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

Lucas FERNÁNDEZ NÚÑEZ[a], *Josefina OCAMPO*[a,1], *Alexandra M. GOTTLIEB*[b], *Silvia ROSSI*[a], *Silvia MORENO*[a,*]

[a]*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IQUIBICEN-CONICET, Intendente Güiraldes 2160 – Ciudad Universitaria – C1428EGA, Buenos Aires, Argentina*
[b]*Departamento de Ecologia, Genética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IEGEBA-CONICET, Intendente Güiraldes 2160 – Ciudad Universitaria – C1428EGA, Buenos Aires, Argentina*

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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

* *Corresponding author*. Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160 – Ciudad Universitaria – C1428EGA, Buenos Aires, Argentina.
E-mail address: smoreno@qb.fcen.uba.ar (S. Moreno).
[1] Present address: Division of Developmental Biology, Eunice Kennedy Shriver National Institute for Child Health and Human Development, National Institutes of Health, Bethesda, USA.
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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

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