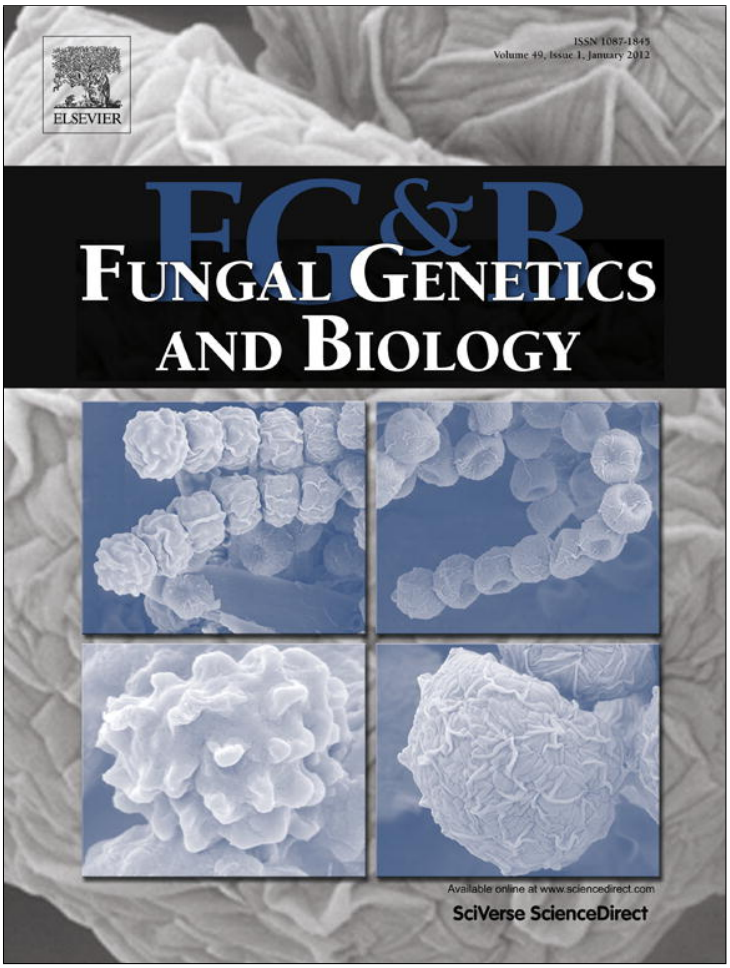

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Cross regulation between *Candida albicans* catalytic and regulatory subunits of protein kinase A

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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 Clp10 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*::Clp10	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*::Clp10	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*::Clp10	This study
RGS3.BCY	Same as RGS3.1 but *RPS10*::Clp10-*BCY1*	This study
RGS3.TPK1	Same as RGS3.1 but *RPS10*::Clp10-*TPK1*	This study

Table 2
Primers used in this study.

Name	Sense	Sequence 5′- 3′
TPK1KO5	Forward	GGAACCAGCAGACACAAGCATCAGGTCATTAAACG ACATCAACTTACAAGAACTTGCCAAGTTTTCCCAGT CACGACGTT
TPK1KO3	Reverse	GATAAAGATTTGGATTATGGTATAAGTGGAGTTGAA GACCCATATCGTGATCAATTCCATGTGGAATTGTGA GCGGA
URA3ver5	Forward	TTCCGAGCTTGGCGTAATCAT
TPK1ver3	Reverse	TAATACATAATAGTTCAATA
5UTR-TPK1	Forward	GAACAA*GAGCTC*GCCGAGGTAGTTGGTGTGGAAA GAC
3UTR-TPK1	Reverse	GAAGAA*GCGGCCGC*ACCAAATCATCCCAAATATCA AGTT
TPK1ver	Forward	TTGGCTTCCTTGTTAAATTGATC
RPS10ver	Reverse	CCCACACTCATTTATATTACTTAT
5UTR-BCY1	Forward	TTAGTA*TCTAGA*ATTAATTGTGCTTACGTGGC
3UTR-BCY1	Reverse	TTAGTA*GGATCC*CTGTTCAAAATAGGGTGTGC
BCY1ver	Forward	CCCCCTCTCGCTCTCTGTCTC
RT1-TPK1	Forward	AGAAGTTCAAGATGTGACTTAT
RT2-TPK1	Reverse	ACAAGGTGGTTCTGATGATG
RT1-TPK2	Forward	GAAGTTAGTACCGTTACATGG
RT2-TPK2	Reverse	ACTGCTGATTTGACAAGAAG
RT1-BCY1	Forward	ATGTCTAATCCTCAACAACA
RT2-BCY1	Reverse	TTAATGACCAGCAGTTGG
RT1-ACT1	Forward	CCCAAGCTTGCCGGTGACGACGCT
RT2-ACT1	Reverse	GTGGTGAACAAATGGATGGACCA
RT1-18S	Forward	ACTTTCGATGGTAGGATAG
RT2-18S	Reverse	TGATCATCTTCGATCCCCTA
ACT1pF	Forward	AACTGCAGCCTCGTTTATAATAAACTTAGTC
ACT1-TPK1ver	Reverse	AAAAGTCCTGGAATTGATCACGATA
ACT1-TPK2ver	Reverse	CAAAAGTCAAGGAAATACAGAGC

2.4. Heterozygous disruption of C. albicans TPK1

C. albicans knockout of the *TPK1* gene was generated using the PCR-based adaptation (Wilson and Davis, 1999) of the sequential URA-Blaster technique (Fonzi and Irwin, 1993) that has been previously described in Giacometti et al. (2009). Specific primers listed in Table 2, TPK1KO5/TPK1KO3, were designed to generate the PCR deletion construct *TPK1::URA3-dpl200.* The products of ten PCR reactions were pooled and used to transform CAI4 derived strain *tpk2Δ/tpk2Δ BCY1/bcy1Δ*a following the protocol described by Wilson and Davis (1999). This technique allowed us to obtain strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* (RGS3). *URA* transformants were grown on uridine deficient SD solid medium, and proper genomic insertion of the transforming cassette was determined by a PCR-based analysis of transformed colonies using a set of primers combining a forward oligo internal to the *URA3* cassette (URA3ver5) and a reverse one external to the modified region (TPK1ver3). From 15 independent isolations, 9 colonies showed heterozygous loss of the *TPK1* allele. All positive clones rendered identical phenotypes in the characterization assays.

2.5. C. albicans TPK1 and TPK2 expression under ACT1 promoter

The plasmids pACT-TPK1 and pACT-TPK2 (Huang et al., 2010) were digested with AscI and integrated into the *ADE2* locus by homologous recombination in strain *tpk2Δ/tpk2Δ* (AS1). The uridine positive colonies were analyzed by PCR with primers set ACT1pF/ACT1-TPK1ver and ACT1pF/ACT1-TPK2 (see Table 2), and the new mutants were named RGHG1 and RGHG2, respectively.

2.6. TPK1 and BCY1 insertion and expression from the neutral locus RPS10

To fully address the transcriptional cross regulation event, 0.5-Kb upstream and 1-Kb downstream of *BCY1* and *TPK1* ORF were independently integrated into the *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* mutant strain. The wild-type *BCY1* and *TPK1* genes were amplified from genomic DNA from strain CAI4 using primers 5UTR-TPK1/3UTR-TPK1 and 5UTR-BCY1/3UTR-BCY1 (Table 2) and Platinum High Fidelity polymerase (Invitrogen). The PCR fragments were digested with restriction enzymes SacI and NotI, and the digested fragments were purified on a column (QIAGEN) and cloned into the same sites of the CIp10 vector (Murad et al., 2000). After sequencing to confirm the integrity of both genes, the plasmids were named CIp10-*BCY1* and CIp10-*TPK1*, respectively. In order to transform strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* (RGS3) with each vector we recycled the *URA3* marker by selection on SD medium plus 5-FOA (1 mg/ml) and uridine (50 μg/ml); obtaining an *ura3* strain named RGS3.1. Empty control CIp10 plasmid was digested with StuI restriction enzyme and used to transform *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* (RGS3.1) strain, the new mutant was named RGS3.1C. Transformation of *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* (RGS3.1) strain with CIp10-*BCY1* and CIp10-*TPK1* linearized vectors rendered the reintegrated versions named RGS3.BCY1 and RGS3.TPK1. The uridine positive colonies were analyzed by PCR with primer sets TPK1ver-F/RPS10ver-R and BCY1ver-F/RPS10ver-R (see Table 2), and the presence of the 2.4-Kb wild-type fragment confirmed the integration of the *BCY1* gene, and the 2.7-Kb wild-type fragment confirmed the reintegration of the *TPK1* gene.

2.7. Crude extracts preparation, PKA activity and cAMP binding measurement

Yeast cells ($1–2 \times 10^7$) from stationary and logarithmic phase were suspended in 500 μl of 10 mM sodium phosphate buffer (pH 6.8) containing 1 mM EGTA, 1 mM EDTA, 10 mM b-mercaptoethanol and one tablet of 'Complete mini' protease mix per 10 ml. All manipulations were thereafter performed at 4 °C. Cells were lysed by disruption with glass beads as described previously (Cassola et al., 2004). The resulting suspension was spun down in a microfuge at maximum speed for 30 min to sediment unbroken cells and cellular debris and the supernatant was used immediately for enzymatic assays.

PKA activity was measured as previously described (Zelada et al., 1998). Briefly, the standard assay mixture contained 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mM kemptide, 0.1 mM [γ-^{32}P]ATP (0.1 to 0.5 Ci/mmol), and 10 μM cAMP when required, in a final volume of 60 μl. After incubation for 10 min at 30 °C, 50-μl aliquots were spotted on phosphocellulose papers and dropped into 75 mM phosphoric acid for washing (Roskoski, 1983). PKA specific activity was expressed as pmoles of ^{32}P incorporated to kemptide per min and per mg of protein. Since strain *tpk2/tpk2* (AS1) had low activity (Cloutier et al., 2003), to ensure accurate measurement of PKA activity in our set of *tpk2Δ/tpk2Δ* strains, the specific activity of [γ-^{32}P] ATP was raised to $2\text{-}5 \times 10^3$ cpm per pmole in the assays. In addition, in all assays the amount of extract was adjusted in order to minimize endogenous kinase activity and the reactions were carried out under conditions of linearity respect to the amount of extract and the time of incubation. Routinely it was checked that the measured activity was inhibited more than 80% by PKI.

Measurements of cAMP binding were performed as previously described (Zelada et al., 1998). Samples were incubated at 30 °C for 30 min in a final volume of 50 μl 15 mM Tris–HCl pH 7.5, 7 mM $MgCl_2$, 1.4 mM β-mercaptoethanol, 600 mM NaCl and 210 nM [^{3}H]cAMP (250.000 dpm). The reaction was terminated by filtering the samples through 0.45-μm nitrocellulose membranes. Non-specific binding was determined in the presence of 100 μM cAMP.

2.8. Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.9. Western blot analysis

Tpk1p and Bcy1p expression was assessed by Western blot analysis. Proteins from crude or soluble extracts were resolved by 10% SDS–PAGE and transferred to PVDF membranes by semidry electroblotting. The blots were blocked with 5% nonfat dried milk and incubated overnight with anti-*C. albicans* Tpk1p (Kronberg. F., PhD thesis) or anti-*C. albicans* Bcy1p antiserum (Zelada et al., 1998) generated in the laboratory. Immunological detection was performed using anti-rabbit IgG conjugated to alkaline phosphatase. For Tpk1p and Bcy1p expression level analysis loading and transfer were monitored by Ponceau S staining of the membranes. Pre-stained carbonic anhydrase was included as a transference control.

2.10. Phosphorylation of the Bcy1p regulatory subunit

To assess *in vivo* phosphorylation of the regulatory subunit all operations were performed in the presence of protein phosphatase inhibitors (inhibitor cocktail set II 150 mM). Cell extracts were prepared in 20 mM Tris–HCl buffer (pH 7.4) containing 1 mM EGTA, 1 mM EDTA, 10 mM β-mercaptoethanol and one tablet of 'Complete mini' protease inhibitor mix per 10 ml and Bcy1p was purified in a pull-down assay using a cAMP-agarose resin (A0144). The S100 fraction (10 mg protein) was incubated with 0.2-ml cAMP-agarose equilibrated in the same buffer containing 150 mM NaCl for 60 min at 4 °C with gentle stirring. cAMP-agarose beads were successively washed with 2 ml buffer A containing 2 M NaCl, 10 mM AMP and 10 mM ATP. The proteins were eluted by boiling the resin 5 min in 1 volume of 2 × Laemmli buffer. The protein samples were separated in a 12% SDS–PAGE and phosphorylation of Bcy1p was determined by immunoblot analyses with anti-Bcy1p and anti-phospho-PKA substrate (RRXS/T).

2.11. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from cells obtained during stationary growth phase by the hot-phenol method (Ausubel et al., 1994). RNA was DNase treated at 37 °C for 30 min. The SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen) was used to synthesize cDNA according to the kit instructions. OligodT (Invitrogen) was used to prime the cDNA synthesis reaction. RNA concentration was measured spectrophotometrically and 2 μg were added to the cDNA synthesis reaction. One-tenth volume of the final cDNA product was added to PCR reactions specific for each gene. Primer sequences for *BCY1* (RT1-BCY1 and RT2-BCY1), *TPK1* (RT1-TPK1 and RT2-TPK1) and *TPK2* (RT1-TPK2 and RT2-TPK2) are detailed in Table 2. Samples were denatured at 94 °C for 2 min, followed by 15–30 cycles (94 °C for 45 s, 55 °C for 45 s, and 72 °C for 30 s). The levels of amplified products were determined at several cycle intervals to ensure that samples were analyzed during the exponential phase of amplification. We performed reactions without reverse transcriptase to control for the presence of contaminating DNA. A 900 bp PCR product amplified with RT1-ACT1 and RT2-ACT1 primers from *C. albicans ACT1* and a 687 bp PCR product amplified from 18*S* rRNA gene was used as a probe for internal mRNA loading control (Bahn and Sundstrom, 2001). Transcripts were quantified using ImageJ (Abramoff et al., 2004).

3. Results

3.1. Expression of TPK1 or TPK2 in strains tpk1Δ/tpk1Δ and tpk2Δ/tpk2Δ increased the levels of Bcy1 protein

The fact that in *C. albicans* strains lacking both *TPK2* alleles we detected a downregulation of *BCY1* expression (Giacometti et al., 2006, 2009) led us to test whether an increased expression of *TPKs* upregulated Bcy1p expression. To do so, we assessed the relationship of PKA activity (Fig. 1) with cAMP binding and Bcy1p levels (Fig. 2) 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 from the *ADE2* locus.

PKA activity was routinely measured at the stationary phase since maximal levels of *TPK1* and *TPK2* mRNA are expressed at this stage, allowing the most discriminating comparisons (Souto et al., 2006; Giacometti et al., 2009). Fig. 1 shows that in a background of high PKA activity (*tpk1Δ/tpk1Δ* mutant), reintegration of a *TPK1* allele resulted in a similar kinase activity than that of the parental strain *tpk1Δ/tpk1Δ*; whereas *TPK2* reintegrated strain exhibited a two-fold increase in kinase activity compared to that of the parental *tpk2Δ/tpk2Δ* (65 vs. 137 pmoles P incorporated min^{-1} mg^{-1} respectively). These results are in line with our previous findings showing that in the homozygous *tpk* mutants the loss of one catalytic isoform is not compensated by overexpression of the other (Souto et al., 2006); and that Tpk2p isoform is the responsible for most of PKA enzymatic activity (Cloutier et al., 2003; Souto et al., 2006).

Fig. 1 also shows that *TPK1* expression from the constitutive *ACT1* promoter in a background of low kinase activity (*tpk2Δ/tpk2Δ* strain) led to two-fold increase of the activity in comparison with the parental strain *tpk2Δ/tpk2Δ* (from 65 to 145 pmoles P incorporated min^{-1} mg^{-1}); whereas *TPK2* expression in the *tpk2Δ/tpk2Δ* strain resulted in four-fold increase of the kinase activity (65 vs. 255 pmoles P incorporated min^{-1} mg^{-1}). It is worthwhile to mention that the kinase level detected in the reintegrated *tpk2Δ/tpk2Δ::TPK2* strain nor that of the *tpk2Δ/tpk2Δ::pACT1-TPK2* never reached that of the heterozygous *TPK2/tpk2Δ* strain, and although

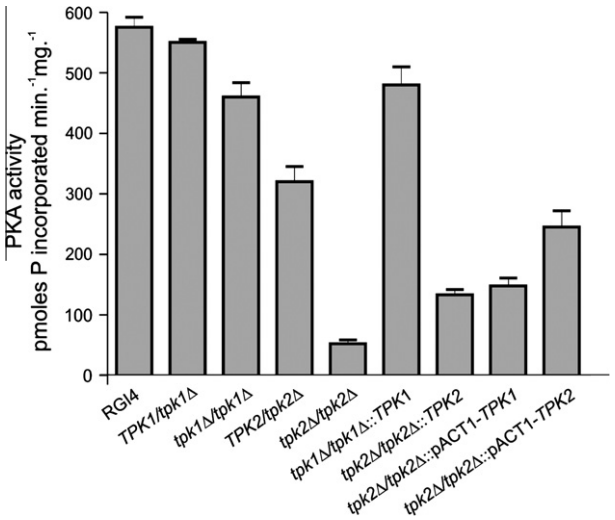

Fig. 1. PKA specific activity of soluble extracts from *TPK* reintegrated strains *tpk1Δ/tpk1Δ::TPK1*, *tpk2Δ/tpk2Δ::TPK2* and overexpressing mutants *tpk2Δ/tpk2Δ::pACT1-TPK1*, *tpk2Δ/tpk2Δ::pACT1-TPK2* in comparison to control *TPK1/tpk1Δ*, *tpk1Δ/tpk1Δ*, *TPK2/tpk2Δ*, *tpk2Δ/tpk2Δ* and wild type RGI4 strains. Phosphotransferase activity measured in soluble extracts from stationary phase cells in the presence of 10 μM cAMP as described in Materials and methods. Values are means ± SD from five independent experiments.

TPK1 and *TPK2* were placed under the actin constitutive strong promoter the expression levels of both *TPK* were quite moderate probably due to a regulatory mechanism responsible for maintaining optimal concentration of catalytic subunits.

In the *tpk1Δ/tpk1Δ::TPK1* mutant, we did not detect any significant variation in the level of cAMP binding activity (Fig. 2, panel A) nor in Bcy1p levels (panels B and C) in comparison to its parental strain *tpk1Δ/tpk1Δ*, a result quite expected since the contribution of Tpk1p to the total PKA activity is very low (Souto et al., 2006). In agreement with our previous results, strain *tpk2Δ/tpk2Δ* presented less than half of the cAMP binding activity levels of the wild type RGI4 strain (7.5 vs. 23 pmoles [^{3}H] cAMP incorporated min^{-1} mg^{-1} respectively), reinforcing the notion of the downregulation of *BCY1* in this genetic background. However in the *tpk2Δ/tpk2Δ::TPK2* restored strain, cAMP binding and Bcy1p levels increased two-fold compared to those of parental *tpk2Δ/tpk2Δ*. The same results were obtained with strains expressing *TPK1* since in *tpk2Δ/tpk2Δ::pACT1-TPK1* binding and Bcy1p expression augmented moderately, while in *tpk2Δ/tpk2Δ::pACT1-TPK2* strain higher levels of the regulatory subunit were expressed. We conclude that cells compensate the increased levels of either Tpk1p or Tpk2p subunits by elevation of Bcy1p levels. Yet could cells balance holoenzyme formation under increased expression of *BCY1*?

3.2. Heterozygous disruption of TPK1 in the strain tpk2Δ/tpk2Δ BCY1/bcy1Δ revealed a novel mechanism of regulation of the activity of Tpk1p

Since *BCY1* reintegration in its own locus or expression from a different locus in a high phosphotransferase activity background were unsuccessful, we performed the heterozygous disruption of *C. albicans TPK1* in the strain *tpk2Δ/tpk2Δ BCY1/bcy1Δ*, which has limited amounts of regulatory subunit in a low phosphotransferase background (Giacometti et al., 2006) obtaining the mutant strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* (strain RGS3, Table 1). Viability of this strain was analyzed in liquid YPD medium at 30 °C (Fig. 3, panel A), the growth curve of strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* showed a lag phase that endured more than 30 h, being also unable to reach the same OD of its parental strain *tpk2Δ/tpk2Δ BCY1/bcy1Δ* and controls *tpk2Δ/tpk2Δ* and *tpk2Δ/tpk2Δ TPK1/tpk1Δ* after 72 h of incubation; not even that of double mutant *tpk2Δ/tpk2Δ bcy1Δ/bcy1Δ*. The *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* mutant also presented a very defective phenotype in comparison to its parental *tpk2Δ/tpk2Δ BCY1/bcy1Δ* and to strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ*, including an atypical reddish colony color and the inability to germinate under any of the conditions tested (see Supplementary data and Supplementary Figs. 1 and 2), suggesting that in a background of very low PKA activity partial regulation limits morphogenesis.

In order to investigate the possible relationship between phosphotransferase activity and the severe phenotype of *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* mutant strain, PKA specific activity and cAMP dependence were assessed in soluble extracts from stationary yeast cells, stage at which maximal specific activity occurs (Souto et al., 2006). Kinase activity was measured in strains *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* and in *tpk2Δ/tpk2Δ TPK1/tpk1Δ* carrying both *BCY1* alleles. Strains *tpk2Δ/tpk2Δ, tpk2Δ/tpk2Δ BCY1/bcy1Δ* and *tpk2Δ/tpk2Δ bcy1Δ/bcy1Δ* were used as controls. The PKA specific activity could be accurately measured in all strains (Fig. 3, panel B) since phosphotransferase activity could be completely inhibited by the inclusion of PKI in the assay (not shown). The kinase activity levels of the control strains were within the expected values according to their genotypes (Fig. 3, panel B). However, it was repeatedly observed that mutant *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* had a significantly diminished specific activity at the stationary phase. Since the mutant *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* has only one copy of *TPK1*, the total Tpk1p specific activity in the presence of cAMP was expected to be similar to that of *tpk2Δ/tpk2Δ TPK1/tpk1Δ*, but it was 3.8 fold lower (9 vs. 35 pmoles min^{-1} mg^{-1}, respectively). This low specific activity was not related to a difference in total protein content in the extract (not shown). The lower specific kinase activity of the strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* could be due to the fact that the loss of one *BCY1* allele downregulates the levels of Tpk1p.

Since *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* mutant had difficulties in entering the stationary stage of growth (Fig. 3, panel A), it was of interest to test kinase specific activity of this strain at different time points of the vegetative growth in comparison to other strains used as controls (Fig 3, panel C). We unexpectedly found that at mid-log phase of growth (24 h) of the *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* strain the phosphotransferase specific activity (in the presence of cAMP) vastly increased, in comparison with control strains at the same growth stage (6 h). To investigate if the rise in the kinase specific activity of the mutant *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* was due to an increase in *TPK1* expression we evaluated *TPK1* mRNA as well as the protein level along the growth curve. As can be seen, in Fig. 4, panels A, B and C we could not detect any significant increase of *TPK1* mRNA nor in Tpk1 protein at this stage of growth. These results suggest that a novel mechanism of regulation of the activity of Tpk1p, besides cAMP could be operating at this stage.

3.3. Expression of BCY1 increased the levels of Tpk1 protein and its catalytic activity

From the generated strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* we obtained two derived strains in which a wild type copy of *BCY1* and *TPK1* could be independently placed under their own promoters inserted in the *RPS10* neutral locus. Their biochemical features are shown in Fig. 5. The new *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* CIp10-*BCY1* and *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* CIp10-*TPK1* strains presented normal growth rates (Fig. 5, panel A) and similar morphogenetic behavior to those of strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ* and to *tpk2Δ/tpk2Δ BCY1/bcy1Δ*, respectively (data not shown).

As part of the characterization of the new strains, PKA activity measured in the absence or presence of cAMP (Fig. 5, panel B) revealed a large increase in kinase activity in the CIp10-*TPK1* strain. More notably in the CIp10-*BCY1* strain it was observed an unexpected raise in PKA activity. This result further supports a cross regulation between catalytic and regulatory subunits; and could explain the failure in obtaining a CIp10-*BCY1* strain in a high phosphotransferase background assuming that the cell could not cope with exceedingly high kinase activity.

C. albicans PKA regulatory subunit belongs to type II class (Zelada et al., 1998) due to the presence of a serine residue (serine 124) susceptible to phosphorylation by the catalytic subunit at the inhibitory site (Zelada et al., 2002). To fully confirm the existence of a crossregulation between *BCY1* and *TPK1* we assessed the degree of Bcy1p phosphorylation in the defective mutant *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* as well as in the versions expressing CIp10-*TPK1* and CIp10-*BCY1*. The regulatory subunit from the strains was enriched from the S100 extract by cAMP-agarose column in the presence of a phosphatase inhibitor cocktail and phosphorylation of Ser124 was revealed with anti-phospho-PKA substrate (RRXS/T) antibody, while total amounts of regulatory subunit were assessed with the anti-Bcy1p antiserum (Fig. 5, panel C). Lanes 1 to 3 showed controls in which protein extracts were preincubated with alkaline phosphatase in the absence of phosphatase inhibitors; as expected no phosphorylation was detected. It was observed an increase in Bcy1p and its degree of phosphorylation in the *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* CIp10-*BCY1*

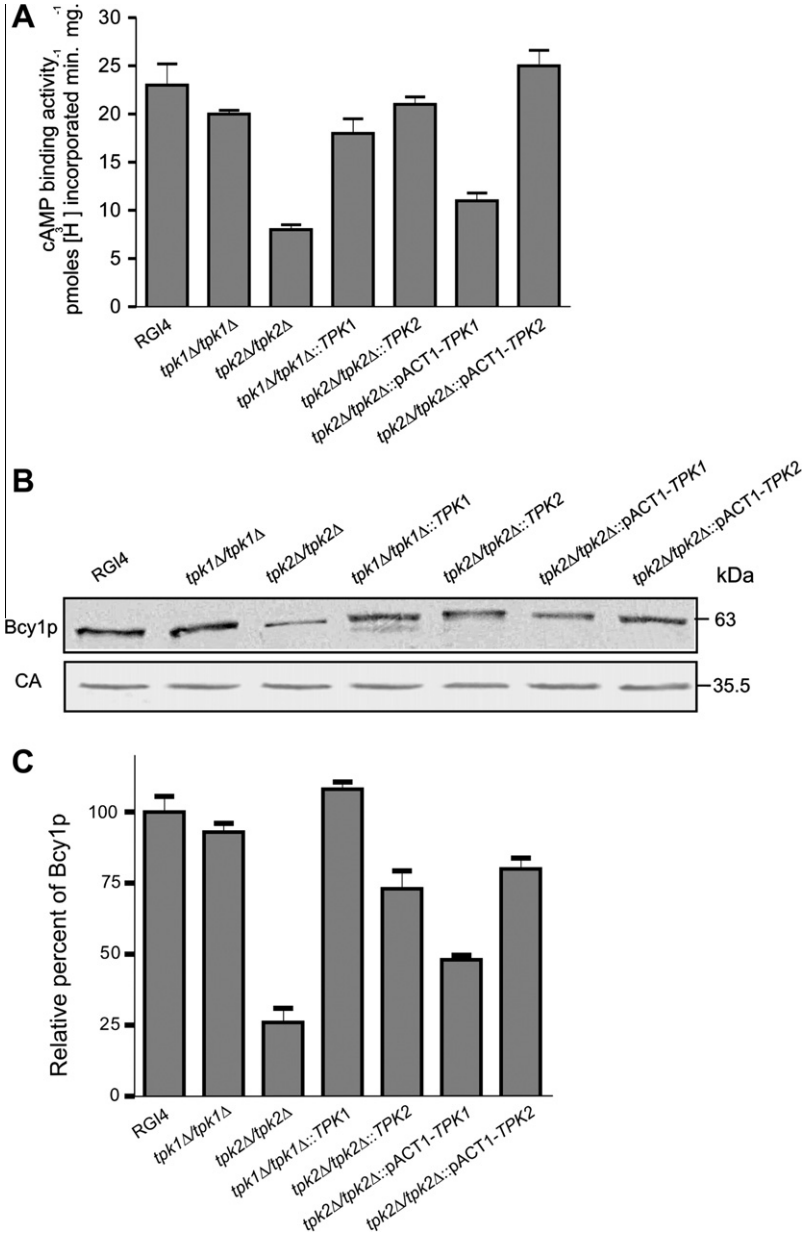

Fig. 2. cAMP binding activity of soluble extracts and Bcy1p levels from *tpk1Δ*/*tpk1Δ*::*TPK1*, *tpk2Δ*/*tpk2Δ*::*TPK2*, *tpk2Δ*/*tpk2Δ*::*pACT1-TPK1*, *tpk2Δ*/*tpk2Δ*::*pACT1-TPK2* in comparison to control RGI4, *tpk1Δ*/*tpk1Δ* and *tpk2Δ*/*tpk2Δ* strains. (A) [^{3}H] cAMP binding activity from soluble extracts. (B) Detection of Bcy1p levels by Western blot analysis. Soluble extracts from stationary phase cells (1 μg protein) were resolved in a 10% SDS-PAGE, transferred to PVDF membranes and developed with anti-*C. albicans* Bcy1p antiserum as described in Materials and methods. The molecular masses of Bcy1p and carbonic anhydrase (CA) are indicated on the right. (C) Densitometry scanning of the blots. Immunoblots were quantified using the GELBASE and SOL (UVPInc.) program. To allow comparison of the samples, data in panel B were expressed as a percentage of the immunoreactive blot detected for wild type strain RGI4, arbitrarily set to 100%. Values are means ± SD from six independent experiments.

strain (panel C, lanes 5 and 8 and panel D) in comparison to its parental *tpk2Δ*/*tpk2Δ* *TPK1*/*tpk1Δ* *BCY1*/*bcy1Δ* strain (panel C, lanes 4 and 7 and panel D), a result in line with the observed upsurge in phosphotransferase activity in CIp10-*BCY1* strain (panel B). We also observed an increase in the levels of Bcy1p and its phosphorylation in the *tpk2Δ*/*tpk2Δ* *TPK1*/*tpk1Δ* *BCY1*/*bcy1Δ* CIp10-*TPK1* strain (panel C, lanes 6 and 9 and panel D), validating our hypothesis on the cross regulation of Bcy1p and Tpk1p expression.

We also tested the cAMP binding capacity of these two strains in comparison to other strains with a high or low kinase background and different degree of *BCY1* regulation (Fig. 6, panel A) as a further biochemical proof of the co-regulated expression of PKA components. The strain lacking kinase regulation, *tpk2Δ*/*tpk2Δ* *bcy1Δ*/*bcy1Δ* mutant was included as a negative control in this assay. We found a very low level of binding activity in the defective mutant *tpk2Δ*/*tpk2Δ* *TPK1*/*tpk1Δ* *BCY1*/*bcy1Δ* in comparison to its *BCY1* isogenic parental strain (*tpk2Δ*/*tpk2Δ* *BCY1*/*bcy1Δ*) that correlated with the low levels of Bcy1p detected as shown in panels B and C. As expected the CIp10-*BCY1* strain exhibited higher levels of cAMP binding activity and of Bcy1p compared to *tpk2Δ*/*tpk2Δ* *TPK1*/*tpk1Δ* *BCY1*/*bcy1Δ* strain, however it should be mentioned that although the construct of CIp10-*BCY1* is under the control of the *BCY1* promoter, Bcy1p levels were higher than expected, being also slightly higher than those detected in the *tpk1Δ*/*tpk1Δ* mutant and in the wild type RGI4 strain. We observed this expression increase in several isogenic clones of CIp10-*BCY1* strain (data not shown). Although we could not attribute it to

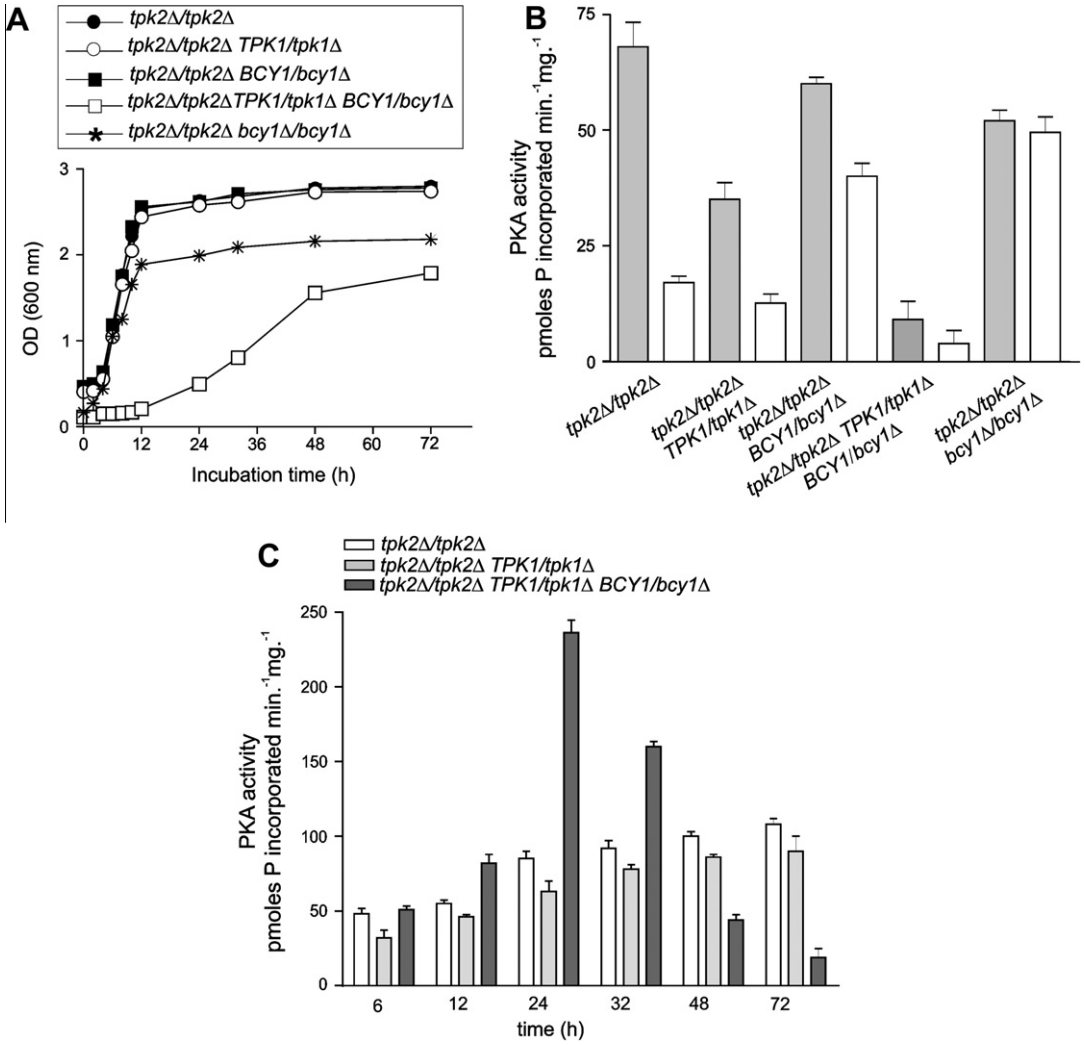

Fig. 3. Effect of *TPK1* heterozygous deletion on *tpk2*Δ/*tpk2*Δ *BCY1*/*bcy1*Δ mutant's viability and PKA specific activity of soluble extracts from *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ and from control strains *tpk2*Δ/*tpk2*Δ, *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ, *tpk2*Δ/*tpk2*Δ *BCY1*/*bcy1*Δ and *tpk2*Δ/*tpk2*Δ *bcy1*Δ/*bcy1*Δ. (A) Strains were grown in liquid YPD at 30 °C and the density of the cultures was measured at 600 nm at different time points. (B) Phosphotransferase activity and cAMP dependence were measured in soluble extracts from stationary phase cells (72 h for the defective mutant and 48 h for the other strains) in the presence (gray bars) and in the absence (empty bars) of 10 μM cAMP as described in Materials and methods. (C) PKA specific activity during vegetative growth measured in soluble extracts from *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ, *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ and control strain *tpk2*Δ/*tpk2*Δ at 6, 12, 24, 32, 48 and 72 h of growth in liquid YPD at 30 °C. Kinase activity was measured in the presence of 10 μM cAMP as described in Materials and methods. Values are means ± SD from three independent experiments. PKA specific activity of RGI4 strain was 598 (not shown).

any particular cause, we are confident in discarding the in tandem insertion of the construct (data not shown).

It is important to emphasize that we detected an increase in binding activity and Bcy1p levels in the CIp10-*TPK1* strain in comparison to the defective mutant *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ, a result conducive to our idea of the balanced expression of PKA components.

The results shown above indicating *BCY1* and *TPK* cross regulation were addressed at the protein level, cAMP binding capacity and PKA specific activity. To investigate if these cross regulation events were at the transcriptional level, we measured mRNA levels of PKA coding genes in the whole set of strains by semi-quantitative RT-PCR. Fig. 7, panels A and B showed that all strains of *tpk2*Δ/*tpk2*Δ genotype exhibited lower *BCY1* levels, validating our previous results (Giacometti et al., 2006, 2009). We also observed that mutant *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ exhibited a significant diminished of *BCY1* mRNA levels in comparison to its parental strain *tpk2*Δ/*tpk2*Δ *BCY1*/*bcy1*Δ. The CIp10-*TPK1* version showed not only an increase in *TPK1* but also in *BCY1* levels, a gene that was not modified in this strain. Similar results were obtained with the CIp10-*BCY1* mutant, in which the levels of *TPK1* transcript were 2-fold higher in comparison with those of its parental *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ.

Altogether these results suggest that a transcriptional regulatory mechanism between *TPK1* and *BCY1* could be operating to restore kinase homeostasis.

4. Discussion

Very little is known about the cross regulation of gene isoforms in *C. albicans*. In this regard a cross regulation among the different isoforms of mannosyltransferases, has been demonstrated since lack of one of them influenced transcript levels of the others, both positively and negatively, suggesting that cross-regulation events operate in *C. albicans* (Cantero et al., 2007).

In this work we explored this possibility with an appropriate set of PKA mutants. We showed that in a set of strains having a *TPK* allele reintegrated in its own locus, as well as in strains expressing one of the *TPK*s under the control of the *ACT1* promoter elicited

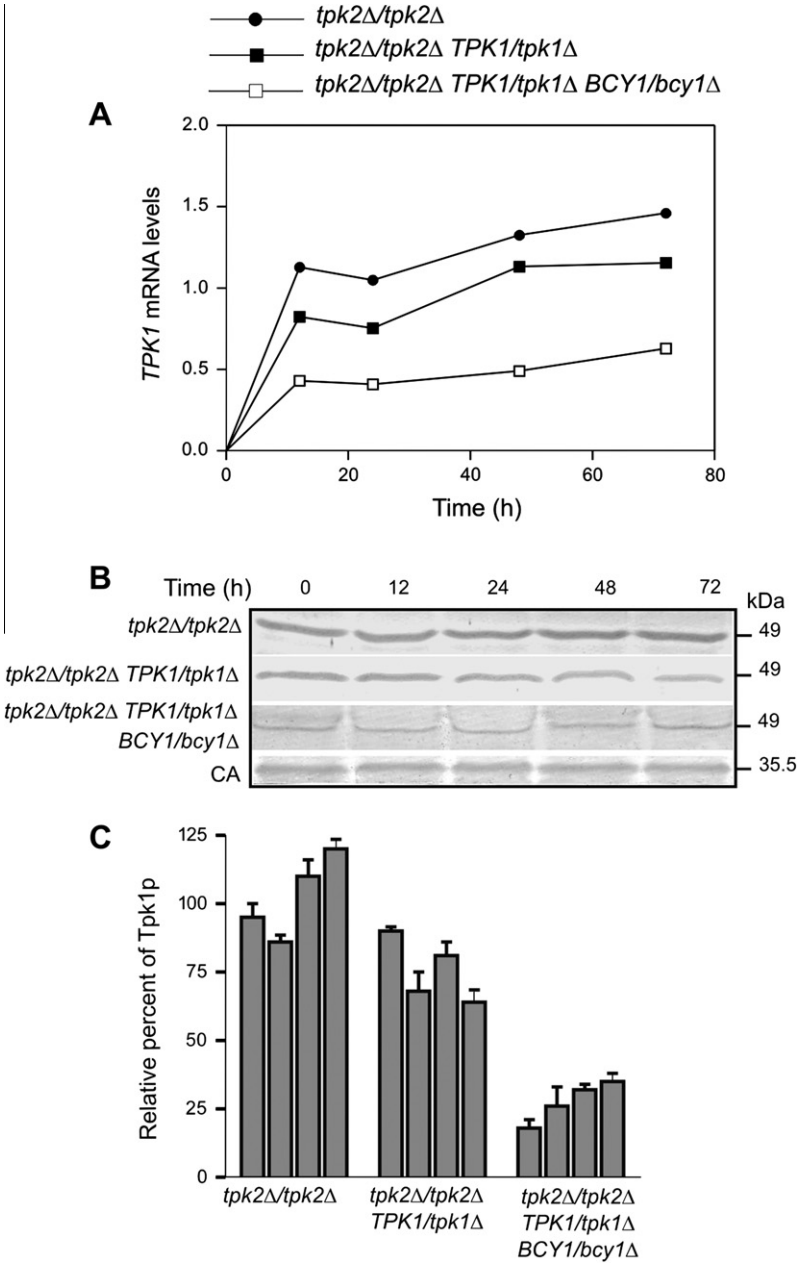

Fig. 4. *TPK1* mRNA and Tpk1p levels during vegetative growth of *tpk2*Δ mutants. (A) Fluctuations of *TPK1* transcript along growth curve of *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ, *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ and control strain *tpk2*Δ/*tpk2*Δ. RT-PCR data were expressed in arbitrary units and values were normalized to 18*S* rRNA. (B) Comparison of Tpk1p levels in the mutant strain *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1* and *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ with those of parental strain *tpk2*Δ/*tpk2*Δ by Western blot analysis. Crude extracts from stationary phase cells were resolved in a 10% SDS-PAGE, transferred to PVDF membranes and developed with anti-*C. albicans* Tpk1 antiserum as described in Materials and methods. The molecular masses of Tpk1p and carbonic anhydrase (CA) are indicated on the right. (C) Densitometry scanning of the blots. Immunoblots were quantified using the GELBASE and SOL (UVPInc.) program. To allow comparison of the samples, data in panel B were expressed as a percentage of the immunoreactive blot detected at time 0 h for parental strain *tpk2*Δ/*tpk2*Δ, arbitrarily set to 100%. Values are means ± SD from six independent experiments.

the upregulation of Bcy1p expression (Figs. 1 and 2). It was found that higher levels of Bcy1p were observed when *TPK2* was the isoform reintegrated or expressed (Fig. 2). These results are in line with previous reports showing that in *C. albicans* strains lacking both *TPK2* alleles *BCY1* mRNA and protein expression are strongly downregulated in comparison with the slightly decrease observed in a *tpk1*Δ/*tpk1*Δ strain (Giacometti et al., 2006, 2009). We can conclude that the up or downregulation of *BCY1* is related to the presence or the absence of the more abundant Tpk2p isoform.

We also evaluated the possibility that an increase in Bcy1p could regulate Tpk1p expression and vice versa. To do so, we cloned the *BCY1* and *TPK1* ORFs with their own promoters in the CIp10 vector (Murad et al., 2000). Starting from a new strain *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ we obtained two derived strains in which the CIp10-*BCY1* or a CIp10-*TPK1* construct were placed 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 (Figs. 5 and 6), suggesting cells attempt to maintain the normal status of substrate phosphorylation through a still unknown transcriptional or translational mechanism.

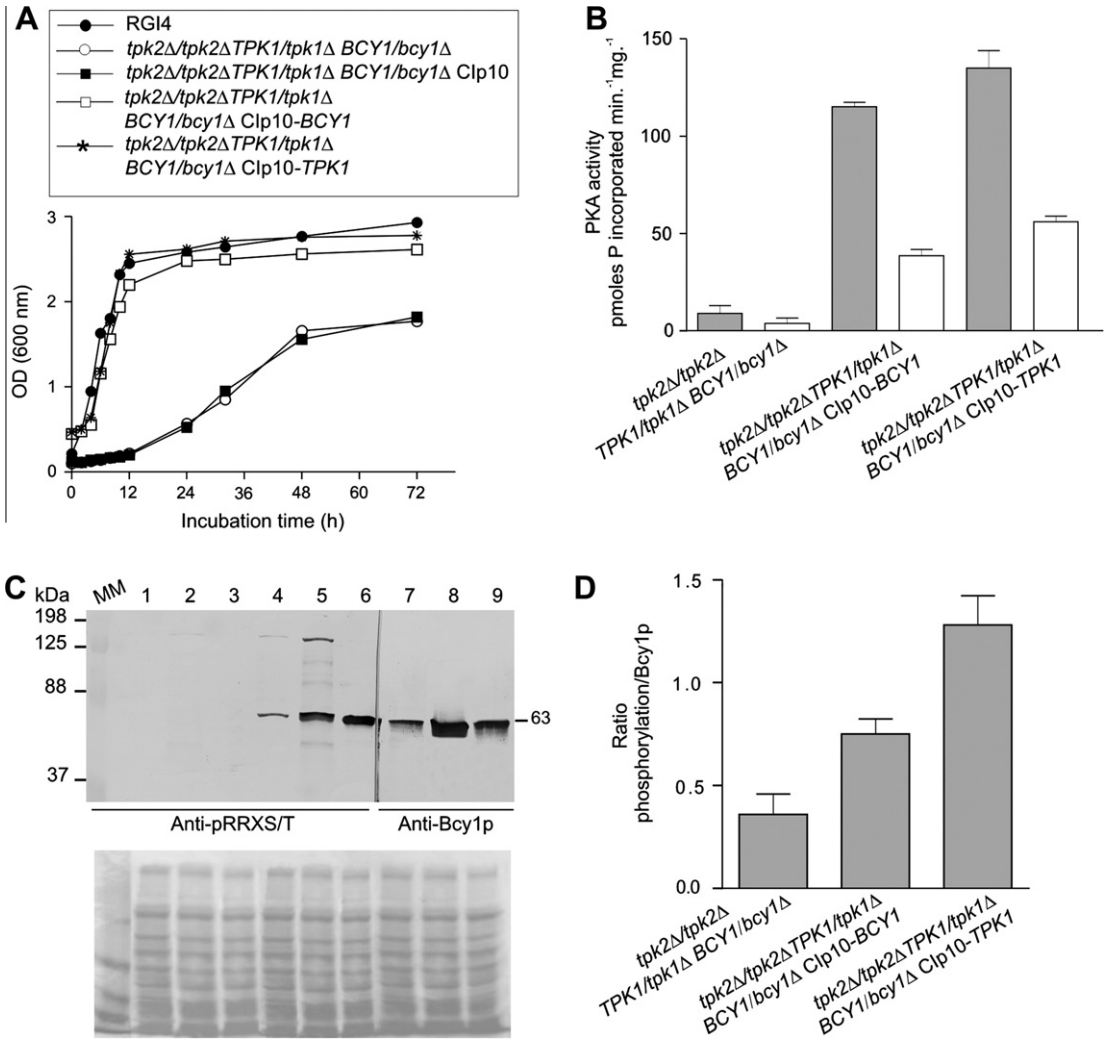

Fig. 5. Cell viability, PKA specific activity and *in vivo* autophosphorylation of Bcy1p of soluble extracts from *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* and from derived strains *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10-*BCY1* and *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10-*TPK1*. (A) Strains were grown in liquid YPD at 30 °C and the density of the cultures was measured at 600 nm at different time points. Strains *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10 (RGS3.1C) and wild type RGI4 were included as controls. (B) Phosphotransferase activity and cAMP dependence measured in soluble extracts from stationary phase cells (72 h for the defective mutant and 48 h for the other strains) in the presence (gray bars) and in the absence (empty bars) of 10 μM cAMP as described in Materials and methods. Values are means ± SD from five independent experiments. PKA specific activity of RGI4 strain was 578 (not shown). (C) *In vivo* phosphorylation was assessed by purification of Bcy1p in the presence of phosphatase inhibitors from *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* (lanes 4 and 7), *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10-*BCY1* (lanes 5 and 8) and strain *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10-*TPK1* (lanes 6 and 9); and in the absence of phosphatase inhibitors plus 10U *E. coli* alkaline phosphatase (lane 1: *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ*, lane 2: Clp10-*BCY1* version and lane 3: Clp10-*TPK1* version). The samples were submitted to 12% SDS-PAGE, blotted to a PVDF membrane, and phosphorylation was determined by immunoblot analysis with anti-phospho-PKA substrate (RRXS/T) antibody (lanes 1–6) and specific anti-*C. albicans* Bcy1p antiserum (lanes 7–9). The image below corresponds to the Ponceau S staining of the membrane as a loading and transfer control. (D) Ratio of phosphorylated Bcy1p to Bcy1p content assessed by densitometry scanning of the blots.

In previous works we have provided evidence that a tight regulation of PKA activity is necessary for true hyphal growth, since mutant cells devoid of the two alleles of *BCY1* gene, in a *tpk2Δ/tpk2Δ* background displayed pseudohyphal growth (Cassola et al., 2004), while mutant cells lacking one *BCY1* allele produced a mixture of true hyphae and pseudohyphae (Giacometti et al., 2006, 2009). We also found that heterozygous and homozygous *BCY1* mutants were highly sensitive to heat treatment (Giacometti et al., 2006). It is possible that these phenotypes are consequence of a down regulation of *TPK1* mRNA transcription.

In the *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* strain we unexpectedly found a significant increment in specific kinase activity at mid-logarithmic phase (24 h) of growth (Fig. 3), which could not be explained by an increase of *TPK1* mRNA nor in Tpk1 protein (Fig. 4). PKA regulation is given by multiple factors including the subcellular localization, the levels of cAMP, and the presence of a high affinity PKA inhibitor, PKI, which is found in many eukaryotes but it is absent in fungal genomes of *S. cerevisiae* (http://www.yeastgenome.org/) and of *C. albicans* (http://www.candidagenome.org/). However, in the absence of a regulatory subunit or PKI, the activity of the catalytic subunit is not known to be regulated. Our present results are compatible with a model in which Tpk1p activity, known to be modulated by cAMP, is also regulated by a second still uncharacterized mechanism.

All PKA catalytic subunits require a phosphorylation at the activation loop site. Since the PKA catalytic subunits tested readily autophosphorylate in bacteria, it was widely considered that this phosphorylation is an autophosphorylation event (Yonemoto et al., 1997). However, there is evidence that PKA catalytic subunits can be phosphorylated by different protein kinases (Cauthron et al., 1998). One could be the phosphoinositide-dependent protein kinase 1, PDK1, well known as the activator of AGC kinases (Moore

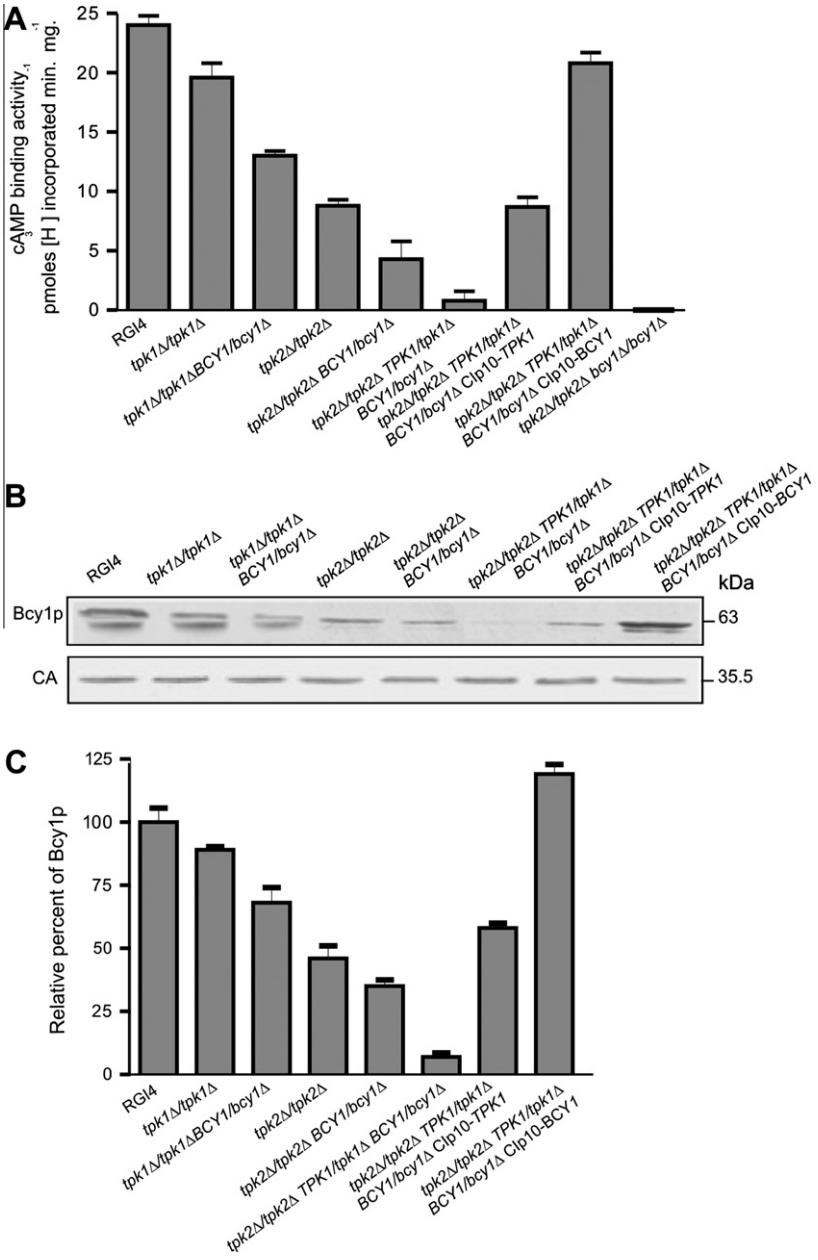

Fig. 6. cAMP binding activity of soluble extracts and Bcy1p levels from wild type RGI4, *tpk1Δ/tpk1Δ*, *tpk1Δ/tpk1Δ BCY1/bcy1Δ*, *tpk2Δ/tpk2Δ*, *tpk2Δ/tpk2Δ BCY1/bcy1Δ*, *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ*, *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10-*TPK1*, *tpk2Δ/tpk2Δ TPK1/tpk1Δ BCY1/bcy1Δ* Clp10-*BCY1* and *tpk2Δ/tpk2Δ bcy1Δ/bcy1Δ* mutant strains. (A) [^{3}H] cAMP binding activity from soluble extracts. (B) Detection of Bcy1p levels by Western blot analysis. Soluble extracts from stationary phase cells (1 μg protein) were resolved in 10% SDS-PAGE, transferred to PVDF membranes and developed with anti-*C. albicans* Bcy1p antiserum as described in Materials and methods. The molecular masses of Bcy1p and carbonic anhydrase (CA) are indicated on the right. (C) Densitometry scanning of the blots. Immunoblots were quantified using the GELBASE and SOL (UVPInc.) program. Double bands detected for Bcy1p corresponding to limited proteolysis were included in the quantification. To allow comparison of the samples, data in panel B were expressed as a percentage of the immunoreactive blot detected for wild type strain RGI4, arbitrarily set to 100%. Values are means ± SD from five independent experiments.

et al., 2002). As previously reported by Biondi et al. (2001) docking of substrates to PDK1 is mediated by binding of a hydrophobic motif (PIF) to an allosteric site on PDK1 (termed "PIF-binding pocket"). Since three ORFs coding for putative PDKs are present in the *C. albicans* genome (http://www.candidagenome.org/), and the putative hydrophobic sequence of interaction with PDK is present in the C-terminal deduced amino acid sequence of Tpk1p and Tpk2p, the possibility that Tpk1p activity in *C. albicans* might also be regulated by PDK1 is an attractive hypothesis. A recent report of Voordeckers et al. (2011) revealed that in *S. cerevisiae* Tpk1p is phosphorylated by Pkh1p, the yeast ortholog of mammalian PDK1, and that this phosphorylation occurs during or shortly after synthesis of the PKA catalytic subunit. Mutagenesis of the PDK1 phosphorylation site in Tpk1p abolishes binding of the regulatory subunit and cAMP dependency. It is tempting to speculate that a similar mechanism is operating in *C. albicans*. Studies on this subject are now in progress in our laboratory.

In this work we performed the determination of the phosphotransferase activity in strains with diverse expression levels of *TPK* genes under different promoters, which was not accomplished before. The kinase activity data reinforced previous findings showing that the *TPK2* transcript is the most abundant, while *TPK1* transcript levels are low (Souto et al., 2006). Increased Bcy1p levels consistent with a higher cAMP binding capacity were observed in

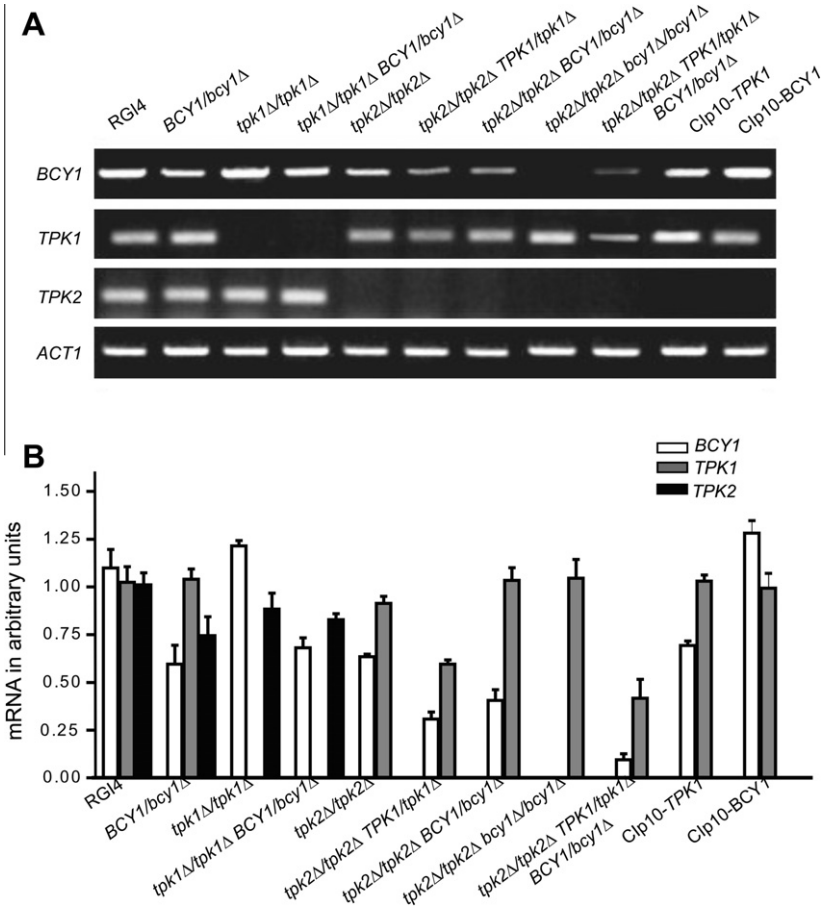

Fig. 7. Semi-quantitative RT-PCR analyses of *TPK* and *BCY1* mRNAs. (A) Agarose gels were stained with ethidium bromide to visualize *BCY1*, *TPK1* and *TPK2* transcripts from the stationary phase of wild-type RGI4 and mutant strains. (B) RT-PCR data were expressed in arbitrary units and values were normalized to actin (*ACT1*) transcript as described in Materials and methods.

mutants whether a wild type copy of *TPK2* was expressed from its own promoter or from the *ACT1* promoter. Another finding was the fact that expression of Clp10-*BCY1* (which could only be achieved in a very low kinase activity background) just as in the *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ *BCY1*/*bcy1*Δ mutant, altered positively the Tpk1p levels and therefore its activity. These results suggest a complex interdependence of the biosynthesis of Tpk isoforms as well as of the regulatory subunit Bcy1p, whose molecular basis remains to be established.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.12.001.

References

Abramoff, M.D., Magelhaes, P.J., Ram, S.J., 2004. Image processing with image. J. Biophoton. Int. 11, 36–42.
Adams, A., Gottschling, D.E., Kaiser, C.A., Stearns, T., 1997. In: Dickerson, M.M. (Ed.), Techniques and Methods in Yeast Genetics. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 9–102.
Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Struhl, K., 1994. In: Chanda, V.B. (Ed.), Current Protocols in Molecular Biology. John Wiley and Sons, NY.
Bahn, Y.-S., Sundstrom, P., 2001. *CAP1*, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth, and cyclic AMP levels and is requires for virulence of *Candida albicans*. J. Bacteriol. 183, 3211–3223.
Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M., Alessi, D.R., 2001. The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. EMBO J. 20, 4380–4390.
Bockmühl, D.P., Krishnamurthy, S., Gerads, M., Sonneborn, A., Ernst, J.F., 2001. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. Mol. Microbiol. 42, 1243–1257.
Bruno, K.S., Aramayo, R., Minke, P.F., Metzenberg, R.L., Plamann, M., 1996. Loss of growth polarity and mislocalization of septa in a *Neurospora* mutant altered in the regulatory subunit of cAMP-dependent protein kinase. EMBO J. 15 (589), 5772–5782.
Cantero, P.D., Lengsfeld, C., Prill, S.K., Subanović, M., Román, E., Pla, J., Ernst, J.F., 2007. Transcriptional and physiological adaptation to defective protein-O-mannosylation in *Candida albicans*. Mol. Microbiol. 64, 1115–1128.
Cassola, A., Parrot, M., Silberstein, S., Magee, B.B., Passeron, S., Giasson, L., Cantore, M.L., 2004. *Candida albicans* lacking the gene encoding the regulatory subunit of the cAMP-dependent protein kinase displays a defect in hyphal formation and an altered localization of the catalytic subunit. Eukaryot. Cell 3, 190–199.
Cauthron, R.D., Carter, K.B., Liauw, S.Steinberg, Steinberg, R.A., 1998. Physiological phosphorylation of protein kinase A at Thr-197 is by a protein kinase A. Mol. Cell. Biol. 18, 1416–1423.

Cloutier, M., Castilla, R., Bolduc, N., Zelada, A., Martineau, P., Bouillon, M., Magee, B.B., Passeron, S., Giasson, L., Cantore, M.L., 2003. The two isoforms of the cAMP-dependent protein kinase catalytic subunit are involved in the control of dimorphism in the human fungal pathogen *Candida albicans*. Fungal Gen. Biol. 38, 133–141.

Cutler, N.S., Pan, X., Heitman, J., Cardenas, M.E., 2001. The TOR signal transduction cascade controls cellular differentiation in response to nutrients. Mol. Biol. Cell 12, 4103–4113.

Davis, D.A., Bruno, M.V., Lozab, L., Filler, S.G., Mitchell, A.P., 2002. *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. Genetics 162, 1573–1581.

Fonzi, W.A., Irwin, M.Y., 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 134, 717–728.

Giacometti, R., Souto, G., Silberstein, S., Giasson, L., Cantore, M.L., Passeron, S., 2006. Expression levels and subcellular localization of Bcy1p in *Candida albicans* mutant strains devoid of one *BCY1* allele results in a defective morphogenetic behavior. Biochim. Biophys. Acta 1763, 64–72.

Giacometti, R., Kronberg, F., Biondi, R.M., Passeron, S., 2009. Catalytic isoforms Tpk1 and Tpk2 of *Candida albicans* PKA have non-redundant roles in stress response and glycogen storage. Yeast 26, 273–285.

Giacometti, R., Kronberg, F., Biondi, R.M., Passeron, S., 2011. *Candida albicans* Tpk1p and Tpk2p isoforms differentially regulate pseudohyphal development, biofilm structure, cell aggregation and adhesins expression. Yeast 28, 293–308.

Huang, G., Yi, S., Sahni, N., Daniels, K.J., Srikantha, T., Soll, D.R., 2010. N-Acetylglucosamine induces white to opaque switching, a mating prerequisite in *Candida albicans*. Plos. Pathog. 6, e1000806.

Jung, W.H., Warn, P., Ragni, E., Popolo, L., Nunn, C.D., Tumer, M.P., Stateva, L., 2005. Deletion of *PDE2*, the gene encoding the high-affinity cAMP phosphodiesterase, results in changes of the cell wall and membrane in *Candida albicans*. Yeast 22, 285–294.

Kumamoto, C.A., Vinces, M.D., 2005. Contributions of hyphae and hypha-coregulated genes to *Candida albicans* virulence. Cell Microbiol. 7, 1546–1554.

Liu, H., 2001. Transcriptional control of dimorphism in *Candida albicans*. Curr. Opin. Microbiol. 4, 728–735.

Lo, H.J., Kohler, J.R., DiDomenico, B., Loebenber, D., Cacciapuoti, A., et al., 1997. Nonfilamentous *C. Albicans* mutants are avirulent. Cell 90, 939–949.

Lowry, O.H., Rosebrough, N.J., Farr, A., Randall, R.J., 1951. Protein measurements with the phenol reagent. J. Biol. Chem. 193, 265–275.

Mazón, M.J., Behrens, M.M., Morgado, E., Portillo, F., 1993. Low activity of the yeast cAMP-dependent protein kinase catalytic subunit Tpk3 is due to the poor expression of the *TPK3* gene. Eur. J. Biochem. 213, 501–506.

Monge, R.A., Román, E., Nombela, C., Pla, J., 2006. The MAP kinase signal transduction network in *Candida albicans*. Microbiology 152, 905–912.

Moore, M.J., Kanter, J.R., Jones, K.C., Taylor, S.S., 2002. Phosphorylation of the catalytic subunit of protein kinase A: autophosphorylation versus phosphorylation by phosphoinositide-dependent kinase-1. J. Biol. Chem. 277 (2002), 47878–47884.

Murad, A.M., Lee, P.R., Broadbent, I.D., et al., 2000. CIp10, an efficient and convenient integrating vector for *Candida albicans*. Yeast 16, 325–327.

Park, H., Myers, C.L., Sheppard, D.C., et al., 2005. Role of the fungal Ras protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell Microbiol. 7, 499–510.

Roskoski, R., 1983. Assay of protein kinase. Methods Enzymol. 99, 3–6.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sherman, F., Fink, G.R., Hicks, J.B., 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sonneborn, A., Bockmühl, D.P., Gerards, M., Kurpanek, K., Sanglard, D., Ernst, J.F., 2000. Protein kinase A encoded by *TPK2* regulates dimorphism of *Candida albicans*. Mol. Microbiol. 35, 386–396.

Souto, G., Giacometti, R., Silberstein, S., Giasson, L., Cantore, M.L., Passeron, S., 2006. Expression of *TPK1* and *TPK2* genes encoding PKA catalytic subunits during growth and morphogenesis in *Candida albicans*. Yeast 23, 591–603.

Staab, J.F., Bahn, Y.S., Sundstrom, P., 2003. Integrative, multifunctional plasmids for hypha-specific or constitutive expression of green fluorescent protein in *Candida albicans*. Microbiology 149, 2977–2986.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., Wigler, M., 1985. In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell 40, 27–36.

Uhler, M.D., McKnight, G.S., 1987. Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. 262, 15202–15207.

Vazquez, J.A., Sobel, J.D., 2003. Candidiasis. In: Dismukes, W.E., Pappas, P.G., Sobel, J.D. (Eds.), Clinical Mycology. Oxford University Press, Oxford, pp. 143–188.

Voordeckers, K., Kimpe, M., Haesendonckx, M., Louwet, W., Versele, M., Thevelein, J.M., 2011. Yeast 3-Phosphoinositide-Dependent Protein Kinase-1 (PDK1) orthologs, PKH1–3, differentially regulate phosphorylation of PKA and the PKB/S6K ortholog, SCH9. J. Biol. Chem. 286, 22017–22027.

Wilson, B., Davis, D., 1999. A Mitchell, Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. 181, 1868–1874.

Yonemoto, W., McGlone, M.L., Grant, B., Taylor, S.S., 1997. Autophosphorylation of the catalytic subunit of cAMP-dependent protein kinase in *Escherichia coli*. Protein Eng. 10, 915–925.

Zelada, A., Castilla, R., Passeron, S., Giasson, L., Cantore, M.L., 2002. Interactions between regulatory and catalytic subunits of the *Candida albicans* cAMPdependent protein kinase are modulated by autophosphorylation of the regulatory subunit. Biochim. Biophys. Acta 1542, 73–81.

Zelada, A., Passeron, S., Lopez Gomes, S., Cantore, M.L., 1998. Isolation and characterization of cAMP-dependent protein kinase from *Candida albicans*, purification of the regulatory and catalytic subunits. Eur. J. Biochem. 252, 245–252.