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Multiple isoforms for the catalytic subunit of PKA in the basal fungal lineage *Mucor circinelloides*

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ABSTRACT

Protein kinase A (PKA) activity is involved in dimorphism of the basal fungal lineage *Mucor*. From the recently sequenced genome of *Mucor circinelloides* we could predict ten catalytic subunits of PKA. From sequence alignment and structural prediction we conclude that the catalytic core of the isoforms is conserved, and the difference between them resides in their amino termini. This high number of isoforms is maintained in the subdivision Mucoromycotina. Each paralogue, when compared to the ones form other fungi is more homologous to one of its orthologs than to its paralogs. All of these fungal isoforms cannot be included in the class I or II in which fungal protein kinases have been classified. mRNA levels for each isoform were measured during aerobic and anaerobic growth. The expression of each isoform is differential and associated to a particular growth stage. We reanalyzed the sequence of PKAC (GI 20218944), the only cloned sequence available until now for a catalytic subunit of *M. circinelloides*. PKAC cannot be classified as a PKA because of its difference in the conserved C-tail; it shares with PKB a conserved C2 domain in the N-terminus. No catalytic activity could be measured for this protein nor predicted bioinformatically. It can thus be classified as a pseudokinase. Its importance can not be underestimated since it is expressed at the mRNA level in different stages of growth, and its deletion is lethal.

Introduction

Protein kinase A (PKA) is the principal mediator of the second messenger cAMP in fungi (Shemarova 2009; McDonough & Rodríguez 2012). In the inactive state, PKA is a holoenzyme comprised by a dimer of regulatory subunits (R) that inhibits two monomers of catalytic subunits (C) (Kim *et al.* 2006). Activation is achieved by the binding of two molecules of cAMP per R subunit monomer, decreasing the R–C interaction affinity leading to dissociation of active C subunits (Kim *et al.* 2006). In fungi, cAMP levels -regulated by external agents such as nutrients, light, pheromones and stress-have an impact in

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multiple processes such as mating, morphology, virulence, nutrient and stress sensing (Borges-Walmsley & Walmsley 2000; D'Souza & Heitman 2001; Harris 2006; Hogan & Sundstrom 2009; Kronstad et al. 2011; Fuller & Rhodes 2012; Jiménez et al. 2012).

A recurrent and yet unsolved theme in cAMP signaling is how specificity is being achieved as the same second messenger molecule, depending on the stimulus that triggered its synthesis, transduces the signal to different final targets. Some mechanisms to attain specificity have been proposed and even some have been demonstrated. The main ones are: a) localized cAMP synthesis and degradation, yielding cAMP microdomains (Antoni 2012; Lefkimmiatis & Zaccolo 2014); b) differential subcellular localization of PKA holoenzyme through interaction of R subunits isoforms with AKAPs (A Kinase Anchoring Proteins) (Scott et al. 2013); c) different isoforms of PKA with differential properties such as transcriptional regulation of its subunits, subcellular localization, substrate selectivity, or differential sensitivity to activation by cAMP (Skålhegg & Taskén 2000).

We are particularly interested on the subject of specificity and have been using eukaryotic microorganisms as models, particularly the dimorphic fungi *Mucor circinelloides* belonging to the class Zygomycetes, one of the basal fungal lineages (Tanabe et al. 2005). Mucor has been an excellent model in which cAMP has a strong physiological effect on its polarized growth (Larsen & Sypherd 1974; Pereyra et al. 1992).

In mammals, there are four isoforms for R subunits (RIα, RIβ, RIIα, RIIβ) and three for C subunits (Cα, Cβ, Cγ) plus splice variants for both R and C (Taylor et al. 1990). Fungi of the phyla Ascomycota and Basidiomycota have only one R isoform and one to three isoforms for the C subunit. In these fungi, the generation of mutants with disruption of the gene/s encoding the C or R subunit/s of cAMP-dependent protein kinase A results in variable alteration of morphogenesis, growth and pathogenicity (some examples described in Borges-Walmsley & Walmsley 2000; D'Souza & Heitman 2001; Harris 2006; Hogan & Sundstrom 2009; Kronstad et al. 2011; McDonough & Rodríguez 2012; Fuller & Rhodes 2012; Jiménez et al. 2012).

The recent sequencing of *M. circinelloides* genome gave us the possibility to begin a molecular genetics approach in the study of PKA in the Mucor model. We have recently shown that *M. circinelloides* genome encodes for four isoforms of R subunit that are differentially expressed during cell growth (Ocampo et al. 2009). It is interesting to mention that one of the differences in the isoform sequences resides in the domain surrounding the inhibitory site, where R subunits interact with C subunits through the substrate site. This domain strongly participates in the overall affinity of R–C interaction (Ocampo et al. 2007). Phenotypic and biochemical consequences of the individual deletion of each subunit point to a specific role for each isoform (Ocampo et al. 2012).

Herein, we report the identification of 10 genes encoding the catalytic subunit protein of PKA (PKAC) and their characterization for mRNA expression levels and phylogenetic relationships. This family of PKAC isoforms differs mainly in their amino termini and in their mRNA expression during growth and differentiation. Until the present work, and before the genome sequencing, only one PKAC subunit has been reported for *M. circinelloides* (Wolff et al. 2002). In this regard, we demonstrate that the PKAC gene deposited by Wolff et al. (2002) in NCBI (GI:20218944) does not correspond to a PKA, since it is similar to a PKB, does not have a PKA catalytic activity, and its sequence suggests it is a pseudokinase. However, it is interesting that this gene is highly expressed and that its deletion is deleterious.

While this work was being submitted, the comparative sequencing of *Phycomyces blakesleeanus* and *M. circinelloides* was published (Corrochano et al. 2016). Our findings are in complete agreement with the general conclusion of this publication which describes an expansion of signal transduction pathways in both of these genomes.

Materials and methods

Strain and growth

Mucor circinelloides strain R7B, a *leuA* mutant strain derived from *M. circinelloides* forma lusitanicus CBS277.49 (Heeswijck & Roncero 1984) was used throughout, except for the transformation experiments. Strain MU402, a uracil and leucine auxotroph derived from R7B (Nicolas et al. 2007), was used as the recipient strain in transformation experiments to knock out the *pkaC* gene. Cultures were grown aerobically or anaerobically at 28 °C in YPG, pH 4.5, at a spore density of 2×10^6 spores ml^{-1}. Aerobic growth was followed up to the stages of isodiametric growth (3 h), germlings (5 h), and mycelial growth (8 h). In some cases spores were cultured overnight under anaerobic conditions yielding yeast-like cells; an aliquot of yeast-like cells was shifted to aerobic conditions for 5 h. For the PKAC knock-out construction, cultures were grown at 28 °C in MMC or YNB medium supplemented with uridine (200 μg ml^{-1}) or leucine (20 mg ml^{-1}) when required. The pH was adjusted to 4.5 or 3.2 for mycelial or colonial growth, respectively. Transformation was carried out as described previously (Quiles-Rosillo et al. 2003).

Deletion of pkaC *gene*

Plasmid pUC18C harboring the *pkaC* gene from position −453 to position 2007 (GenBank accession no. AJ431364.1) was generated by molecular subcloning into the pUC18 vector of a 2460 bp EcoRI DNA fragment, obtained by PCR using genomic DNA as template and specific primers, including the EcoRI restriction sites. Plasmid pCpyrG, containing the *Mucor circinelloides* pyrG gene (Benito et al. 1995) flanked by *pkaC* sequences, was constructed to disrupt *pkaC*. To generate this plasmid, the pUC18C was PCR amplified by using the primers pkaC-p1 (5′-TGCTA***AGATCT***CCCAAGAAGCGATCTTCACC-3′) and pkaC-p2 (5′- GTTTG***AGATCT*** AAGCGTTGCCAATTCTT TGC-3′); both include Bgl II restriction sites (in bold type and italics). These primers amplify outwardly from the *pkaC* gene toward the vector sequence, producing a deletion of 0.537 kb of the *pkaC* coding region. The PCR product digested with Bgl II was ligated with a pyrG 3.2-kb BamH I fragment from pEPM1 (Benito et al. 1995) to produce pCpyrG plasmid. The plasmid was linearized with EcoRI and introduced into MU402 protoplasts by transformation. Primers used for screening *pkaC* null mutant clones: Sec2F 5′-AATCATGAT

GAGCGCTAGTTCTCCC-3′; CaR 5′-ATCACATTGGCATTGGTA-GACCGG-3′; PyrGZ 5′-GTGGCTGATTGGACTCAAACAGG-3′; MC6 5′-CTCTGATCGTATGACTAGCTGGCCC-3′.

PKAC subunits sequence prediction and confirmation

Access to the information of *Mucor circinelloides* genome in its 4 and 8 assemblies was generously facilitated by the DOE Joint Genome Institute, and the *Mucor* Genome project. Putative C genes were predicted through Blast search using a 171 amino-acid sequence derived from a PKA gene fragment cloned from *Mucor rouxii* (LGRVHLSQSKHNGRYYAIKVLKK SEVVRLKQVEHT NSEKHILESAANPFMVNLWGTFQDDINLYMVMDYVPGGELFSIL RKNQRFPDHIAKFYAAEVLLAIEYFHSKRIVYRDLKPENLLIDAQG HIKITDFGFAKHVPDITWTLCGTPDYLAPEVIQSKGYGLAVD WWTL). This fragment has already been proved adequate to pick PKA; when blasted to mammalian genomes it yielded ≥500 hits with the highest score, corresponding exclusively to PKA proteins. cDNA and protein sequences were derived after intron predictions and sequence similarity search. For a confirmation of the predictions and final sequence curation, all PKACs cDNA were subcloned into a pGEM-T vector and sequenced. Primers used for subcloning are listed in Table S1 in the supplemental material.

Phylogenetic analyses

Two protein datasets were employed, one comprised 118 terminals and another 81 (datasets 1 and 2 in Table S3). The smaller dataset comprises some protein sequences that were classified as class I or II PKAC in the literature (Ni *et al.* 2005; Fuller *et al.* 2011).

Protein alignments were performed in MEGA 6.0 (Tamura *et al.* 2013) using MUSCLE with default settings. Pairwise distance values (uncorrected p-distances) and average distance values were calculated using MEGA. The best-fitting substitution models were determined for both matrices using the Bayesian and corrected Akaike information criteria and the Maximum Likelihood value (lnL), as implemented in MEGA. The highly variable amino terminal was trimmed for all terminals before further analyses. Phylogenetic analyses were carried out under the Maximum Likelihood (ML) criterion in MEGA, with the following settings: the heuristic search was obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model (Jones *et al.* 1992), and then selecting the topology with superior log likelihood value; the heuristic method was Subtree Pruning and Regrafting (SPR level 3) and a strong swapping filtering. Bootstrap support values (BSV) were estimated with 500 pseudoreplicates in MEGA. For rooting purposes we included protein sequences from *Rozella allomycis* which, according to James *et al.* (2013), belongs to the basal lineage Cryptomycota.

Semiquantitative RT-PCR

RNA was prepared by standard procedures from 100 ml cultures of spores, grown aerobically up to 3, 5 or 8 h (mycelial growth), anaerobically until logarithmic growth (yeast-like cells), and from cultures shifted for 5 h from anaerobiosis to aerobiosis, as well as from the equivalent number of uncultured fresh spores. Semiquantitative reverse transcription-PCR (RT-PCR) of each *pkaC* RNA was performed using the elongation factor EF-1a gene (*tef-1*) as an internal standard. The *tef-1* gene has been demonstrated -since long-to be constitutively expressed throughout germination in *Mucor circinelloides* (Linz & Sypherd 1987) and very recently, it was verified as a suitable marker to standardize mRNA level measurements (Valle-Maldonado *et al.* 2015a). The RT-PCR amplification reaction, using Superscript II transcriptase reverse from Invitrogen, was calibrated in order to determine the optimal number of cycles that allowed detection of the appropriate mRNA transcripts while still keeping amplification for these genes in the log phase. Specific sense and antisense primers were designed so as to identify specifically each mRNA isoform (Table S2 in the supplemental material). 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.

Standard PKA assay

The PKA catalytic activity was determined by assay of its phosphotransferase activity with kemptide as substrate. The phosphorylation of kemptide was performed by adding an aliquot of PKA, partially purified by 60 % $(NH_4)_2SO_4$ precipitation, to a standard incubation mixture containing 15 mM $MgCl_2$, 0.1 mM [γ^{32}P]ATP (700 dpm pmol^{-1}), 200 μM kemptide, and 10 μM cAMP (when added). After 15 min at 30 °C, aliquots were processed according to the phosphocellulose paper method (Roskoski 1983). PKA activity was expressed in units defined as picomoles of phosphate incorporated into substrate min^{-1} at 30 °C.

PKA expression and purification in yeast

pkaC and *pkaC5* cDNA were subcloned in pESC-ura yeast expression plasmid (Stratagene) under the control of Gal1 promoter as recombinant proteins with a c-myc tag in its N-terminal side. PKA proteins were expressed in the *tpk1^{w1}BCY1-* strain containing a deletion of *TPK2* and *TPK3* genes and a weak version of Tpk1 with no detectable PKA activity (genotype MATα his3 leu2 ura3 trp1 ade8 tpk^{1w} tpk2::HIS3 tpk3::TRP1). Transformed strains were grown overnight in minimal medium YNB without uracil, with 2 % glucose as carbon source, and transferred to YPgal (2 % galactose) for 16 h at 30 °C. Protein expression was confirmed by western blot using anti-c-Myc antibody (Covance Inc.).

Results

Identification of ten genes encoding catalytic subunits of PKA in Mucor circinelloides

The database from the recently sequenced genome from *Mucor circinelloides* CBS277.49, v2.0 (http://genome.jgi-psf.org/Mucci2/Mucci2.home.html) was Blasted in search of sequences coding for PKA C, using as a template a predicted 171 aminoacid sequence derived from a fragment of PKA

gene cloned from *Mucor rouxii* using degenerate primers from conserved sequences bridging the amino terminal part of the conserved core of PKAs (see sequence in M & M). Ten putative ORFs coding for sequences highly similar to PKA C were retrieved from Blast search, and designated PKC1 to PKC10. The criterion used for accepting a sequence as PKA was that the C-terminus ended in the hydrophobic motif FXXF, a characteristic only shared by PKAs. An exception was made for a sequence having one extra aminoacid in the C-terminus (see below). The sizes of the predicted proteins range from 408 to 525 aminoacids. As shown schematically in Table 1 and further detailed in Fig 1, predicted proteins share a catalytic core domain (shaded in dark grey) and an amino-terminal extension of variable length. The introns of the conserved catalytic domain were predicted so as to fulfill the majority of the following criteria (our own statistics from inspection of *M. circinelloides* genome, and personal communication of S. Torres Martínez): GTAA as a 5′-splice donor site, AG as 3′-acceptor site, a total intron length of around 60–80 bp, a branching site at around 15–30 bp upstream of the 3′-acceptor site that best matched the eukaryotic branching consensus sequence YNCURAY (Kupfer *et al.* 2004), and at a time that its removal maintained the similarity between the sequences when compared to known fungal and metazoan C subunits. The number of introns in the core domain of the C subunits was very variable (1–6). Using as reference the three introns in the core domain of PKAC2, it can be seen that the first intron is shared by six isoforms (PKAC1 to PKAC5 plus PKAC8), the second is only shared by four isoforms (PKAC2, PKAC5, PKAC7 and PKAC8) and the third one is shared by seven isoforms (PKAC1 to PKAC4, PKAC6, PKAC8 and PKAC9). PKAC10 shares none of these introns, but instead has three different introns in the catalytic domain, only shared by PKAC9, which is thus the isoform with the largest number of introns. All of the introns correspond to regions between domains with conserved secondary structure, either α-helix or β-sheet.

The first ATG and the introns of the non-conserved amino termini of each C subunit were predicted according to the following rules: first ATG within the context CA[C/A][A/C]ATGNC (Balance 1991), preceded by a CT rich region (V. Garre, personal communication); sequence similarity between isoforms; intron 5′ donor and 3′ acceptor sites within the rules mentioned for the core domain, and maintenance of the reading frame with the conserved catalytic core domain. Certain degree of similarity was found at the very amino terminus of PKAC1, PKAC2, PKAC5, PKAC6, and PKAC7, which helped with the prediction (Fig 1). It has to be noted that the differences in the amino termini amongst the sequences of the PKACs from the JGI *M. circinelloides* database, and those we deposited in the NCBI database, are probably due to an inadequate intron prediction within non-conserved domains.

Table 1 – *Mucor circinelloides* pkaC genes: length and intron distribution.

Gene name	Scaffold[a]	Sequence location (bp)[a]	cDNA with introns	pI	Protein (aa)
PKAC1	4	1984208–1985987		8.90	434
PKAC2[b]	1	2456584–2458372		9.26	453
PKAC3	3	1146580–1147982		7.10	420
PKAC4	5	1529305–1531072		9.12	525
PKAC5	1	208177-209857		7.00	476
PKAC6	6	527630–529275		8.47	491
PKAC7	4	3174371–3175879		9.26	464
PKAC8	6	2741124–2742829		6.29	420
PKAC9	6	2023560–2025121		8.73	408
PKAC10	3	1230714–1232132		7.42	413

a Obtained from *Mucor circinelloides* Genome Database, assembly version 2.0 (http://genome.jgi.doe.gov/Mucci2/Mucci2.home.html).
b A splicing variant of this gene was found, with the last intron 9 bp larger than the one predicted.

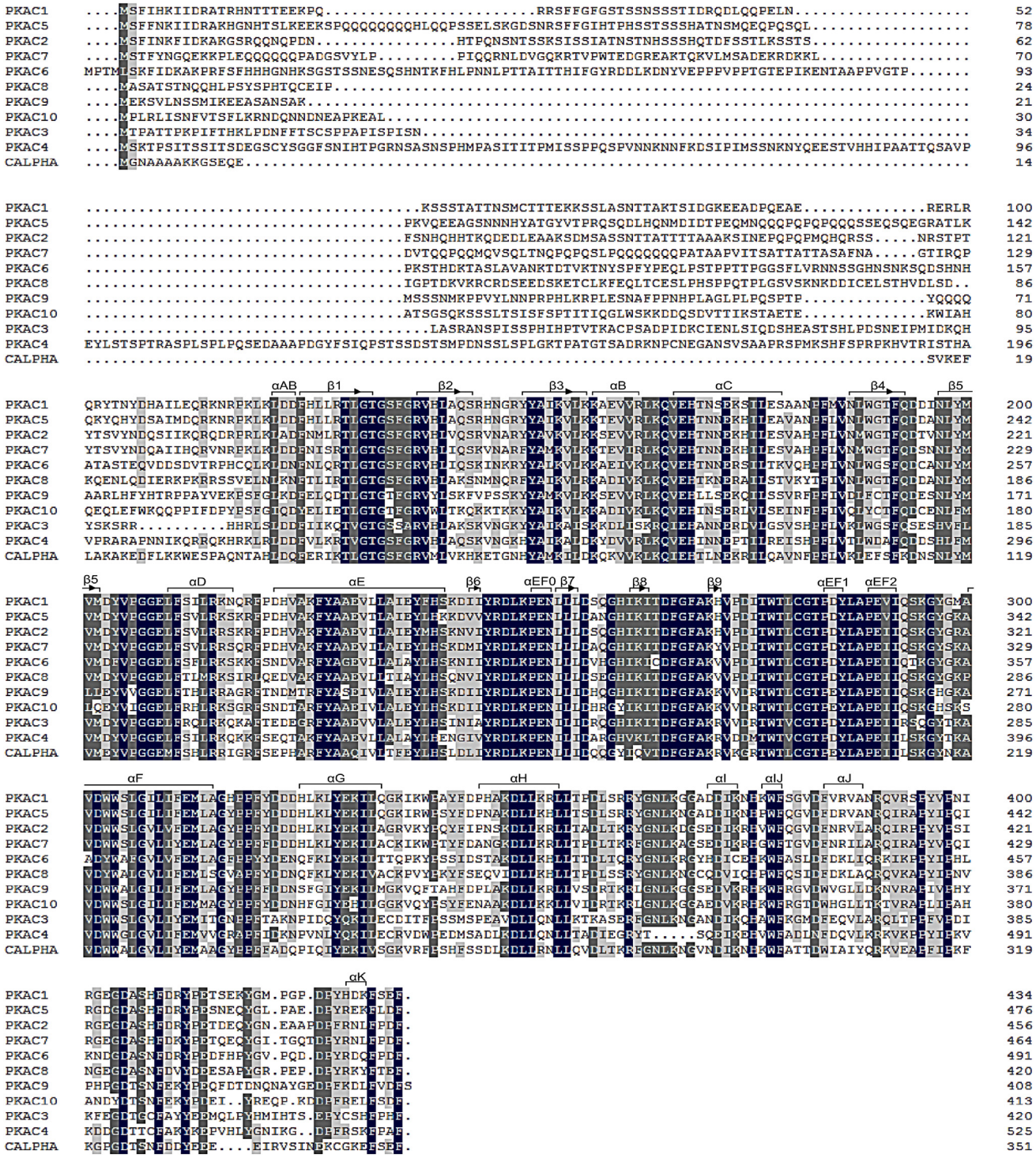

Fig 1 – Sequence alignment of predicted PKA catalytic subunits from *Mucor circinelloides*, including the human Cα isoform as a reference. Alignment was performed using the DNAMAN software (Lynnon Corp., Canada) and manual curation. Identical residues are shaded in black and similar amino acids are shaded in gray. Conserved secondary structures (α-helix or β-sheet), detected from structural studies from mammalian C subunits, are underlined.

When human C_α was used as a reference of mammalian PKAC, a clear conservation of around 54–60 % is observed within the core domain beginning at the αAB helix of each fungal subunit; a clear divergence in the N-terminal domains is also apparent (Fig 1). Fungal C subunits have a general similarity of around 20 % with a variable length of 90–220 aminoacids, far larger than the very short 40 aminoacid of mammalian C_α N-terminus. Estimated evolutionary

divergence amongst PKACs shows that the most similar pair of sequences is C2–C7 (bold type, Fig 2A), whereas the pair C3–C10 is the most divergent when the whole protein is considered. When the conserved region is examined the divergence amongst C9, C3 and C4 is evident (italics, Fig 2A). The core structure of the 10 isoforms was predicted using the Phyre2 server (**P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine V 2.0 (Kelley *et al.* 2015) that works based on homology modeling against a database of proteins of known 3D structure. The results of this prediction are considered to be accurate by this program, since all of the core sequences show high alignment coverage, high confidence (>90 %) and high sequence identity (>35 %). Fig. 2B shows the modeled structure of PKC5 as an example (the models of the rest of the isoforms are highly similar to the one of PKC5; data not shown). The suggested fold is the one of a typical eukaryotic protein kinase (EPK) with a well defined two-lobe structure. The larger C-lobe mainly formed by helices, while the smaller N-lobe formed by a five stranded β-sheet linked to the C-lobe through the C-helix. The aminoacid sequence of the more important functional domains in the C subunit is absolutely conserved. In the N-lobe the most important elements are the Gly-rich loop (between β1 and β2) which positions the γ-phosphate of ATP for catalysis, the AxK motif (β3) coupling ATP phosphates to C-helix, and the C-helix itself which has been considered a 'signal integration motif' (Taylor & Kornev 2011), occupying a strategic position in the integration of the two lobes. All the important residues in this lobe are conserved in the 10 kinases, with the exception of PKAC3 that has an Ala instead of a Gly in the third Gly of the Gly-rich loop. The

A

	PKAC1	PKAC2	PKAC3	PKAC4	PKAC5	PKAC6	PKAC7	PKAC8	PKAC9	PKAC10
PKAC1	---	0.340	0.551	0.533	0.279	0.468	0.379	0.464	0.478	0.491
PKAC2	0.201	---	0.547	0.559	0.382	0.470	**0.338**	0.498	0.479	0.500
PKAC3	0.447	0.452	---	0.508	0.540	0.571	0.548	0.567	0.591	*0.603*
PKAC4	0.416	0.406	0.383	---	0.570	0.585	0.551	0.573	0.580	0.582
PKAC5	0.137	0.185	0.444	0.393	---	0.484	0.387	0.484	0.498	0.506
PKAC6	0.329	0.319	0.466	0.406	0.310	---	0.508	0.479	0.532	0.518
PKAC7	0.217	**0.146**	0.430	0.412	0.195	0.332	---	0.519	0.484	0.527
PKAC8	0.319	0.345	0.479	0.429	0.310	0.307	0.351	---	0.536	0.524
PKAC9	0.367	0.373	*0.500*	*0.500*	0.390	0.428	0.373	0.450	---	0.379
PKAC10	0.402	0.417	0.497	0.471	0.395	0.408	0.413	0.424	0.279	---

B

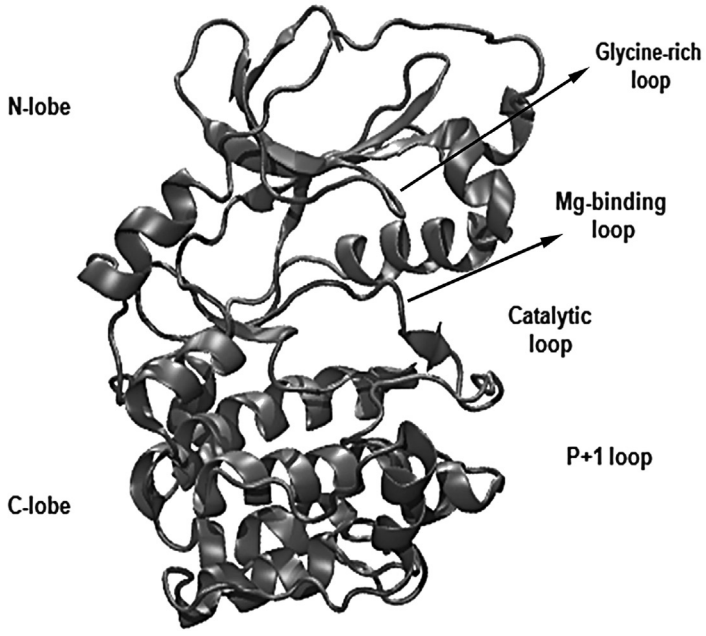

Fig 2 – (A) Estimates of evolutionary divergence amongst PKAC aminoacid sequences from *M. circinelloides*. Below diagonal, pair-wise genetic distance values (uncorrected p-distance) calculated for the conserved C-terminal region (369 aligned positions); above diagonal, distance values for the whole protein (540 aligned positions); ambiguous positions were removed between pairs compared. (B) Modeled structure of PKAC5 based on mammalian PKA catalytic subunit Cα, was performed using the Phyre2 web portal for protein modeling, prediction and analysis (Kelley *et al.* 2015). The principal structural lobes and functional motifs are indicated.

Lys of the AxK motif, a key residue absolutely conserved in the entire protein kinase family, is also conserved in the *Mucor* PKA family. This Lys forms a salt bridge with a Glu in the C-helix and its mutation is widely known since it turns the protein in a kinase-dead enzyme. The C-lobe contains the principal elements of the catalytic machinery: the catalytic loop, between β6 and β7, involved in the catalytic step itself; the conserved DFG motif, flanked by β8 and β9, participating in the recognition of one of the ATP-bound Mg^{++} ions and the activation segment, and finally extending from the DGF motif to the F-helix. The internal and dynamic architecture of C subunits, in its inactive and active forms, is guided by the conserved and harmonic interaction of two hydrophobic spines: the regulatory (R) spine and the catalytic (C) spine The R-spine is defined by four non-consecutive hydrophobic residues located in both lobes and the C-spine by eight residues also distributed in both lobes, completed by the adenine ring of ATP. The F-helix in the C-lobe completes this hydrophobic assembly by coordinating the R- and C-spines with the whole molecule (Taylor & Kornev 2011). Assembly of C subunit into its active form is dependent on the phosphorylation of a Thr residue in the activation loop which is conserved in the ten C subunits (TWTL/V motif after β9). This mechanism of folding of PKs into active conformation is conserved in all the protein kinases, and is mediated either by an autophosphorylation process or mediated by an external activating kinase (Kornev *et al.* 2008). Finally, there is a conserved C-terminal tail of variable length, an element considered as a cis-regulatory domain for all the AGC kinases. Particularly in PKAs this C-tail ends right after a conserved hydrophobic motif FxxF. This tail is absolutely conserved in the 10 PKACs, with the exception of PKAC9 which has an unusual extra aminoacid after the hydrophobic motif.

Phylogenetic analysis of PKAC isoforms

Until now such a large amount of predicted C subunits of PKA had never been reported. Ascomycota and Basidiomycota have been reported to have one to three PKA catalytic subunits.

The phylogenetic analysis of 118 PKACs (dataset 1, in Table S3) shows that, on one hand, each isoform from *Mucor circinelloides* shares a most recent common ancestor with a protein derived from another species and another taxonomic family (v.g., *Backusella* and/or *Rhizopus*, but also *Phycomyces*) (Fig 3). In most cases (90 %), these relationships are strongly supported by the data at hand (BSV $\geq$ 88 %). Solely the placement of PKAC5 remains uncertain (BSV $\leq$ 50 %). Likewise, the location of PKA proteins from the basidiomycetes and ascomycetes (*Ustilago* and *Neurospora,* respectively) and from blastocladiomycetes (*Blastocladiella*) is unclear. On the other hand, the topology in Fig 3 shows that the PKAC from Mucoromycotina clustered in five scattered clades (BSV $>$ 73 %), the Kickxellomycotina in two strongly supported clades, whereas the Entomophthoromycotina failed to form a single clade. Yet, the relationships amongst the PKACs from the Zygomycota are not fully resolved.

Fungal PKAs in general are being classified as belonging to Class I or Class II. Particularly these classes are being distinguished in fungi containing two isoforms of PKA, such as *Aspergillus nidulans* (Ni *et al.* 2005) and *Aspergillus fumigatus* (Fuller *et al.* 2011). Class I fungal enzymes are described as the ones more similar in sequence to canonical PKAs; and class II as those uniquely found in filamentous fungi. Class I enzymes are the ones contributing mostly to the total PKA activity in the cell, and are those, that when disrupted, display stronger phenotypes. The analysis of a subset of 64 PKAC isoforms together with 17 additional PKAC from ascomycetes and basidiomycetes (dataset 2 in Table S3) shows two particular clades highly supported (BSV $\geq$99), which correspond to Class I and Class II PKACs (Fig 4). However, the sister group relationships of these two clades are not resolved, as is the case for all deeper branches.

Expression of PKAC genes

The expression at the mRNA level of each PKAC gene was measured at different developmental stages from spores to mycelium under aerobic growth and to yeast-like cells under anaerobiosis with further shift to aerobiosis and consequent mycelial growth. The mRNA level expression of each PKAC in spores, shows that all the isoforms were expressed with the exception of PKAC7, PKAC9 and PKAC10 (Fig 5 left panel). PKAC5 expression was more or less stable along germination. For isoforms PKAC2 and PKAC7, the mRNA levels increase early at 3 h during vegetative growth; PKAC9 increases later at 5 h (germ tube emission) and then three isoforms remain stable at levels similar to those of PKAC5 during mycelial growth. PKAC4 and PKC6 steadily decrease their levels along germination; PKAC1 decreases abruptly at 3 h of growth, and then remains at very low levels. The mRNA levels for PKAC3 and PKAC8 decrease during spore isodiametric growth stage (3 h), but then an important up-regulation is shown by PKAC3 at 5 h, and at 8 h (mycelial growth) by PKAC8. PKC10 expression is only observed at 8 h during aerobic mycelial growth; it is not observed either in spores or in yeast-like cells. The expression pattern of the PKAC isoforms after o.n. anaerobic growth was similar to the aerobic vegetative growth after 3 h (Fig 5 right). After 5 h of shift from anaerobic yeast-like growth to filamentous growth, the mRNA expression profile was very similar to the 5 hs aerobic growth (Fig 5). These results suggest that the expression of each PKAC gene is highly regulated and coordinated in response to the environment, in agreement with the regulated expression of each of the four PKAR (regulatory subunits of PKA) isoforms during growth and differentiation (Ocampo *et al.* 2012).

PKAC: possible pseudokinase

Until the sequencing of *Mucor circinelloides* genome, the only sequence reported as a PKA catalytic subunit from *Mucor* was the one deposited in NCBI under the name PKA catalytic *Mucor racemosus* (GI 20218944; Wolff *et al.* 2002), named here as PKAC. When the PKAC sequence was compared against the ten PKA C subunits predicted by us from the fungal genome, it became apparent that it should not be classified as an active PKA, mainly for two reasons: the core region is not conserved and the C-terminus is much longer than the one from PKAs (data not shown). A careful inspection of the sequence was therefore undertaken to settle the question of

Fig 3 – Maximum Likelihood phylogenetic analysis of 118 PKAC isoforms (dataset 1 Table S3). The topology with the highest log likelihood (−24511.52) was inferred by using the Maximum Likelihood method based on the Le & Gascuel (2008) model. A discrete Gamma distribution was used to model evolutionary rate differences among sites using five categories (+G, parameter = 0.826). The rate variation model allowed for 10.36 % sites to be evolutionarily invariable (+I). Branch lengths are measured in number of substitutions per site. All positions with less than 50 % site coverage were eliminated; therefore 313 positions were involved in the final dataset. Bootstrap values > 60 % (500 pseudoreplicates) are indicated on each branch; full support is emphasized by thickened lines. Red dots show the placement of *Mucor circinelloides* PKAC isoforms. Mucoro, stands for Mucoromycotina (in blue type); Entomo, Entomophthoromycotina (orange); Kickx, Kickexellomycotina (pink). Dashed brackets are used to indicate a non-monophyletic group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

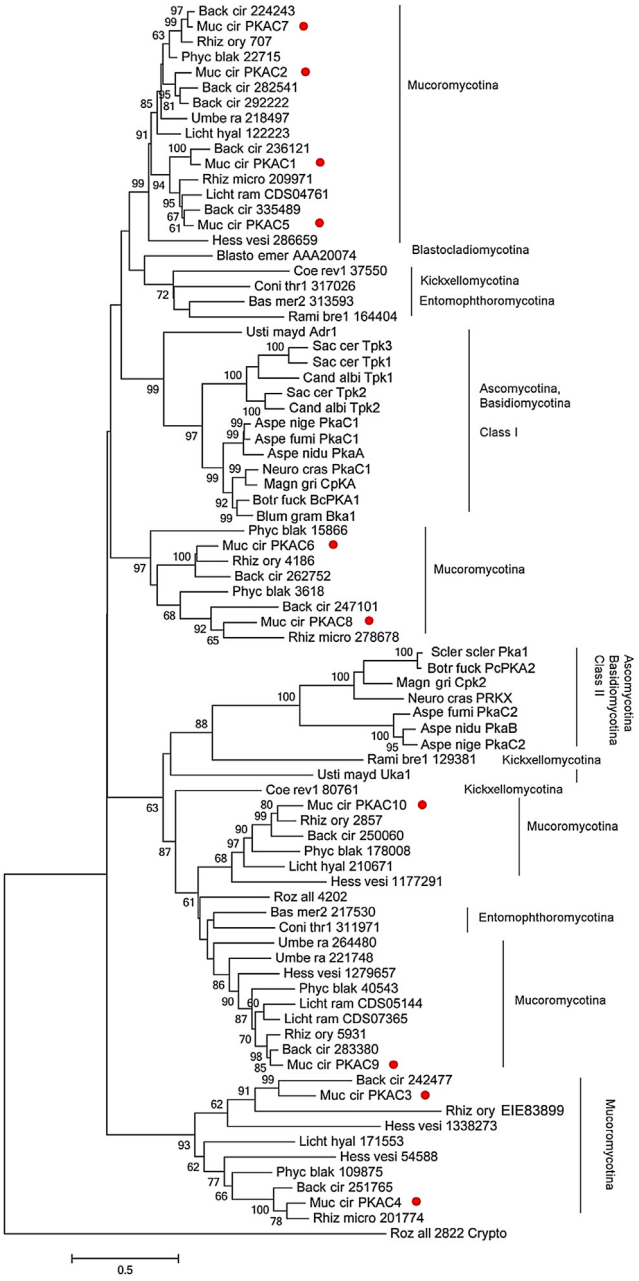

Fig 4 – Phylogenetic relationships amongst PKACs from a diverse arrange of fungal species (dataset 2 Table S3). The topology with the highest log likelihood (−20656.60) was inferred by using the Maximum Likelihood method based on the Le & Gascuel (2008) model. A discrete Gamma distribution was used to model evolutionary rate differences among sites using five categories (+G, parameter = 1.03). The rate variation model allowed for 9.72 % sites to be evolutionarily invariable (+*I*). Branch lengths are measured in number of substitutions per site. All positions with less than 50 % site coverage were eliminated thus, 313 positions were involved in the final dataset. Bootstrap values > 60 % (500 pseudoreplicates) are indicated on each branch. Red dots show the placement of *Mucor circinelloides* PKAC isoforms. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whether this sequence corresponded to a PKA or not. Through a domain search, in Pfam (pfam.xfam.org), we found that two domains could be predicted for PKAC: a C2 domain (87–197 aa) and a protein kinase domain (Pkinase; 247–508 aa). The C2 domain is located in the amino terminal part of the molecule, where all *M. circinelloides* PKA isoforms diverge. This is a structural domain involved in targeting proteins to cell membranes; in its classical version it has a beta-sandwich of 8 strands. Originally it was identified as a calcium binding domain in the PKC family; it has been found coupled to several enzymatic domains in a Ca^{+2}-independent form (Zhang & Aravind 2010). The BLAST search of C2 domain from PKAC yielded significant hits with the Sch9 type protein kinases (PKB family of AGC group) (Fig 6A). Within the domain we could predict seven well defined beta strands (according to Psipred secondary structure prediction; data not shown) for all the proteins in the alignment, including PKAC, and a not so clear third beta strand in Sch9 proteins, not shared by PKAC. One striking observation is that PKAC differs in the core catalytic domain, highly conserved in all the protein kinase family. The major differences are the presence of an Asn instead of an Asp in the YRD motif in the subdomain VIb (from the end of αE to the beginning of αF, see Figs 1 and 6B), and the replacement of an absolutely conserved Asp by a Ser in the catalytic loop. The Asp is crucial for kinase activity because of its function as a base acceptor to achieve proton transfer. According to Boudeau *et al.* (2006), a mutation in these two residues is enough to suggest that PKAC (GI 20218944) might be a pseudokinase. Within the activation segment -necessary to coordinate Mg^{2+} ions-the DFG motif is conserved. However, the phosphorylatable Thr residue (Thr 197 in human PKA), which needs to be phosphorylated in all the protein kinases in order to attain an active state, has a non-conserved upstream immediate sequence. Moreover, the PKAC C-terminus has a characteristic PxxP motif (within the C-lobe tether, CLT), and an FxxF hydrophobic motif (within the N-lobe tether, NLT), conserved in all AGC kinases. Still, the C terminus of PKAC (GI 20218944), unlike real PKAs, extends beyond the FxxF motif, as in Sch9 kinases (Fig 6C). The active site tether (AST) domain in this PKAC, essential for PKA activity, diverges from canonical PKAs and PKBs (Yang *et al.* 2009). The mRNA level of PKAC (GI 20218944) was measured. As shown in Fig 7A and B, its mRNA is expressed in spores, during aerobic germination, anaerobic growth and during the shift from anaerobic to aerobic growth. In aerobic conditions, the mRNA levels decreased during the first hours and increased after germ tube emission at 5 h of growth. The changes in the mRNA expression levels suggest that this isoform might have a role during *M. circinelloides* differentiation. In order to discard whether PKAC displays PKA catalytic activity, using kemptide as substrate, this isoform as well as a canonical PKA from *M. circinelloides* (PKAC5) were cloned as myc-tag fusion proteins. These clones were expressed in a yeast strain with an attenuated PKA catalytic activity background (*tpk1*^{w1}*BCY1*); an untransformed WT yeast strain was used as control. The correct expression of both proteins was verified by Western blot (Fig 7C bottom panel). Results indicate that PKAC5 showed cAMP dependent kinase activity, while PKAC activity could not be detected with the same substrate (kemptide; Fig 7C). Kemptide has been also used to measure

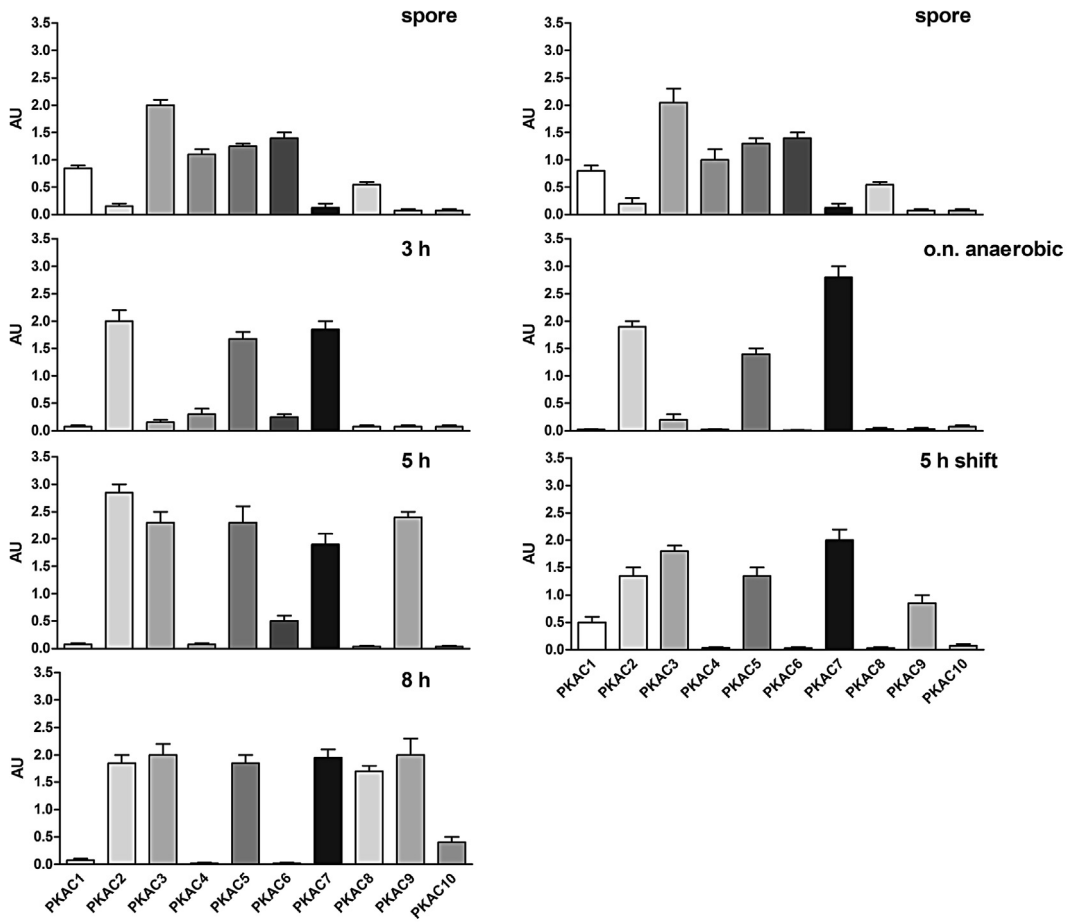

Fig 5 – Expression levels of *pkaC* genes were determined in spores at 0, 3, 5 and 8 h of growth under aerobic conditions (left panel) and in spores grown overnight (o.n.) under anaerobiosis and after a further 5 h shift to aerobiosis (right panel). Relative mRNA levels of each *pkaC* were analyzed by semi-quantitative PCR and quantified by digital imaging. The elongation factor EF-1a gene was used (*tef*-1) as an internal standard. Each value represents the mean ± SEM of four independent experiments.

PKB activity, although it is not the optimal peptide substrate (Kim *et al.* 2005). PKAC activity was also assessed in the presence of Mn^{+2} and using Nth1, a substrate peptide derived from neutral trehalase described to have a higher *Vmax* and *Km* parameters for yeast PKA activity than canonical kemptide (Galello *et al.* 2010). PKAC did not show a measurable activity under any of these conditions (data not shown). These results support our proposal that PKAC is a pseudokinase. To further analyze whether PKAC has a role *in vivo*, we generated a *pkaC* null mutant strain by gene replacement using a knockout vector that contains de *pyrG* gene used as a selective marker, flanked by sequences of the *pkaC* gene and adjacent regions (Fig 8A). A restriction fragment from this plasmid containing the *pyrG* gene and sufficient sequences of the *pkaC* gene to allow homologous recombination was used to transform the strain MU402, which is wild-type (wt) for PKA but auxotrophic for uracil and leucine. Several transformations were performed without success until we could obtain some transformants. The transformant clones were difficult to obtain, but we ended with four clones showing a high average growth. The disruption of the *pkaC* gene was confirmed in the four clones by PCR using a primer that hybridizes in a *pkaC* genomic region outside of the replacement fragment (MC6) and another primer that hybridizes inside the *pyrG* sequence (pyrGZ). An expected 0.8 kb DNA fragment was obtained in all four mutant clones, but not in the WT cells (Fig 8B, lanes 1 to 5). Gene disruption was also confirmed with a second pair of primers hybridizing inside the *pyrG* gene and inside the *pkaC* sequence upstream of the region cloned in the knockout vector (data not shown). Finally a pair of primers that hybridizes in the deleted region of *pkaC* (Sec2F) and in the 3′ region of *pkaC* (CaR) was used as an additional control to detect a 1.1 kb product in the WT strain and its absence in the disrupted clones. However, not only a 1.1 kb fragment was observed in the WT strain Mu402, as expected, but also in the four deletion clones (Fig 8B, lanes 6–10). This indicates that the clones are heterokaryons having both types of nuclei, WT and *pkaC* null, and that after 15 cycles of selection WT nuclei are maintained. In a heterokaryon system, when a gene is disrupted the wt nuclei are normally lost after two or three growth cycles in selective medium (Osmani *et al.* 2006). Thus the percentage of growth in selective medium relative to non selective medium should increase. In the particular case of *pkaC* disruption, after fifteen cycles the presence of

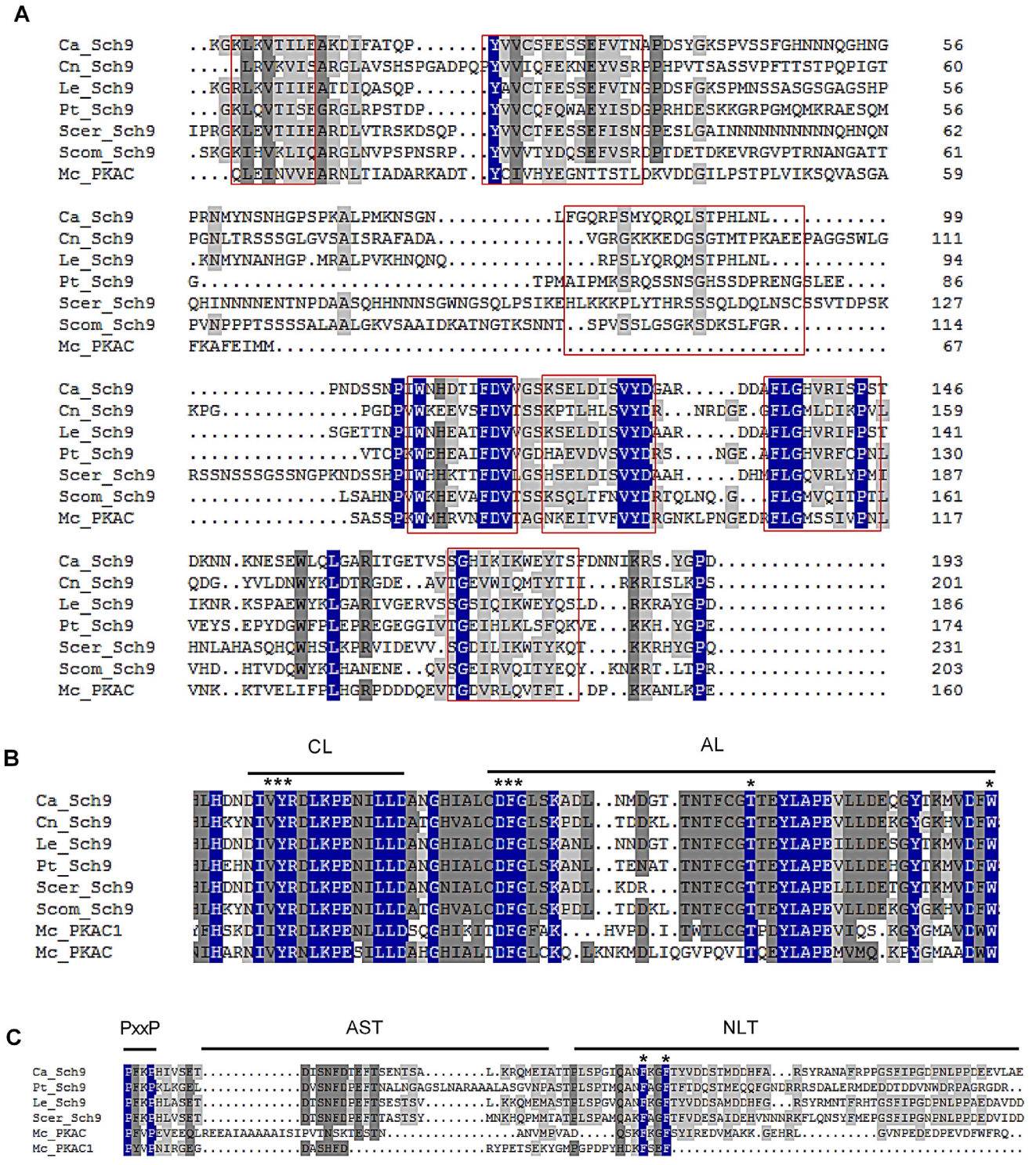

Fig 6 – (A) Sequence alignment showing the N-terminal region of Sch9 corresponding to the predicted C2 domain from *Candida albicans* (Ca; GI 74587222), *Cryptococcus neoformans* (Cn; GI 57230732), *Lodderomyces elongis* (Le; GI 149248728), *Pyrenophora tritici* (Pt; GI 187985597), *Saccharomyces cerevisiae* (Scer; GI 5279) and *Schizophyllum commune* (Scom; GI 300111898), in comparison with the PKAC from *M. circinelloides* (Mc; GI 20218944). Red boxes indicate the eight structurally predicted β-strands, (B) Sequence alignment showing the differences in the core catalytic loop (CL) and activation segment (AS) from six Sch9 members of fungal PKAs plus one *bonafide* Mc_PKAC1 in comparison with Mc_PKAC. The asterisks indicate YRD and DFG motifs, the conserved Asn and the phosphorylatable Thr residue; (C) C-terminal region of PKAC, NLT corresponds to N-lobe tether and AST to active site tether. PxxP conserved motif in CLT, C-lobe tether. The asterisk indicates the FxxF hydrophobic motif. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

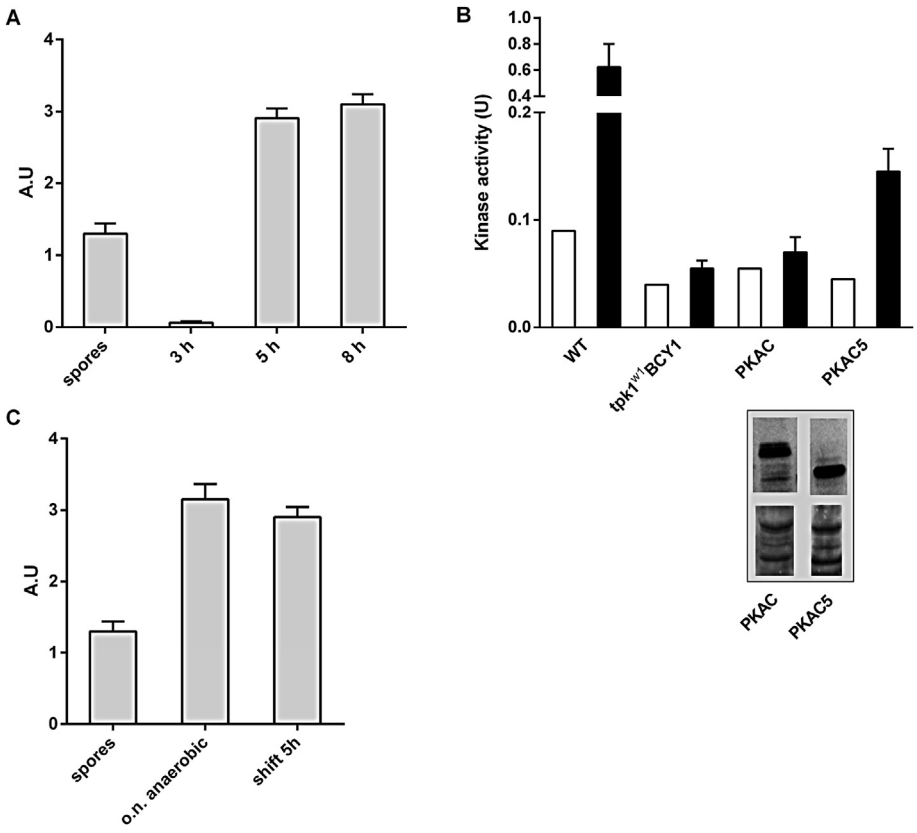

Fig 7 – PKAC mRNA expression level was determined as in Fig 5, under aerobic growth (A) or under anaerobiosis and shift from anaerobic to aerobic growth conditions (B); the values are expressed in arbitrary units (a.u.), normalized to *tef-1* mRNA levels. Each value represents the mean ± SEM of four independent experiments; (C) PKAC and PKAC5 kinase activity. The kinase activity was measured from a semipurified extract of a WT (SP1), *tpk1*^{w1}*BCY1*, and *tpk1*^{w1}*BCY1* expressing *pkaC-c-myc* and *pkaC5-c-myc* strains. PKA activity was assessed with (black bars) and without (white bars) cAMP using kemptide as substrate. PKA activity units are defined in materials and methods. Each value represents the mean ± SEM of three independent experiments. Bottom panel (upper part) Western blot α-c.Myc from the same extracts used to measured kinase activity; bottom panel (lower part), total protein visualized with Ponceau Red.

WT nuclei was still detected and the percentage of growth in selective medium over non selective medium decreased or remained the same. We thus conclude that *pkaC* deletion mutant is viable only as an heterokaryon containing wild type nuclei and that, therefore, *pkaC* is essential for viability in *M. circinelloides*.

Discussion

Ten genes coding for the catalytic subunit of PKA have been predicted from the recently sequenced genome of *Mucor circinelloides*. The gene organization seems to be very similar, even

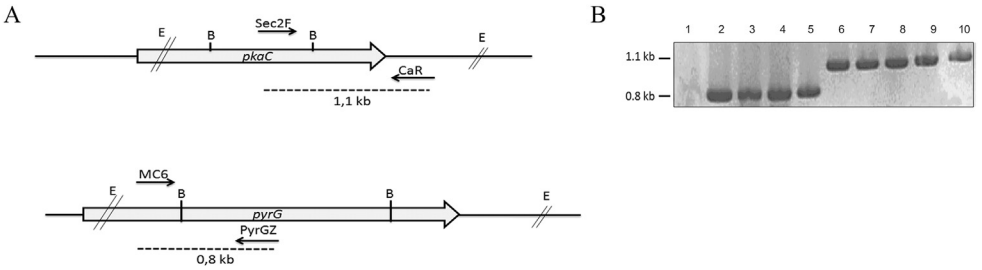

Fig 8 – (A) Genomic structures of the WT (top) *pkaC* locus and of the locus after the homologous recombination of the replacement fragment (bottom). The primers used in panel B are indicated. E, EcoRI; B, Bam HI. (B) PCR from genomic DNA was performed in WT (lanes 1 and 6) and 4 different clones (lanes 2–5 and 7–10) using primers MC6-PyrGZ (lanes 1–5; 0.8 kb product) and with primers SeC2F-CaR (lanes 6–10; 1.1 kb product).

with such small number of genes, to the statistics derived for the recently published genomes of *Phycomyces blakesleeanus* and *M. circinelloides* (Corrochano *et al.* 2016). The average lengths for introns and exons are, respectively, 62 (93) and 323 (311) nucleotides, with around 3.3 (3.8) introns/gene (number in parenthesis are from the work of Corrochano *et al.* 2016). The conservation of splicing 5′ donor, 3′ acceptor and branching sites has already been systematically studied for a selected group of diverse fungi (Kupfer *et al.* 2004). The 36 introns predicted in this study for a basal fungus, fit into the general scheme, although with a stronger bias to GTAA in the first four positions of the 5′-donor site in 70 % of the introns; this is more conserved than the predicted consensus sequence in our model fungi: GURWGU. The 3′ acceptor site of the predicted introns fit perfectly well (92 %) into the YAG consensus for fungi. The consensus sequence for the branching site for metazoan introns is YNCURAY, while *Saccharomyces cerevisiae* introns show a highly conserved sequence of UACUAAC. The predicted branching sites for half of the PKA introns fit into the consensus metazoan consensus, with slight variations for the remaining 50 %.The ten predicted PKA catalytic subunits (PKAC1-PKAC10) from *M. circinelloides* have a highly conserved core domain, even when compared to the core from mammalian PKA C subunits. This domain contains all the predicted structural domains that give rise to the different functional domains, the ones shared by all protein kinases in general, and those particular of PKA (Figs 1 and 2B). The difference amongst PKA isoforms resides mainly in the amino terminal sequence. We have recently reported (Ocampo *et al.* 2009) that *M. circinelloides* codes for four regulatory subunits of PKA (PKAR1-PKAR4), which are highly similar in the cAMP binding domains (at the C terminus) and differ in their amino termini. Taking into consideration this large number of PKA subunits we predict the existence of at least 40 varieties of holoenzymes (R_2C_2) by combination of each R subunit dimer with two C subunits, or even a larger number if a R_2 interacts with two different C subunits at a time. The comparison of the ten PKA C subunits from *M. circinelloides* with those predicted for zygomycetes showed a high number of paralogues for the subdivision Mucoromycotina (Table S4). The average genetic distance value estimated amongst the complete *M. circinelloides* isoforms is 0.499; this value drops when the highly variable N-terminal region is excluded, becoming closer to those obtained for other zygomycetes with multiple PKACs (Table S4).

Until now no such large number of PKAC subunits had been reported. A very recent paper studying PKA in animal evolution made a thorough bioinformatic search of C and R subunits in organisms originated from single-cell eukaryotic ancestors, reported a high number of isoforms in several basal fungal lineages such as the zygomycetes *P. blakesleeanus* and *Rhizopus oryzae* and the blastocladiomycete *Allomyces macrogyunus* (Peng *et al.* 2015). Our phylogenetic tree compares the PKAC subunits from *M. circinelloides* with PKACs from a large number of fungal species, mainly zygomycetes (Fig 3). We can conclude that the paralogous copies of *M. circinelloides* pre-date the divergence of Mucoromycotina. Indeed, the evidence suggests that PKAC duplication even pre-dates the divergence of the Entomophthoromycotina, Kickxellomycotina, Mucoromycotina and Zoopagomycotina, the four currently accepted subdivisions. It is well known that genome duplication is a common feature of fungi (Cornell *et al.* 2007). *Rhizopus oryzae* was the first basal fungal lineage fully sequenced in which WGD (whole genome duplication) was demonstrated (Ma *et al.* 2009). The recent genomic comparison between *P. blakesleeanus* and *M. circinelloides* has demonstrated a WGD for these two fungi (Corrochano *et al.* 2016). Therefore the origin of gene expansions, such as the ones for PKA C subunits, could be a consequence of either gene duplication or tandem duplication, followed by divergence. An expansion of the total number of kinases (kinome) and proteins involved in signal transduction in fungi has already been described (Kosti *et al.* 2010), and do not correlate with the expected number predicted from the overall duplications. Corrochano *et al.* (2016) stress the flexibility gained by the signal transduction pathways with such an expansion. Recently, Valle-Maldonado *et al.* (2015b) had reported the existence of an unusually large number of G proteins in *M. circinelloides* (10 Gα, 4 Gβ and 4 Gγ), which are also involved in signal transduction and are highly related to the cAMP pathway.

Present results also suggest that the current classification of PKACs in class I and II (Ni *et al.* 2005; Fuller *et al.* 2011) is pertinent mainly to Ascomycete isoforms and does not apply to the rest of the fungal groups assayed, particularly Mucoromycotina (Fig 4). The correspondence between each PKAC isoform in *Mucor* with an ortholog in the other mucoromycotina fungi, suggests essential signaling functions common to all these species, and the difference in the total number of isoforms indicates specific adaptations in cAMP signaling. The isoforms expression was very selective and differential (Fig 5), with some isoforms expressed at the isodiametric vegetative stage of growth (PKAC2, PKAC5 and PKAC7), others appearing at the beginning of the differentiation stage, upon germ tube emission (5 hs, PKAC3 and PKAC7), while others appearing only upon hyphal growth (PKAC8 and PKC10). Our previous studies measuring PKA catalytic activity and R subunit cAMP binding activity, during growth and differentiation, were performed ignoring the existence of multiple isoforms. Those results are in accordance with present outcomes in the sense that PKA activity was evidenced in spores; the activity decreased upon spore swelling and isodiametric growth, and then increased steadily upon germ tube emission and hyphal growth (Rossi & Moreno 1994). We have proposed that PKA activity is involved in shaping (morphology) and branching in *M. circinelloides* (Lübbehüsen *et al.* 2004) and that a threshold activity of PKA must be attained in *Mucor rouxii* in order to trigger polarized aerobic growth (Pereyra *et al.* 2000). At present, we consider that certain PKA (both R and C isoforms) are involved in this polarized growth. It is interesting that the expression pattern after overnight anaerobic growth of spores is almost identical to the one displayed by isodiametric cells (3 hs) in aerobiosis (Fig 5, right and left panel). This agrees with our hypothesis that dimorphism in *Mucor* corresponds to either a polarized growth (mycelium) or an isodiametric growth (yeast-like cells) (Pereyra *et al.* 1992). During our previous works we had purified PKAC from hyphal growing cells and measured kinetic parameters with several substrate peptides (Pastori *et al.* 1985; Zaremberg *et al.* 2000; Rinaldi *et al.* 2008). Currently, we know that partially purified

preparations from the mycelial stage must have had -at least- 5 PKAC isoforms. However, no evidence of a mixture of PKACs had emerged from the kinetic curves. It is tempting to conclude that the kinetic parameters of the isoforms towards different substrates are very similar, as we have shown in *S. cerevisiae* for Tpk1 and Tpk2 catalytic subunits of PKA (Galello *et al.* 2010). If this is so, then the difference in their functional roles and their specificity must reside in their divergent amino termini. It is well known, that the amino termini of the R dimers form a docking domain for anchoring proteins, in mammals (Scott *et al.* 2013), and it has also been shown that free catalytic subunits can interact with localizing proteins through their N-termini (Sastri *et al.* 2005). It will be interesting to investigate why are so many PKAs coexisting in these zygomycetes; what is their individual role and —particularly- to understand the molecular mechanism through which the N-terminus might be involved in the specificity. This question is also pertinent for understanding the specificity of the three PKA isoforms from *S. cerevisiae*.

Finally, we have to highlight the importance of the re-characterization of the PKAC subunit accomplished herein. Before the genomic era it was proposed to be the paradigmatic PKA catalytic subunit. There is only one protein isoform with these characteristics in the genome of *M. circinelloides*: it cannot be classified as a PKA because of the difference in the C-tail; it can be compared to a PKB because of its C2 domain in its N-terminus.). Although the hydrophobic spines necessary for a protein kinase to adopt an active conformation (34, 35) is conserved in PKAC, no catalytic activity can be expected for this protein, as judged by the differences in its catalytic core regarding conserved residues in the catalytic loop and can therefore can be classified bioinformatically as a pseudokinase (Reiterer *et al.*, 2014. However, PKAC must have an important role as it is expressed differentially during growth and differentiation, and its deletion is lethal. It would be of interest to understand the role of PKAC and its mechanism of action. At present, more attention is being paid to pseudokinases, considered as essential components of signaling pathways and consequently more information on their mechanism(s) of action is now available (Reiterer *et al.* 2014).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.07.013.

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