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 asexual 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—Rα, Rβ, R1α, and R1β—and three genes coding for the C subunit—Cc, 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 pseudo-hyphal differentiation was altered by deletion of the gene coding for the R subunit, and each of the three C subunits have 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 of 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 a 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 pkC 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 expressed throughout germination in M. circinelloides (43). The RT-PCR amplification of some proteins, manual spectrum interpretation was carried out by the DOE Joint Genome Institute and the Mucor Genome project. Putative additional R subunit sequences from M. circinelloides were calculated by analyzing the amount of cAMP binding in picomoles. The cAMP binding assay. cAMP binding was measured by nitrocellulose filter assay. Crude extracts from the wild type, the ΔR (ΔpkaR1) strain, and the ΔR + R strain (i.e., the ΔpkaR1 strain transformed 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 dpm pmol−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 ATP (700 dpm/pmol), 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 R7B and strain ΔR (105 spores/ml) were cultivated in liquid YPG medium (pH 4.5). Cultures were grown for 3 to 4 h until germ tube emergence. 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 (105 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 EGTA (pH 8.5), 5 mM NaCl, 0.5 mM EDTA, 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 purified R subunit from M. circinelloides (E1) and used at a dilution of 1/20,000. Secondary antibodies were used at 1/10,000 (anti-rabbit immunoglobulin G-peroxidase conjugated). The blots were developed with Luminol chemiluminescence reagent.

Radial growth assay and sporulation. Portions (10 μl) of a spore suspension (105 spores/ml) were spotted onto leucine-supplemented or unsupplemented minimal medium (pH 4.5) plates in triplicate. Radial colony growth was monitored at 30°C every 12 h from 24 to 72 h by colony diameter measurement. Growth rates are expressed in μm h−1. All experiments were performed a minimum of three times, and the results from representative experiments are presented.

For comparison of sporulation, 100 spores of each strain were spread on medium plates and incubated at 30°C for 5 days, and then a plug of agar (1 cm2) was removed for spore calculation. The results shown are averages of three replicates.

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.

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 CEQUIBEM in Argentina. For the identification of some proteins, manual spectrum interpretation in Argentina was carried out by comparison of the experimental MS and tandem MS (MS/MS) data with the in silico digestion of the predicted new R coding genes.

Semiquantitative RT-PCR. R1 was prepared from 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 (ef-1) as an internal standard. The ef-1 gene has been demonstrated to be constitutively expressed throughout germination in M. circinelloides (45). The RT-PCR ampli-
A phylogenetic analysis. A phylogenetic tree was generated by using the CLUSTAL W2 alignment results. The sequence accession numbers are as follows: gi 110293154 gb ABG66306.1, Blastocladiella emersonii; gi 400120 sp P31320.1, Coccidioides immitis; gi 68472545 ref XP_719591.1, C. albicans; gi 112093154 gb ABG66306.1, Colletotrichum gloeosporioides; gi 156043067 ref XP_001397686.1, A. niger; gi 119187827 ref XP_001244520.1, Coccidioidea immittis; gi 39972039 ref XP_367410.1, Magnaporthe grisea; gi 85100016 ref XP_961532.1, Neurospora crassa; gi 400120 sp P31320.1, Blastocladia emersonii; gi 238842 gb AAB20314.1, Schizosaccharomyces pombe; gi 71024735 ref XP_762597.1, Ustilago maydis; gi 11096028 gb AAG10916.1, Cryptococcus neoformans; gi Physbl 41174 estEst_Genewise1Plus.C_180005 R3, Phycomyces sp.; gi Physbl 70276 fgeneshPB_pg_33_11 R2, Phycomyces sp.; gi Physbl 74992 fgeneshPB_pm.C_180005 R3, Phycomyces sp.; gi Physbl 69062 fgeneshPB_pg25_230 R4, Phycomyces sp.; RO3G_07551.1, Rhizopus sp. strain R6; RO3G_15555.1, Rhizopus sp. strain R4; RO3G_16579.1, Rhizopus sp. strain R5; RO3G_00025.1, Rhizopus sp. strain R3; and RO3G_14827.1, Rhizopus sp. strain R2.

RESULTS

Construction of a knockout strain for the pkaR gene. It has been reported that a M. circinelloides transformant overexpressing the pkaR1 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 pkaR1 gene to determine in a direct way its role on the morphology adopted by spores upon germination. One null mutant for the pkaR1 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 pkaR1 gene and adjacent regions. A restriction fragment from this plasmid containing the pyrG gene and sufficient sequences of the pkaR1 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 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
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 PCR-Ks 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, /H9004R, 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 ($\Delta R+R$) 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 $\Delta R$ 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 $\Delta R+R$ 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.

**TABLE 1. Number of spores and corresponding growth rates for three $M. circinelloides$ strains**

| Strain              | Mean ± SEM   |
|---------------------|--------------|
|                     | No. of spores/cm² | Growth rate (µm/min) |
| Mutant ($\Delta R$) | $(7 \pm 0.7) \times 10^6$ | $15.85 \pm 0.9$ |
| Wild type           | $(8.9 \pm 0.9) \times 10^6$ | $23.25 \pm 0.8$ |
| Mutant ($\Delta R+R$)| $(3.1 \pm 0.5) \times 10^6$ | $23.46 \pm 1.2$ |

*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 *pkaR1* using a high-copy-number plasmid under the control of the *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 *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 *pkaR1* gene affects growth, germ tube emergence, and yeast-to-hyphal morphogenesis.

**Identification of other *pkaR* genes in *M. circinelloides*.** Since the disruption of *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 *M. circinelloides* genome sequence, generated using 8× sequence coverage, was made available through the *Mucor* genome project developed at the Joint Genome Institute [http://genome.jgi-psf.org/Mucci/Mucci1.home.html](http://genome.jgi-psf.org/Mucci/Mucci1.home.html). A BLAST search using the available *pkaR1* sequence found three different sequences, in addition to *pkaR1*, in the *M.
**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 **pkaR1**, **pkaR2**, and **pkaR3** (accession numbers fgeneshMC_pm.13.1.2_1, 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 8.6e−08, 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 repres-
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 intron-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 aerobic and anaerobic conditions 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 aerobiosis 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 over-expression 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 sporangiospore (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 conidiate (*A. niger*) to delayed (*A. fumigatus*) or decreased (*C. lagenarium*) conidiation, also appear to be common to PKA regulatory subunit mutations in filamentous fungi, supporting a key role for 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-
tivity (28). 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-hypha 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 duplication did not occur in Mucor nor in Phycomycetes (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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