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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—RIα, RIβ, RIIα, and RIIβ—and three genes coding for the C subunit—Cα, Cβ, and Cγ.

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 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 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 *Mucormycotina* 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

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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 R7B (33), was used as the recipient strain in transformation experiments to knock out the *pkaR1* gene. The leucine auxotroph R7B (wild type) (33) is a *leuA* mutant strain derived from *M. circinelloides* forma *lusitanicus* 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 65°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 $[\alpha^{-32}P]dCTP$ 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. AJ400723) 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 pRpyrG, which contains the *M. circinelloides* pyrG 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′-CCACG*AGATCT*GTTTGTGGGAGGCAGCGAGG-3′) and pkaR-p2 (5′-ATTTT*AGATCT*GGACAGGAGGTTGTGAAGC-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 0.561 kb of the *pkaR1* coding region. The PCR product digested with BglII was ligated with a *pyrG* 3.2-kb BamHI fragment from pEPM1 (3) to produce pRpyrG plasmid. The plasmid was linearized with EcoRI digestion and introduced into MU402 protoplasts by transformation. The *pkaR2* GenBank accession no. is bankit1191938 FJ800364.

cAMP binding assay. cAMP binding was measured by nitrocellulose filter assay. Crude extracts from the wild type, the ΔR (Δ*pkaR1*) strain, and the ΔR+R strain (i.e., the Δ*pkaR1* 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 saturating. 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 [γ³²P]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 (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 N⁶-cAMP-agarose from BioLog (35). Briefly, crude extracts from both strains were loaded onto the resin; the resins were 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 4 to 5 h until the emission of germ tube. PKA holoenzyme was prepared as described previously (27) using buffer A (25 mM Tris-HCl [pH 8], 5 mM EDTA, 3 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 purified R subunit from *M. rouxii* in rabbits. The primary antibodies were 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 (10³ 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.

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 spectrometric (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 new R coding genes.

Semiquantitative RT-PCR. RNA 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* RNA was performed using the elongation factor EF-1a gene (*tef-1*) as an internal standard. The *tef-1* gene has been demonstrated to be constitutively expressed throughout germination in *M. circinelloides* (43). The RT-PCR ampli-
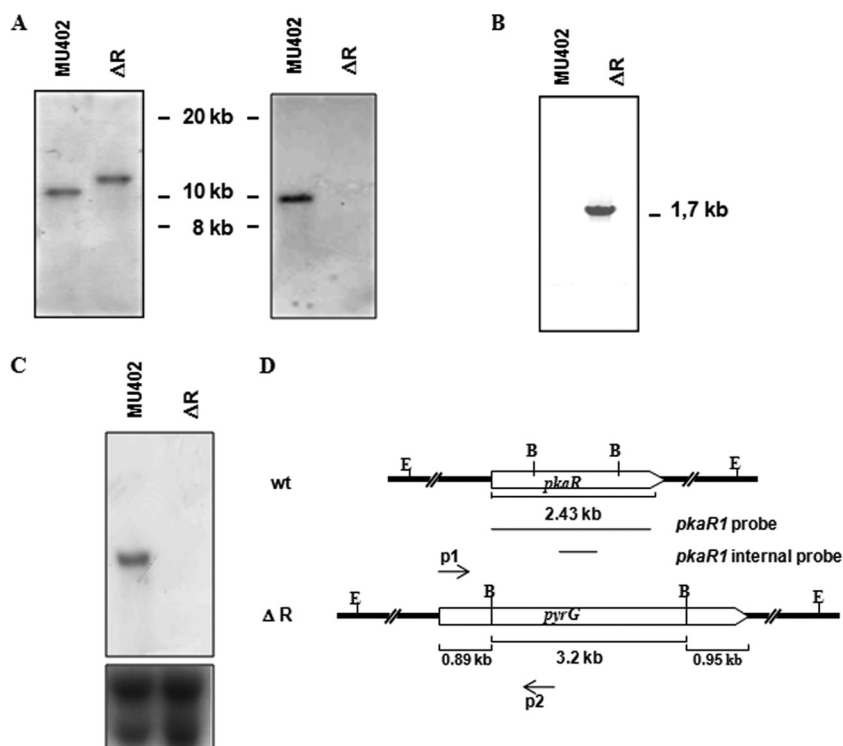
FIG. 1. Disruption of *pkaR1* gene. (A) Genomic DNA from the wild-type strain, MU402, and the *pkaR1* knockout mutant (ΔR) was digested with EcoRI and hybridized with the *pkaR1* probe (left panel) and the *pkaR1* internal probe that only hybridized with the fragment replaced in the disruption (right 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).

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 for these genes in the log phase. Specific oligonucleotide sense and antisense primers were designed so as to identify specifically each mRNA isoform. These primers included TEF-F (5′-TCACGTCGATTCCGGTAAGT C-3′), TEF-R (5′-TATCACCGTGCCAGCCAGA-3′), pkaR1-F (5′-CTTGTTTGATACCAA TGATACCAGTAATG-3′), pkaR1-R (5′-CTCCTGTCCATCTTCAAAATAA ACT-3′), pkaR2-F (5′-AGACAAGTAGTTGAACATCAGCCTG-3′), pkaR2-R (5′-TTGCTGAGAGCCGTCAGGCAGCTTCTT-3′), pkaR3-F (5′-CATCCCA TGTGGGCGGCATTGGCCC-3′), pkaR3-R (5′-CCTTGCTTCATTTCATTG ACCAACTG-3′), pkaR4-F (5′-GATGTGTACTGCAAAGATCAGCCTCA-3′), and pkaR4-R (5′-CTGCAAGATAACATGCTCTCCATCATTG-3′). The expected PCR product lengths were 775, 955, 817, and 994 bp for pkaR1, pkaR2, pkaR3, and pkaR4, respectively, and 550 bp for TEF1. The PCR bands were analyzed and quantified by digital imaging (Bio-Imaging Analyzer Bas-1800II and Image Gauge 3.12; Fujifilm) expressing the pixel intensities in arbitrary units.

Phylogenetic analysis. A phylogenetic tree was generated by using the CLUSTAL W2 alignment results. The sequence accession numbers are as follows: gi 6322156 ref NP_012231.1, *Saccharomyces* sp.; gi 150863720 ref XP_001382284.2, *Pichia pastori*; gi 68472545 ref XP_719591.1, *C. albicans*; gi 110293154 gb ABG66306.1, *Colletotrichum gloeosporioides*; >gi 156043067 ref XP_001588090.1, *Sclerotinia sclerotiorum*; gi 145252346 ref XP_001397686.1, *A. niger*; >gi 119187827 ref XP_001244520.1, *Coccidioides immitis*; gi 39972039 ref XP_367410.1, *Magnaporthe grisea*; gi 85103516 ref XP_961532.1, *Neurospora crassa*; gi 400120 sp P31320.1, *Blastocladiella emersonii*; gi 238842 gb AAB20314.1, *Schizosaccharomyces pombe*; gi 71024735 ref XP_762597.1, *Ustilago maydis*; gi 11096028 gb AAG30146.1, *Cryptococcus neoformans*; jgi Phybl1 41174 estExt_Genewise1Plus.C_290035 R1, *Phycomyces* sp.; jgi Phybl1 70276 fgeneshPB_pg.33_11 R2, *Phycomyces* sp.; jgi Phybl1 74992 estExt_fgeneshPB_pm.C_180005 R3, *Phycomyces* sp.; jgi Phybl1 69062 fgeneshPB_pg.25_230 R4, *Phycomyces* sp.; RO3G_05751.1, *Rhizopus* sp. strain R1; RO3G_06050.1, *Rhizo-*

pus 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
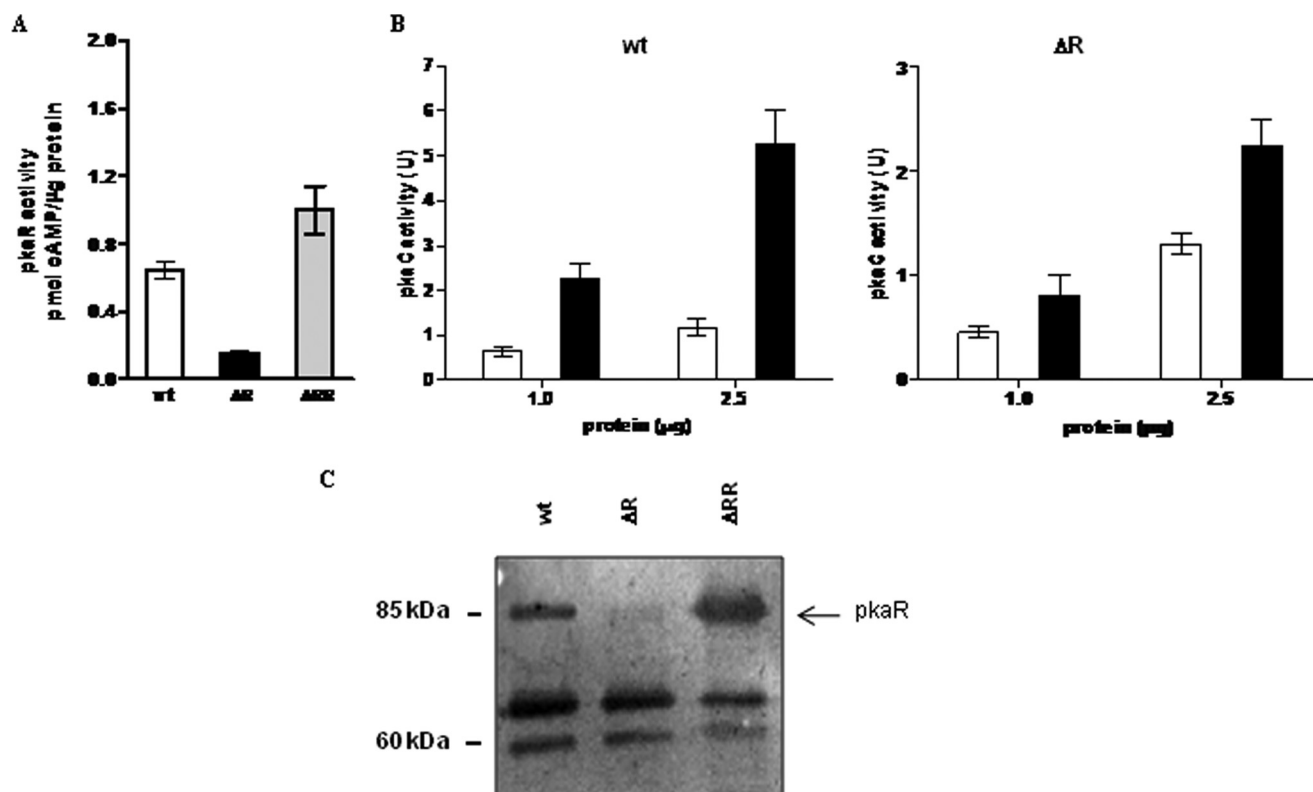
FIG. 2. Biochemical characterization of *pkaR1* mutant strain. (A) Binding activity was measured in crude extracts from the wild-type strain (WT), mutant strain (ΔR), and complemented strain (ΔR+R) after anaerobic-aerobic shift. (B) *pkaC* activity was measured in semipurified samples from wild-type and ΔR strains in the absence (□) or in the presence of cAMP (■). The data shown in panels A and B correspond to five assays, performed with independent preparations. Values are represented as means ± the SEM from four replicates for each assay. (C) Western blot analysis of semipurified samples using a polyclonal antibody raised against *M. rouxii pkaR*.

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 PCRs 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
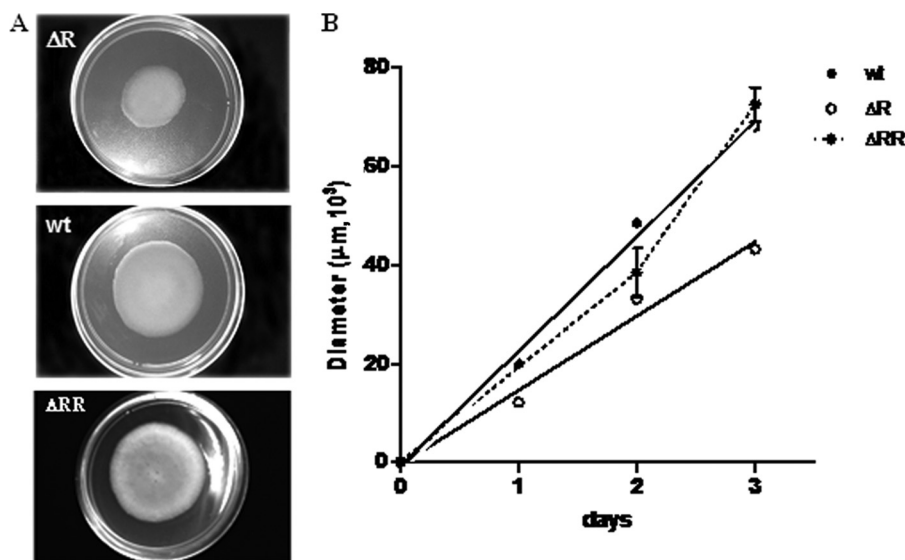
FIG. 3. Growth rate and sporulation of the *pkaR1* mutant. (A) Aliquots with the same concentration of spores from the indicated strains were spotted onto minimal medium plates pH 4.5 in triplicate. (B) The growth was monitored for 3 days, and a graph of the calculated radial growth rates from three representative assays is shown. wt, wild type.

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 aerobiosis 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[a]

Strain	Mean ± SEM	
	No. of spores/cm^2	Growth rate (μm/min)
Mutant (ΔR)	$(7 \pm 0.7) \times 10^5$	15.85 ± 0.9
Wild type	$(8.9 \pm 0.9) \times 10^6$	23.25 ± 0.8
Mutant (ΔR+R)	$(3.1 \pm 0.5) \times 10^7$	23.46 ± 1.2

[a] 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 cm^2) were removed for spore calculation. The calculated growth rates correspond to the experiment illustrated in Fig. 3A.
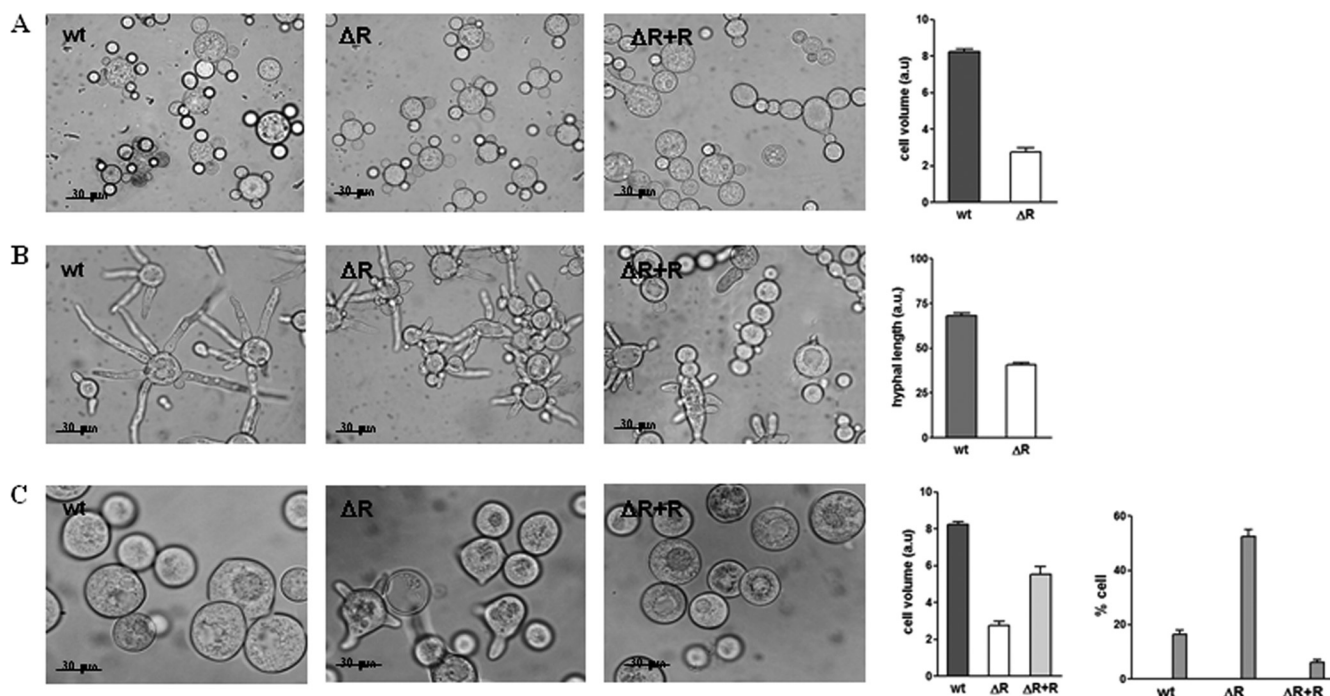
FIG. 4. Morphology and germination of *pkaR1* mutant. (A) Microscopic examination of cells of indicated strains grown overnight in anaerobic conditions. Average volumes of the mother cell are shown in the figure, each value representing the mean ± the SEM for three independent experiments. The volumes were estimated from the diameters measured in arbitrary units (a.u.). (B) Cells from cultures grown for 4 h in aerobic conditions after a shift from overnight anaerobic growth. The average length of the germ tubes is represented on the bar graphic, with each value representing the mean ± the SEM for three independent experiments. (C) Cells from aerobic cultures grown for 3 h. The average volume of mother cell is represented on the left graphic, with each value representing the mean ± the SEM. The left graph shows the germ tube emergence analysis of the indicated strains after 3 h of aerobic growth in minimal medium. The numbers of cells with germ tube emitted were determined and are expressed as the percentage of the total cells (right graph). Each value represents the mean ± the SEM for three independent experiments.

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 par-

tially 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/Mucci1/Mucci1.home.html). A BLAST search using the available *pkaR1* sequence found three different sequences, in addition to *pkaR1*, in the *M.*

A

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PKAR1 : ................MITDEHPFEFAP.QQDEYTQLLTELHNEYCAEQPLDVLQFCSNFFIRKLEEQRLEHRNNHHSRNNLFDTNDTSNDL : 75
PKAR4 : MTFTFGEPSSQEQQHQDQQQPFSFS..YADEYTQLLTELHDVYCKDQPHDVLQYCANFFNRKLEEQRTSYFREQHGLVETEH........ : 80
PKAR3 : ...........MPDSVHPFSTQPALSQDYQNLIHDLSQQVKANNPEDVLQFFD.FFLDKLMQERSHNRSH................... : 58
PKAR2 : .............MSHPQDIHPFAQV..DNEEYNMLISELNRQVVEHQPEDLLQFCTTFFLKRKLEDERAESRQYDQ............... : 61
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PKAR1 : HPLCEQPQEDFSQQQGIQWETTHMGHPNDHGALHDDDDDPLEDEDDEEFD....KFSTEPLPSLPP.TNYNRGRRTSVSAESMAPS.... : 156
PKAR4 : HPLAENGATSSMFEPAYSSFSSNSSSHHYENDQDIDDDILLDEEDEDDDDEERDNFSTTPLSSIPAAANYNRGRRTSVSAESMAPS.... : 166
PKAR3 : .LQQQQPHNTSHVGGIGPYSTEQESHAIPPHPTIHEEDEDEDMVSD........HLPNFQKTSNFRNRRVSVSAESLCPS.... : 129
PKAR2 : HPLATNQQH..........QSLGPDFHPSLDQDMHDDEERDTISDD........MPSLSVPPPSRGRRTSVSAESLCPS.... : 122
                                       IS
```
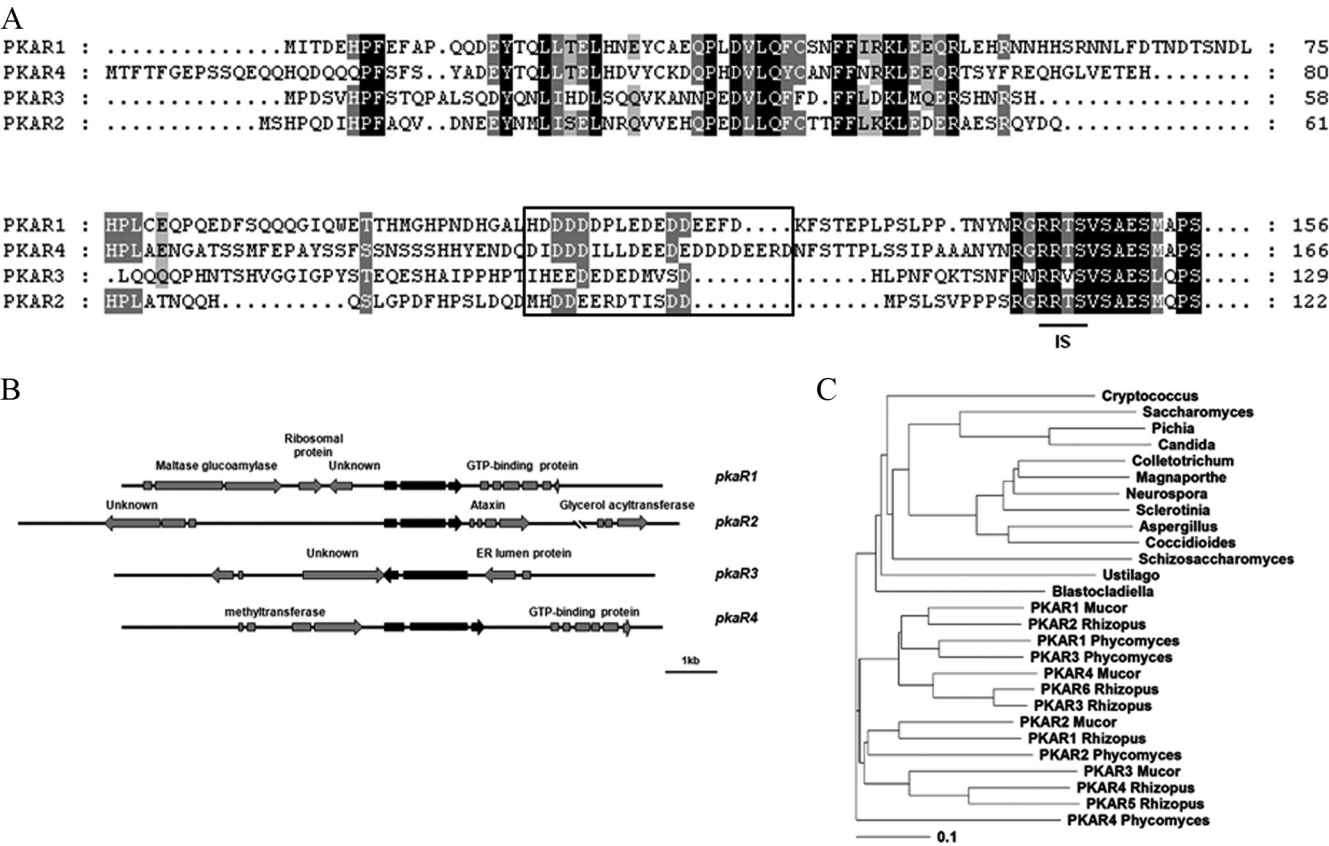
B

C

FIG. 5. *pkaR* genes and proteins. (A) Sequence alignment of N termini of *M. circinelloides* PKAR proteins. Only the N-terminal portions of PKAR isoforms are shown. The acidic cluster is boxed. Inhibitor site (IS) is underlined. Identical (white letters in a black background) or similar (shaded) amino acids are indicated. (B) Genomic structure of *M. circinelloides* genes with their flanking genes, introns, and exons indicated. (C) Phylogenetic tree of PKAR proteins. Bars represent the number of substitutions per site (for the accession numbers, see Materials and Methods).

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 phosphorylable Ser; however, RI subunits display a pseudosubstrate IS with a nonphosphorylable 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 repre-
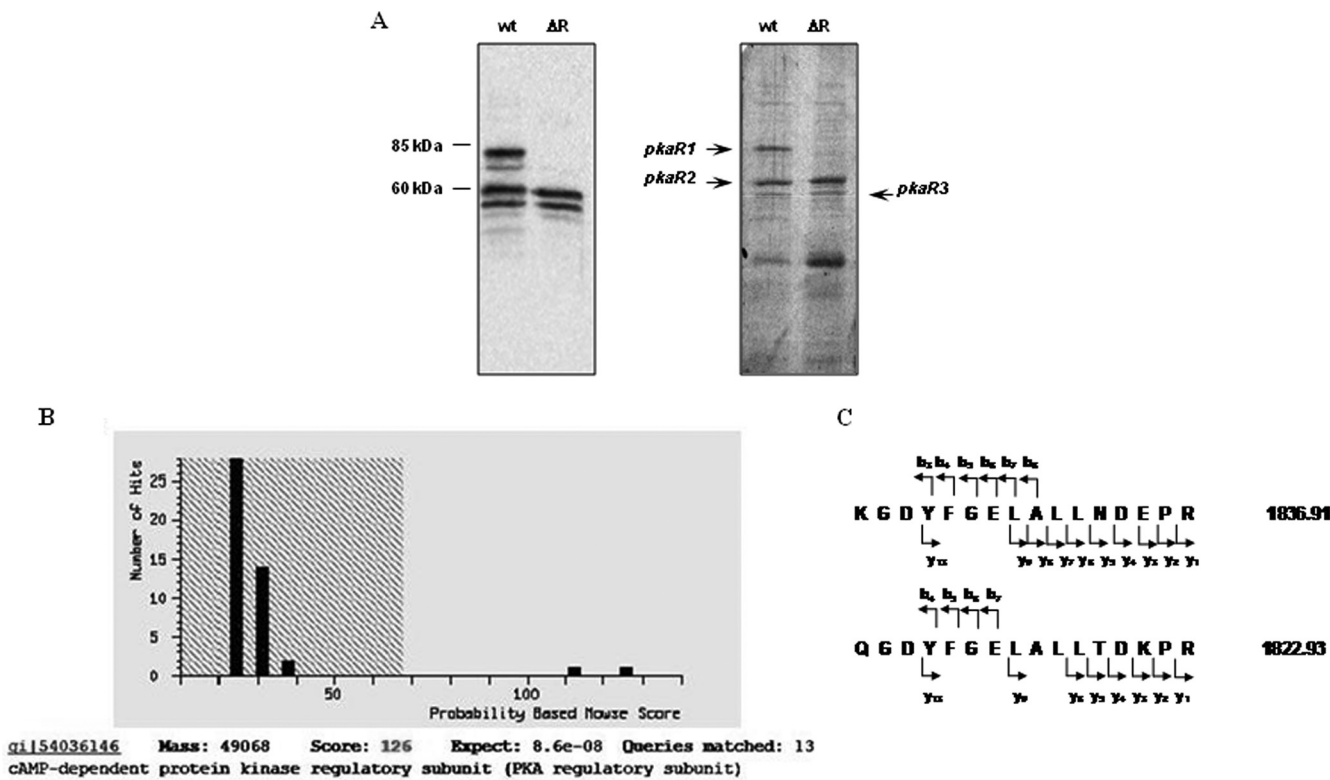
FIG. 6. Identification of *pkaR* genes. (A) cAMP-agarose purification of cAMP-binding proteins. The left panel shows a Western blot of the purified samples using polyclonal antibody raised against *M. rouxii* PKAR. The right panel shows a purified sample analyzed by SDS-PAGE and colloidal Coomassie blue staining. The arrows indicate the identified *pkaR* proteins. (B) Result of the MASCOT search of the MS analysis obtained by MALDI-TOF of PKAR1. (C) *b* and *y* ions identified by MS/MS from one peptide (1,836.91 from PKAR2 and 1,822.93 from PKAR3) from each MALDI-TOF spectrum.

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 aerobiosis 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 aerobiosis (Fig. 7A). In spores, the *pkaR3* mRNA levels were higher than those for the other
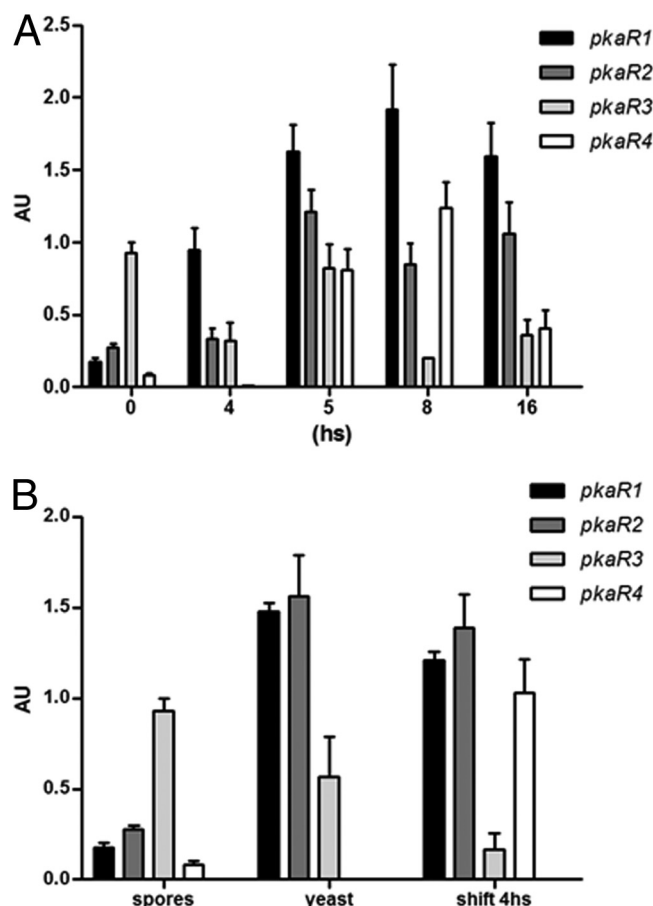
FIG. 7. Expression of *pkaR* genes. Semiquantitative RT-PCR was used to measure the relative abundance of mRNA from each *pkaR* using *tef-1* mRNA to normalize the data. Total RNA was extracted from cell cultures grown for 0 (spores), 4, 8, and 16 h under aerobic conditions (A) and cell cultures in anaerobic conditions shifted to aerobiosis for 4 h (B). PCR bands were analyzed and quantified by digital imaging expressing the pixel intensities in arbitrary units (a.u.) relative to the *tef* band intensity. Each value represents the mean ± the SEM of four independent experiments.

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 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 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-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 duplication did 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.

ACKNOWLEDGMENTS

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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.