP dependence, has less inhibited catalytic activity and suggest the existence of extra R subunits for PKA.

Growth and morphology of the pkaR1-null mutant. The role of pkaR1 in morphology and hyphal development was monitored by analyzing the phenotypes of the wild type, mutant, and complemented transformant growing on solid medium and in liquid cultures under aerobicosis and anaerobiosis shifted to aerobic growth conditions. The radial growth rate on solid medium of the wild-type strain was 1.5-fold higher than that of the mutant strain (Fig. 3), whereas the mutant strain showed a significant reduction in spore formation (Table 1), although the size of the spores were the same in both strains (data not shown). The altered phenotypes of the mutant were restored by the introduction of the wild-type pkaR1 allele into the null mutant. Thus, the complemented transformant had a radial growth rate similar to that of the wild-type strain, while the spore production was greater than that of the wild-type strain.

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<tr>
<th>Strain</th>
<th>Mean ± SEM</th>
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<tr>
<td></td>
<td>No. of spores/cm²</td>
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<tr>
<td>Mutant (ΔR)</td>
<td>(7 ± 0.7) × 10⁶</td>
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<tr>
<td>Wild type</td>
<td>(8.9 ± 0.9) × 10⁶</td>
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<tr>
<td>Mutant (ΔR+R)</td>
<td>(3.1 ± 0.5) × 10⁷</td>
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* Spores (n = 100) of each strain were spread onto MMC plates in triplicate and incubated at 30°C for 5 days. Plugs of agar (1 cm²) were removed for spore calculation. The calculated growth rates correspond to the experiment illustrated in Fig. 3A.
The differences between the wild-type strain and the complemented transformant are probably the effect of overexpression of \textit{pkaR1} using a high-copy-number plasmid under the control of the \textit{gpd1} promoter.

The growth curves of the wild-type, mutant, and complemented strains in liquid medium in aerobic conditions were similar, and no differences in mycelial dry mass were observed after 80 h of growth (data not shown). However, microscopic examination revealed that the germination kinetics, the mother cell volume and the germ tube length were different in the wild-type and mutant strain. Submerged cultures grown overnight under anaerobic conditions and shifted to aerobiosis were used for measurement of the mother cell volume (overnight anaerobic growth) and germ tube length (4 h after aerobic shift) (Fig. 4). The mutant strain showed a different phenotype for both features, since the germ tubes of the mutant were shorter than those from the wild-type strain (Fig. 4B), and the budding yeast volume of the mutant strain was 2.5-fold lower than that of the wild-type strain (Fig. 4A and B). The phenotype of the complemented transformant could not be compared to the wild-type and mutant strain because their anaerobic grown cells had a deformed morphology and appeared in groups, which could be due to the high cAMP binding activity (Fig. 2A).

The same differences in cell volume between the wild-type strain and the mutant strain were observed in cells continuously grown in aerobic conditions; these differences were partially reversed in the complemented transformant (Fig. 4C). We could also observe that the emergence of germ tubes during filamentous growth in aerobic conditions was earlier in the mutant strain than in the wild-type strain (Fig. 4C). Thus, after 3 h of growth, 50% of the mutant cells had emitted germ tubes, whereas only 15% of the wild-type cells had attained this stage of morphology. This defect was also due to the absence of the \textit{pkaR1} gene since it was suppressed in the complemented strain, which showed even a lower percentage of cells with germ tubes than did the wild-type strain (Fig. 4C). This tendency was also observed in the number of emerging buds from yeasts under anaerobic conditions of growth (data not shown).

In summary, the deletion of the \textit{pkaR1} gene affects growth, germ tube emergence, and yeast-to-hyphal morphogenesis.

**Identification of other \textit{pkaR} genes in \textit{M. circinelloides}**. Since the disruption of \textit{pkaR1} gene resulted in a highly decreased but not null cAMP binding activity, in a PKA activity that was still dependent on cAMP, and in the presence of several bands in a Western blot revealed with anti-R antibody (see Fig. 2C), the existence of other R subunit coding genes was hypothesized. While the present study was in progress, a draft assembly of the \textit{M. circinelloides} genome sequence, generated using 8× sequence coverage, was made available through the \textit{Mucor} genome project developed at the Joint Genome Institute (http://genome.jgi-psf.org/Mucci1/Mucci1.home.html). A BLAST search using the available \textit{pkaR1} sequence found three different sequences, in addition to \textit{pkaR1}, in the \textit{M. circinelloides} genome sequence assembly.earnutrients between the wild-type strain and the complemented transformant are probably the effect of overexpression of \textit{pkaR1} using a high-copy-number plasmid under the control of the \textit{gpd1} promoter.

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The *M. circinelloides* genome with a high score and significant E-value. A sequence alignment with the deduced amino acid sequences showed that all of the sequences shared a high degree of similarity with other regulatory subunits and had the highly conserved residues present in R proteins of PKAs (data not shown). Therefore, these putative genes were named *pkaR2*, *pkaR3*, and *pkaR4* (accession numbers fgeneshMC_pm.13_#_2, e_gw1.8.247.1, and e_gw1.2.493.1, respectively, in the *M. circinelloides* genome).

As occurs with all of the R subunits, there is a high degree of similarity in the carboxy-terminal domain of the *M. circinelloides* proteins containing the highly conserved cAMP binding domains A and B (data not shown). Moreover, they show inhibitory sequences (IS) resembling a substrate that would interact with the C subunit. In mammalian systems the RII subunits contain a real substrate homolog in the IS with a phosphorylatable Ser; however, RI subunits display a pseudosubstrate IS with a nonphosphorylatable amino acid. Fungal R subunits described up to now cannot be classified as RI or RII because they share properties of both subunits; however, regarding the IS sequence, they have a Ser in the IS and, therefore, in this property, they resemble RII. The four *Mucor* R subunits all share this characteristic. All of the R subunits thus far differ in their amino termini, which are not conserved except for the more amino-terminal portion that contains the dimerization and docking domain of R. Also, in this case, the amino termini of the four R subunits are completely dissimilar (Fig. 5).

To determine whether the identified *pkaR* genes code for R subunits, cAMP binding proteins were isolated from the wild-type and the mutant strains using cAMP-agarose affinity resin. After exhaustive washing, the purified proteins were analyzed by SDS-PAGE, staining with colloidal Coomassie blue, and Western blotting with anti-R antibody. Three main stained bands were found in the purified fractions from the wild-type strain, all of them positive for the detection with the anti-R antibody (Fig. 6A), suggesting that they corresponded to R subunits. MALDI-TOF spectra from tryptic digests of the protein bands identified the slowest mobility band, which was present in the wild-type strain but not in the mutant strain, as the product of *pkaR1* with a score of 126 and an expected value of $10^{-8}$, the coverage percentage being 40% with 17 peptides matched. To identify the other bands, the masses of the tryptic peptides of PKAR2, PKAR3, and PKAR4 were predicted in silico, allowing one missed cleavage, and compared to the peptides masses obtained from the MALDI-TOF MS spectra. This analysis identified the other two bands as PKAR3 and PKAR2, because we found 12 peptides from PKAR2 repre-
senting 27% sequence coverage, and 11 peptides from PKAR3 representing sequence coverage of 29.1%. To verify the identity of these two bands, we performed MS/MS analysis from one representative peptide from each protein: ion 1,836.91 for PKAR2 and ion 1,822.93 for PKAR3. In both cases several ions from y and b series were identified, confirming the identity of the bands (Fig. 6C). The putative R subunit from PKAR4 was not detected by Western blotting in the analyzed extract (4 h of aerobic growth) or by MS. The highest mobility band stained with colloidal Coomassie blue, which was not detected with antibodies, was identified by MS and corresponded to GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a common contaminant in this type of purifications (unpublished results).

Analysis of the nucleotide sequences of pkaR genes revealed that pkaR1, pkaR2, and pkaR4 genes have two introns in equivalent positions, sharing the same splice sites, whereas pkaR3 has only one intron in a conserved position (Fig. 5B). Moreover, pkaR3 has the lowest sequence homology compared to the four pkaR sequences (data not shown). Therefore, conserved intro-exon boundaries suggest that the M. circinelloides pkaR genes arose by means of gene duplication events, pkaR3 gene being less closely related to the other three. The duplication that gave rise to the pkaR1 and pkaR4 gene pair incorporated a downstream gene encoding protein with a very high identity, the same functional domains predicted (GTP-binding protein), and also the same molecular function predicted. However, the duplications did not incorporate other flanking genes, as indicated by the different genes present upstream and downstream of each pkaR gene (Fig. 5B). These duplications occurred only in the Zygomycetes lineage because an analysis of fungal public genome databases, using M. circinelloides PKAR1 sequence as query in a BLAST search, revealed the presence of more than one pkaR gene only in two other Zygomycetes, Rhizopus oryzae and Phycomyces blakesleeanus (six for the former and four for the latter). Phylogenetic analysis and protein sequences comparison of PKAR proteins from Zygomycetes, Ascomycetes, and Basidiomycetes suggests that the duplication event that gave rise to pkaR genes occurred in the zygomycete ancestor and before species divergence (similar results have been obtained in phylogenetic analyses of the crgA gene [V. Garre, unpublished data]) because each M. circinelloides PKAR homolog is most closely related to R. oryzae and P. blakesleeanus orthologs than to their paralogs (Fig. 5C).

Expression of pkaR genes. The levels of pkaR1, pkaR2, pkaR3, and pkaR4 mRNA were assessed in the wild-type strain during growth in liquid cultures under aerobicosis and anaerobiosis shifted to aerobic growth conditions (Fig. 7). Semiquantitative RT-PCR was used to measure transcript levels using tef-1 mRNA to normalize the results, because its levels show no variation among different morphological type cells (43). Differences in the expression of each pkaR isoform mRNA were observed during growth during aerobicosis (Fig. 7A). In spores, the pkaR3 mRNA levels were higher than those for the other
three isoforms, but the levels decreased when the germ tubes were emitted (4 h) and remained at similar levels after 8 and 16 h of growth. In contrast, pkaR1 mRNA levels started to increase after 4 h and increased still more after 8 h of growth. At that point (5 h of aerobic growth), the levels of pkaR2 and pkaR4 mRNA transcripts were also increased, but the amounts of pkaR1 and pkaR2 mRNA were higher than those of pkaR3 and pkaR4 mRNA. After 16 h of growth, the pkaR4 transcript levels decreased, and the pkaR1 and pkaR2 mRNA levels remained almost without change. The pattern of the mRNA transcript levels was similar to the protein expression pattern obtained in the cAMP binding protein purification (Fig. 6A).

In order to establish whether the expression of the pkaR genes is regulated during yeast-to-mycelial transition, levels of the corresponding mRNAs were also measured in wild-type strain grown overnight in anaerobic conditions and then shifted to aerobic conditions during 4 h of growth (Fig. 7B). Under conditions for anaerobiosis, pkaR1 and pkaR2 mRNA levels were similar and higher than those of pkaR3 and pkaR4, the latter being undetectable. Upon shift to aerobicism for 4 h, the main change was the drastic increase in pkaR4 mRNA.

In summary, pkaR3 was the highest expressed gene in spores, pkaR1 and pkaR2 were highly expressed during growth in both aerobic and anaerobic conditions, and pkaR4 was highly induced in the anaerobic-aerobic transition.

These results lead us to believe that each pkaR isoform would have a differential role during M. circinelloides growth and differentiation.

**DISCUSSION**

Previous results showed that PKA activity plays a role in the induction of filamentous growth in *Mucor* (19, 28). The overexpression of pkaR1 subunit and the consequent cAMP-dependent PKA downregulation, in *M. circinelloides*, results in an increased branching and a reduction in hyphal extension rate in liquid medium growing conditions (19). Previous results also established, in *M. rouxii*, that the concentration of PKA seems to be critical for the differentiation of the germinated spores (29).

In the present study, we analyzed the role of the PKA pathway in the dimorphic fungus *M. circinelloides*, by generation of a deletion mutant in the pkaR1 gene. This mutant was phenotypically different from the wild-type strain in both macroscopic and microscopic features. On solid medium, the mutant showed a reduced spore formation and slow radial growth. The latter defect appears to be a common feature of regulatory subunit mutants of filamentous fungi, since it has also been reported for *A. niger*, *N. crassa*, and *Colletotrichum lagenarium* (1, 42, 44). Sporulation defects, ranging from failure to conidiation of PKA signaling in asexual development of these organisms (42, 46, 44).

During submerged growth the mutant and the wild-type strains had different germination kinetics. The germ tube emergence in the mutant was earlier than in the wild-type strain, although the germ tubes of the mutant were shorter than those of the wild-type strain. In anaerobic culture, the mother budding yeast cells from the mutant strain had a lower volume than those from the wild-type strain, this difference in volume being maintained after the shift to aerobic conditions.

All of these phenotypic defects observed in the mutant strain are less severe than those observed in mutants of other filamentous fungi, such as *N. crassa* mutants defective in the regulatory subunit of PKA that show a complete loss of growth polarity (1). Other filamentous fungi, such as *A. fumigatus*, showed weak morphological changes upon pkaR disruption as occurs in this case with *M. circinelloides*: reduced growth, germination defects, and increased hyphal diameter with no evidence of loss of polarity in submerged culture (46). On the other side, the deletion of the gene encoding the regulatory subunit in *C. albicans* and *Y. lipolytica* is lethal (7, 8). All of these results indicate that the PKA pathway exerts different roles in fungal morphology.

Previous reports from our laboratory demonstrated that the time of germ tube emergence and the size of the cell at this time depend on the threshold level of PKA total specific ac-
The biochemical characterization of the mutant strain indicated that the lack of pkaR1 resulted in a highly decreased but not null cAMP binding activity and in a kinase activity that was not independent of cAMP but with a −/+ cAMP activity ratio higher than that of the wild-type strain. The PKA activity is less inhibited and therefore is more active in mutant strains, and it is probable that the critical PKA activity necessary for germination is attained at a smaller cell volume and earlier than in wild-type cells. In the mutant strain complemented with pkaR1, there should be an excess of regulatory subunit over PKA holoenzyme as indicated by the cAMP binding activity in this strain. The PKA activity should be downregulated under this condition. The phenotypes displayed by this strain are in agreement with a lower PKA activity versus the mutant pkaR1 strain. The results obtained here indicate that PKA activity affects the growth and germ tube emergence and yeast-to-hyphal morphogenesis.

There are differences in the published results regarding the PKA activity in mutants of the regulatory subunit. In an A. niger mutant, the PKA activity did not show any change with respect to the wild-type strain, suggesting the possible existence of another gene carrying out similar functions (42). In other fungi the PKA activity in deleted R subunit strains was higher or lower than in the wild-type strain (7, 10, 17). The observed decrease in pkaC activity in the mutant strain was consistent with the previously reported regulation of the expression of the catalytic and regulatory subunits; the expression of both pkaR and pkaC was found to be highly regulated and coordinated in response to environmental factors, leading to morphogenesis (19).

The biochemical and morphological results obtained in M. circinelloides made us predict the existence of more than one gene coding for regulatory subunits of PKA. A BLAST search of the genome sequence of M. circinelloides predicted the existence of four R subunits: pkaR1, pkaR2, pkaR3, and pkaR4. The pkaR1 was the gene disrupted in here, the only gene that had been previously cloned. We confirmed this prediction by purifying the cAMP binding proteins and analyzing them by MS and through the detection of mRNA by semiquantitative RT-PCR. The results of RT-PCR indicate that there is differential expression of the four pkaR genes showing a morphology-specific pattern of transcript accumulation. Thus, pkaR3 is mainly expressed in spores, whereas pkaR4 always accompanies polarized mycelial growth and is almost not expressed in other stages. In addition, pkaR1 and pkaR2 expression is associated with both aerobic and anaerobic growth, and these genes show the highest mRNA levels.

Mammalian R subunits consist of the N terminus, through which two monomers of R dimerize and dock to anchoring proteins: the C terminus, which has two tandem cAMP-binding domains and, between these two parts, a flexible linker region that includes a basic substratelike inhibitory site. There is a high global degree of similarity among different R sequences from different species due to the fact that the cAMP-binding domains are highly conserved (6); however, there is striking sequence diversity in the N terminus and the linker regions (45).

The identified proteins in M. circinelloides, also differ in the sequence of the N terminus domain and linker region. In the linker region of these R subunits there is a cluster of contiguous acidic residues (Fig. 5). We have previously demonstrated that the M. circinelloides PKA has an interaction affinity between the holoenzyme R-C subunits higher than in higher eukaryotic holoenzymes but similar to those in M. rouxii PKA. We have demonstrated that this cluster of acidic residues in linker I is partly responsible for the high affinity of the M. circinelloides PKA holoenzyme (24). PKAR1 and PKAR4 have a higher number of acidic residues than PKAR2 and PKAR3 in the linker I region. Therefore, the interaction affinity between C and the latter isoforms should be lower than between PKAR1 and C. Since pkaR4 mRNA was not detected in Mucor extracts by 4 h after germination, the PKA activity measured in the extracts (Fig. 2) should be a consequence of the expression of pkaR1, pkaR2, and pkaR3 in the wild-type strain and of pkaR2 and pkaR3 in the deletion mutant strain. The reduced number of acidic residues in both proteins PKAR2 and PKAR3, and hence their putative lower affinity for C, could explain the lower cAMP dependence on PKA activity in extracts from the mutant strain. The PKA holoenzyme isoforms in the mutant strain are thus predicted to have decreased interaction affinity, resulting in a moderate alteration in differentiation and growth.

A careful analysis of the exon-intron distribution in the four pkaR genes indicates that pkaR1, pkaR2, and pkaR4 have two introns in equivalent positions, whereas pkaR3 has only one intron. This suggests that it is possible that the presence of four pkaR genes is due to multiple rounds of whole-genome duplications, duplication of segments smaller than the entire genome, or duplications in individual genes. Although whole-genome duplication in the Zygomycetes phylogenetic branch is not supported yet by whole-genome analyses, gene duplication is a recurrent theme in Zygomycetes biology, and many examples have been published (16, 18, 39). Moreover, a comparative study of 34 complete fungal genome sequences, representing a broad diversity of Ascomycetes and Basidiomycetes species, and the zygomycete R. oryzae, showed that the multiplicity of isoforms for one protein varies greatly between fungal species, but the zygomycete R. oryzae possesses by far the highest number of duplication clusters of all of the fungi analyzed (9). A recent report (20) indicates that the order and genomic arrangement of the duplicated gene pairs and their common phylogenetic origin provide evidence for an ancestral whole-genome duplication in R. oryzae, but preliminary genome analyses seem to indicate that whole-genome duplicationdid not occur neither in Mucor nor in Phycomyces (V. Garre and L. M. Corrochano, unpublished data). Our results suggest that the duplication events, leading to the four extant copies of pkaR genes, occurred very early in the zygomycete evolutionary lineage because each M. circinelloides PKAR homolog is most closely related to R. oryzae and P. blakesleeanus orthologs than to their paralogs (Fig. 5C). Probably these three duplication events did not incorporate flanking genes, suggesting that pkaR genes were derived from limited local duplications within the genome. However, duplications of larger genome regions cannot be discarded because the same genome arrangement at the pkaR loci could be originated by massive losses and rearrangements occurred after the ancestral duplications, this alternative being consistent with the fact that the duplications arose very early in zygomycete evolution.

The multiplicity of genes seems not only to be related to the
cAMP-PKA signaling pathway, since there have also been found multiple isoforms in proteins not related to this pathway, such as chitin synthase genes (18), white collar-1 genes (39), glycoside hydrolases (mce), etc. The case of white collar-1 genes is of particular interest because functional analyses suggest that they have acquired different function after gene duplication (38, 39), although some low degree of redundancy seems to occur (39). Similarly, evolutionary mechanisms may have retained the multiple PKAR isoforms that may have acquired different specificities in a subfunctionalization process (15), which is supported by the existence of different expression patterns. Moreover, the different isoforms may show differences in the activation by cAMP due to differences in the linker domain, as well as possible different subcellular localization through their N-terminal anchoring domain.

The results, taken as a whole, confirm that PKA is involved in multiple processes in M. circinelloides physiology. It has been clearly shown that in mammalian systems, there is a multiplicity of possible PKA holoenzymes due to the existence of four R subunits and three C subunits. The functional significance of each holoenzyme is being ascertained due to differential expression of the subunits in different tissues and at different stages of development. The discovery of multiple genes for the R and C subunits in a zygomycete produces a situation that seems to be as complex as in mammals and poses the very interesting challenge of studying the different holoenzymes present at each developmental stage, their specificities in substrate phosphorylation, and their roles in differentiation.

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REFERENCES


AUTHOR’S CORRECTION

A Subunit of Protein Kinase A Regulates Growth and Differentiation in the Fungus *Mucor circinelloides*

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Volume 8, no. 7, p. 933–944, 2009. Page 933: The first two paragraphs of this article, from “Filamentous fungi are defined . . .” to “. . . release the active catalytic subunit” are virtually identical to the beginning paragraphs of a previously published paper (Carmit Ziv, Rena Gorovits, and Oded Yarden, “Carbon source affects PKA-dependent polarity of *Neurospora crassa* in a CRE-1-dependent and independent manner,” Fungal Genetics and Biology 45:103–116, 2008). Dr. Rossi admits the plagiarism that she committed and apologizes to the authors Drs. Ziv, Gorovits, and Yarden.