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Cross regulation between *Candida albicans* catalytic and regulatory subunits of protein kinase A

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ABSTRACT

In the pathogen *Candida albicans* protein kinase A (PKA) catalytic subunit is encoded by two genes *TPK1* and *TPK2* and the regulatory subunit by one gene, *BCY1*. PKA mediates several cellular processes such as cell cycle regulation and the yeast to hyphae transition, a key factor for *C. albicans* virulence. The catalytic isoforms Tpk1p and Tpk2p share redundant functions in vegetative growth and hyphal development, though they differentially regulate glycogen metabolism, the stress response pathway and pseudohyphal formation. In *Saccharomyces cerevisiae* it was earlier reported that *BCY1* overexpression not only increased the amount of *TPK3* mRNA but also its catalytic activity. In *C. albicans* a significant decrease in Bcy1p expression levels was already observed in *tpk2*Δ null strains. In this work we showed that the upregulation in Bcy1p expression was observed in a set of strains having a *TPK1* or *TPK2* allele reintegrated in its own locus, as well as in strains expressing the *TPKs* under the control of the constitutive *ACT1* promoter. To confirm the cross regulation event between Bcy1p and Tpkp expression we generated a mutant strain with the lowest PKA activity carrying one *TPK1* and a unique *BCY1* allele with the aim to obtain two derived strains in which *BCY1* or *TPK1* were placed under their own promoters inserted in the *RPS10* neutral locus. We found that placing one copy of *BCY1* upregulated the levels of Tpk1p and its catalytic activity; while *TPK1* insertion led to an increase in *BCY1* mRNA, Bcy1p and in a high cAMP binding activity. Our results suggest that *C. albicans* cells were able to compensate for the increased levels of either Tpk1p or Tpk2p subunits with a corresponding elevation of Bcy1 protein levels and vice versa, implying a tightly regulated mechanism to balance holoenzyme formation.

1. Introduction

Candida albicans is a major fungal pathogen in humans, usually causing superficial infections of mucosal epithelium. The most severe expression of candidiasis occurs in immunocompromised patients including debilitating mucosal disease such as oropharyngeal candidiasis as well as life-threatening infections of the bloodstream and major organs (Vazquez and Sobel, 2003). Animal studies have shown that the pathogenic potential of *C. albicans* is associated with its ability to reversibly alternate between three morphological states: yeast, pseudohyphae and hyphae (Lo et al., 1997; Kumamoto and Vinces, 2005). Morphological transitions in *C. albicans* are regulated by different signal transduction pathways, including cAMP-PKA, MAPK, Rim101, and the TOR pathway (Sonneborn et al., 2000; Cutler et al., 2001; Liu, 2001; Monge et al., 2006).

In *C. albicans* the cAMP-protein kinase A (PKA) cascade mediates besides morphogenesis a wide range of cellular functions (Giacometti et al., 2006, 2011). In this signaling pathway an increase in the cAMP levels leads to PKA activation by releasing the catalytic subunit upon cAMP binding to the regulatory subunit. The *C. albicans* PKA regulatory subunit is encoded by *BCY1* gene while two genes *TPK1* and *TPK2* code for the catalytic subunits. Positive roles have been established for both catalytic isoforms in cell growth and hyphae formation (Bockmühl et al., 2001; Cloutier et al., 2003). Consistent with previous reports (Bockmühl et al., 2001; Huang et al., 2010), our lack of success in generating a double mutant of *TPK1* and *TPK2* suggests that this mutant is not viable. In *Saccharomyces cerevisiae* a constitutively high PKA activity in a strain bearing a deletion in the *BCY1* gene causes a severe decrease in tolerance to heat and starvation stress (Toda et al., 1985). In *C. albicans* high uncontrolled PKA activity is lethal since a mutant strain lacking the regulatory subunit is not viable (Davis et al., 2002; Cassola et al., 2004); however a null *BCY1* mutant could be obtained in a background of low kinase activity, such as the strain *tpk2*Δ/*tpk2*Δ, although it is defective in its morphogenesis in spite of its constitutive PKA cata-

Abbreviations: PKA, cAMP dependent protein kinase; PKI, PKA inhibitor fragment (14–24); PVDF, polyvinylidene difluoride.

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lytic activity. Moreover, in this *tpk2Δ/tpk2Δ bcy1Δ/bcy1Δ* mutant strain, Tpk1p appears dispersed throughout the cell unlike the parental strain bearing Bcy1p, in which Tpk1p was predominantly nuclear (Cassola et al., 2004). We have also shown that heterozygous strains for *BCY1* irrespective of the *TPK* genetic background displayed a mixture of pseudohyphae and true hyphae upon incubation in several inducing liquid media, as well as a more vacuolated phenotype (Giacometti et al., 2006, 2011). Staab et al. (2003) demonstrated that overexpression of *C. albicans* regulatory subunit prevented the release of active catalytic subunits and abrogated the activation of genes involved in germ tube formation; however strains overexpressing *BCY1* were able to produce pseudohyphae. In *Neurospora crassa* there are striking morphological abnormalities associated with mutations in the regulatory subunit conducive to lower expression levels (Bruno et al., 1996). A work of Jung et al. (2005) demonstrated that *C. albicans* mutant cells devoid of Pde2p (one of the cAMP phosphodiesterases), presenting a constitutive activation of the cAMP pathway, have defective cell wall and membrane. Thus, both, the lack or the overexpression of *BCY1* leads to alterations in cell structure, morphogenetic phenotype, and localization of the Bcy1 protein supporting the idea that in *C. albicans*, the existence of a regulated PKA, through the expression of both *BCY1* alleles, is a determinant for the preservation of the cell integrity as well as for normal filamentation.

We previously showed that in *C. albicans* Tpk2p isoform is the most abundant isoform in the cell representing approximately 90% of the total PKA activity (Souto et al., 2006). We also observed in *tpk2Δ* strains a significant decrease in Bcy1p expression (Giacometti et al., 2006, 2009). In NIH3T3 cells expression of the PKA catalytic subunit resulted in an upregulation of expression of the endogenous regulatory subunit (Uhler and McKnight, 1987); while in *S. cerevisiae BCY1* overexpression not only increased the amount of *TPK3* mRNA but also its phosphorylatable activity otherwise negligible (Mazón et al., 1993).

In this work we showed the upregulation in Bcy1p expression in a set of strains having a *TPK1* or *TPK2* allele reintegrated in its own locus, as well as in strains expressing the *TPK1* or *TPK2* sequence under the control of the *ACT1* promoter. To confirm the cross regulation in the expression of Bcy1 and Tpk proteins and since we were not able to express *BCY1* in a high phosphotransferase activity background, we performed a series of biochemical studies in a mutant with the lowest PKA activity carrying one *TPK1* and a unique *BCY1* allele (strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ*) and produced two derived strains in which a wild type copy of *BCY1* or *TPK1* were placed under their own promoters inserted in the *RPS10* neutral locus. Our results showed that placing one copy of *BCY1* upregulated the levels of Tpk1p and its catalytic activity while *TPK1* insertion led to an increase in Bcy1p which was reflected in a high cAMP binding activity, suggesting cells attempt to maintain the normal status of substrate phosphorylation through a still unknown mechanism.

2. Materials and methods

2.1. Chemicals

Reagents were purchased as follows: Calcofluor white (CFW), kemptide (LRRASLG), PKA inhibitor fragment (14–24), cAMP-agarose (A0144), Alkaline Phosphatase from *Escherichia coli* (P5931), Sigma Chemical Co.; phosphocellulose paper P-81 was from Whatman; [^{32}P]ATP and [^{3}H]cAMP from New England Nuclear; pre-stained protein markers from Recom; Polyvinylidenedifuoride (PVDF) membranes (Immobilon-P) from Millipore; restriction endonucleases and pGEM-T easy vector were from Promega; 'Complete mini' protease mix was from Roche. Anti-phospho-PKA substrate (RRXS/T) was from Cell Signaling. Phosphatase inhibitors cocktail set II, was from Calbiochem. All other chemicals were of analytical grade.

2.2. Organisms, strains, media, and culture conditions

All *C. albicans* strains used in this study are derived from the wild type strain CAI4 (Fonzi and Irwin, 1993) and were detailed in Table 1. Yeast cells were cultured at 30 °C in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or in SD minimal medium (Sherman et al., 1986). To allow phenotype comparisons all tests were performed with strains carrying the *URA3* gene re-integrated using the CIp10 vector (Murad et al., 2000) ensuring *URA3* expression at the neutral *RPS10* locus. The genotype of all strains was routinely verified by PCR using the URA3ver5/RPS10ver primers (Table 2).

2.3. DNA manipulations

DNA purifications were performed with Qiagen affinity columns following the manufacturer's recommendations. Bacterial plasmid DNA was isolated by the alkaline lysis method (Sambrook et al., 1989) or using the QIAprep Spin Miniprep Kit (Qiagen). Yeast genomic DNA was isolated according to Adams et al. (1997). DNA modifying enzymes were used according to the manufacturers' recommendations.

Table 1
C. albicans strains used in this study.

Strain	Genotype	Source or reference
CAI4	*ura3::λimm434/ura3::λimm434*	Fonzi and Irwin, 1993
RGI4	Same as CAI4 but *RPS10*::CIp10	Giacometti et al., 2009
R1U1.1	Same as RGI4 but *TPK1/tpk1Δ*	Giacometti et al., 2011
RS1u	Same as RGI4 but *tpk1Δ::hisG/ tpk1Δ::hisG*	Giacometti et al., 2009
RG12.1u	Same as RGI4 but *tpk1Δ::hisG/ tpk1Δ::hisG BCY1/bcy1Δ::dpl200*	Giacometti et al., 2011
R2U2.1	Same as RGI4 but *TPK2/tpk2Δ*	Giacometti et al., 2011
AS1	*ura3::λimm434/ura3::λimm434 tpk2Δ::hisG/tpk2Δ::hisG*	Sonneborn et al., 2000
RS2u	Same as RGI4 but *tpk2Δ::Cat/tpk2Δ::Cat*	Giacometti et al., 2009
RS11u	Same as RGI4 but *tpk2Δ::hisG/ tpk2Δ::hisG TPK1/tpk1Δ::hisG*	Giacometti et al., 2009
*tpk2Δ/tpk2Δ BCY1/bcy1Δ*a	Same as RGI4 but *tpk2Δ::Cat/tpk2Δ::Cat BCY1/bcy1Δ::Cat*	Cassola et al., 2004
BBA1u	Same as *tpk2Δ/tpk2Δ BCY1/bcy1Δ*a but *RPS10*::CIp10	Giacometti et al., 2011
EC1u	Same as RGI4 but *tpk2Δ::Cat/tpk2Δ::Cat bcy1Δ::Cat/bcy1Δ::Cat*	Giacometti et al., 2011
HPY321	*tpk1Δ::hisG/tpk1Δ::hisG::TPK1*-dpl200 *ura3Δ::imm434/ura3Δ::imm434::URA3*	Park et al., 2005
HPY421	*tpk2Δ::hisG /tpk2Δ::hisG::TPK2*-dpl200 *ura3Δ::imm434/ura3Δ::imm434::URA3*	Park et al., 2005
RGHG1	Same as AS1 but *ADE2::pACT1-TPK1*	This study
RGHG2	Same as AS1 but *ADE2::pACT1-TPK2*	This study
RGS3	Same as *tpk2Δ/tpk2Δ BCY1/bcy1Δ*a but *TPK1/tpk1Δ::URA3-dpl200*	This study
RGS3.1	Same as RGS3 but *TPK1/tpk1Δ::dpl200*	This study
RGS3.1C	Same as RGS3.1 but *RPS10*::CIp10	This study
RGS3.BCY	Same as RGS3.1 but *RPS10*::CIp10-*BCY1*	This study
RGS3.TPK1	Same as RGS3.1 but *RPS10*::CIp10-*TPK1*	This study