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Research Article

Catalytic isoforms Tpk1 and Tpk2 of *Candida albicans* PKA have non-redundant roles in stress response and glycogen storage

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Abstract

Candida albicans **cAMP-dependent protein kinase (PKA) is coded by two catalytic subunits (*TPK1* and *TPK2*) and one regulatory subunit (*BCY1*). In this organism the cAMP/PKA signalling pathway mediates basic cellular processes, such as the yeast-to-hyphae transition and cell cycle regulation. In the present study, we investigated the role of *C. albicans* PKA in response to saline, heat and oxidative stresses as well as in glycogen storage. To fine-tune the analysis, we performed the studies on several *C. albicans* PKA mutants having heterozygous or homozygous deletions of *TPK1* and/or *TPK2* in a different *BCY1* genetic background. We observed that *tpk1* Δ/*tpk1* Δ strains developed a lower tolerance to saline exposure, heat shock and oxidative stress, while wild-type and *tpk2* Δ/*tpk2* Δ mutants were resistant to these stresses, indicating that both isoforms play different roles in the stress response pathway. We also found that regardless of the *TPK* background, heterozygous and homozygous *BCY1* mutants were highly sensitive to heat treatment. Surprisingly, we observed that those strains devoid of one or both *TPK1* alleles were defective in glycogen storage, while strains lacking Tpk2 accumulated higher levels of the polysaccharide, indicating that Tpk1 and Tpk2 have opposite roles in carbohydrate metabolism. Copyright © 2009 John Wiley & Sons, Ltd.**

Keywords: ***Candida albicans*****; PKA; stress response; glycogen regulation**

Introduction

The dimorphic opportunistic pathogen *Candida albicans* is able to change its mode of growth from budding yeast (blastospores) to filamentous form (hyphae or pseudohyphae) in response to a wide variety of environmental signals (Sudbery *et al*., 2004). Filamentation and virulence are believed to be associated (Calderone and Fonzi, 2001; Odds *et al*., 2001); therefore, morphogenesis has been a subject of considerable research.

In *C. albicans*, several signalling pathways can regulate the yeast-to-hyphae transition (Whiteway and Oberholzer, 2004). Among them, the cAMP–PKA pathway plays a major role in hyphal development and virulence, since many mutants in the pathway are defective in the dimorphic process and show reduced virulence. Thus, complete inactivation of the pathway by disruption of the gene encoding adenylate cyclase (*CDC35*) causes severely defective phenotypes in both physiologically unperturbed and germ tube-inducing conditions (Rocha *et al*., 2001). The *cdc35* null mutants grow more slowly than wild-type cells in rich media and are severely defective in morphogenetic transitions under all environmental conditions (Rocha *et al*., 2001). Downstream, activation of PKA induces the formation of hyphal

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filaments. Results from our and other laboratories have established that both catalytic isoforms of PKA, Tpk1 and Tpk2 share growth functions (Bockmühl *et al*., 2001; Sonneborn *et al*., 2000; Souto *et al*., 2006) and have positive roles in filamentation (Leberer *et al*., 2001; Mallet *et al*., 2000), although some different functional specificities have been observed, depending on the morphogenesis-inducing conditions and on their capacity to promote agar invasion (Bockmühl *et al*., 2001). Tpk1 seems to mediate hyphal formation on solid media but is not required for agar invasion, while Tpk2 is needed for both hyphal development in liquid media and agar invasion. Furthermore, the homozygous *tpk1* Δ strain adheres to, invades and damages oral epithelial cells *in vitro* similarly to the wild-type strain. In contrast, homozygous *tpk2* Δ strain has reduced capacity to invade and damage oral epithelial cells, suggesting that Tpk2 may be important for governing virulence in oropharyngeal candidiasis (Park *et al*., 2005). In line with previous results, we have shown that the Tpk1 was related to the onset of germ-tube formation, since only an increase in *TPK1* expression was observed at this point (Souto *et al*., 2006). In addition, 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 the *BCY1* gene, coding for the regulatory PKA subunit, in a *tpk2* Δ/*tpk2* Δ 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).

Studies in *S. cerevisiae* have revealed that PKA activity is directly involved in the dimorphic shift and cell growth (Cameron *et al*., 1988; Gimeno *et al*., 1992), while carbon storage and the response to stress are mediated indirectly by PKA through the inhibition of STRE-dependent genes expression (Francois *et al*., 1992; Hardy *et al*., 1994; Varela *et al*., 1995). However, the role of *C. albicans* PKA in response to different stresses has not been thoroughly investigated. To address this point, we studied the response to saline, heat and oxidative stresses as well as glycogen accumulation in a series of *C. albicans* PKA mutant strains devoid of one or the two gene copies of *TPK1* or *TPK2* in a different *BCY1* genetic background. We observed that *tpk1* Δ/*tpk1* Δ strains developed a lower tolerance to saline exposure, heat shock and oxidative stress, while *tpk2* Δ/*tpk2* Δ mutant was resistant to these stresses, like the wild-type strain, indicating that each isoform plays different roles in the stress response pathway. We also found that heterozygous and homozygous *BCY1* mutants, irrespective of the *TPK* background, were highly sensitive to heat treatment. Opposite roles for Tpk1 and Tpk2 isoforms were observed in glycogen storage, suggesting that each of the isoforms has different substrates involved in glycogen regulation.

Materials and methods

Chemicals

Reagents were purchased as follows: kemptide (LRRASLG), PKA inhibitor (PKI) fragment (14–24), cAMP, anti-rabbit IgG (conjugated to alkaline phosphatase) were from Sigma Chemical Co.; phosphocellulose paper P-81 was from Whatman; [γ-^{32}P]ATP was from New England Nuclear; 'Complete mini' protease mix was from Roche; kaleidoscope polypeptide standards were from Bio-Rad; and polyvinylidene difluoride membranes were from Immobilon-P. All other chemicals were of analytical grade.

Strains, media and culture conditions

We performed the studies with both *URA3* and *ura3* newly generated *C. albicans* strains as well as with previously obtained *C. albicans* strains, as detailed in Table 1. Yeast cells were cultured at 30 °C in YPD (1% yeast extract, 2% peptone and 2% glucose). The identities of all strains and the characterization of each new strain, including those in which the *URA3* gene was re-established, were routinely verified by PCR. In comparison to their respective parental strains, prototroph *URA3* strains showed faster growth in liquid or solid media as well as more synchronized cell cultures than auxotrophic strains grown in uridine (50 μg/ml) supplemented media.

Germ-tube formation experiments were performed as described previously (Castilla *et al*., 1998) in two different liquid inducing media: minimal medium plus 10 mM GlcNAc (Shepherd *et al*., 1980) and Spider medium (Liu *et al*., 1994). Cultures were incubated at 37 °C with orbital agitation and samples were removed at various times up

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Table 1. *C. albicans* strains used in this study

Strain	Genotype	Source or reference
CAI4	*ura3*::λ*imm434*/*ura3*:: λ*imm434*	Fonzi and Irwin, 1993
RGI4	Same as CAI4 but *RPS1*/*rps1*Δ::Clp10	This study
LG65	Same as CAI4 but *BCY1*/*bcy1*Δ::*Cat*	Giacometti *et al*., 2006
RG65	Same as LG65 but *RPS1*/*rps1*Δ::Clp10	This study
RG11	Same as LG65 but *TPK1*/*tpk1*Δ::*URA3-dpl200*	This study
RG11.1	Same as RG11 but *TPK1*/*tpk1*Δ::*dpl200*	This study
R1U1	Same as CAI4 but *TPK1*/*tpk1*Δ::*URA3-dp1200*	This study
IIHH6-4a	Same as CAI4 but *tpk1*Δ::*hisG*/*tpk1*Δ::*hisG*	Bockmühl *et al*., 2001
RS1u	Same as IIHH6-4a but *RPS1*/*rps1*Δ::Clp10	This study
R2U2	Same as CAI4 but *TPK2*/*tpk2*Δ::*URA3-dp1200*	This study
H2D	Same as CAI4 but *tpk2*Δ::*Cat*/*tpk2*Δ::*Cat*	Cloutier *et al*., 2003
RS2u	Same as H2D but *RPS1*/*rps1*Δ::Clp10	This study
DB11.1	Same as CAI4 but *tpk2*Δ::*hisG*/*tpk2*Δ::*hisG TPK1*/*tpk1*Δ::*hisG*	Bockmühl *et al*., 2001
RS11u	Same DB11.1 but *RPS1*/*rps1*Δ::Clp10	This study
*tpk2*Δ/*tpk2*Δ *BCY1*/*bcy1*Δa	Same as H2D but *BCY1*/*bcy1*Δ::*Cat-URA3-Cat*	Laboratory collection
*tpk2*Δ/*tpk2*Δ *BCY1*/*bcy1*Δ	Same as H2D but *BCY1*/*bcy1*Δ::*Cat*	Cassola *et al*., 2004
*tpk2*Δ/*tpk2*Δ *bcy1*Δ/*bcy1*Δa	Same as H2D but *bcy1*Δ::*Cat*/*bcy1*Δ::*Cat-URA3-Cat*	Laboratory collection
*tpk2*Δ/*tpk2*Δ *bcy1*Δ/*bcy1*Δ	Same as *tpk2*Δ/*tpk2*Δ *bcy1*Δ/*bcy1*Δa but *bcy1*Δ::*Cat*/*bcy1*Δ::*Cat*	Cassola *et al*., 2004

to 2 h. Cell morphology was examined by light microscopy.

DNA manipulations

DNA purifications were performed using 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.

Heterozygous deletion of *C. albicans TPK1* and *TPK2*

C. albicans knockout of the *TPK1* or the *TPK2* gene was generated using the PCR-based adaptation (Wilson *et al*., 1999) of the sequential URA−Blaster technique (Fonzi and Irwin, 1993). PCR primers were designed to amplify the mini-URA3 cassette in pDDB57 plasmid (Wilson *et al*., 1999) tailed with 60−70 additional nucleotides corresponding to the flanking sequences of the open reading frame of the gene to be knocked out. Specific primers are listed in Table 2. Primers TPK1KO5/TPK1KO3 and TPK2KO5/TPK2KO3 were designed to generate a PCR deletion construct (*TPK1::dpl200-URA3-dpl200* and *TPK2::dpl200-URA3-dpl200*) that, following recombination, would precisely replace the coding sequence of the targeted gene with the coding sequence of *URA3* under control of its own promotor, providing the ability to grow in media lacking uridine. The PCR products were verified by gel electrophoresis of an aliquot of the reaction mixtures. The corresponding products of 10 PCR reactions were pooled and used to delete *TPK1* in strains CAI4 and *BCY1/bcy1*Δ (LG65) and *TPK2* in the CAI4 strain, following the protocol described (Wilson *et al*., 1999). This technique allowed us to obtain strains *TPK1/tpk1*Δ (R1U1), *TPK1/tpk1*Δ *BCY1/bcy1*Δ (RG11) and *TPK2/tpk2*Δ (R2U2), respectively. *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 or TPK2ver3).

To evaluate whether *URA3* auxotrophy affected the observed phenotypes, we performed all tests in the entire set of *URA3* as well as in the *ura3* mutants. To that end, the *URA3* marker was recycled by selection on SD medium plus 5-FOA (1 mg/ml) and uridine (50 μg/ml); and all *ura* mutants were transformed with *Stu*I-digested CIp10 plasmid (Murad *et al*., 2000), ensuring *URA3* expression at the neutral *RPS10* locus, in

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Table 2. Primers used in this study

Name	Sense	Sequence 5′ → 3′
TPK1KO5	Forward	GGAACCAGCAGACACAAGCATCA-GGTCATTAAACG ACATCAACTTACAAGAACTTGCCA-AGTTTTCCCAGT CACGACGTT
TPK1KO3	Reverse	GATAAAGATTTGGATTATGGTATA-AGTGGAGTTGAA GACCCATATCGTGATCAATTCCAT-GTGGAATTGTGA GCGGA
TPK2KO5	Forward	TCGGACAGTAATTCCTTAAACTCA-AACACATCAATG GACAATCATCAACAGTTTTCCCAG-TCACGACGTT
TPK2KO3	Reverse	ATCTTCTCCTTGGCTTCCGTAGTC-TAATTGTTCTTC TGGATAATGGTCAATGTGGAATT-GTGAGCGGA
URA3ver5	Forward	TTCCGAGCTTGGCGTAATCAT
TPK1ver3	Reverse	TAATACATAATAGTTCAATA
TPK2ver3	Reverse	GGGCCATAGCTAGTTTGAGTTT
RT1-BCY1	Forward	ATGTCTAATCCTCAACAACA
RT2-BCY1	Reverse	TTAATGACCAGCAGTTGG
RT1-TPK1	Forward	AGAAGTTCAAGATGTGACTTAT
RT2-TPK1	Reverse	ACAAGGTGGTTCTGATGATG
RT1-TPK2	Forward	GAAGTTAGTACCGTTACATGG
RT2-TPK2	Reverse	ACTGCTGATTTGACAAGAAG
RT1-ACT1	Forward	CCCAAGCTTGCCGGTGACGACGCT
RT2-ACT1	Reverse	GTGGTGAACAAATGGATGGACCA

order to avoid potential problems associated with ectopic expression of *URA3* (Brand *et al*., 2004).

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 analysed during the exponential phase of amplification. We performed reactions without reverse transcriptase as a control for the presence of contaminating DNA. A 900 bp PCR product amplified with RT1–ACT1 and RT2–ACT1 primers from *C. albicans ACT1* was used as internal mRNA loading control. Transcripts were quantified using ImageJ (Abramoff *et al*., 2004).

Crude extracts preparation and PKA activity measurement

Yeast cells ($1–2 \times 10^7$) from stationary phase were suspended in 500 μl 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM EGTA, 1 mM EDTA, 10 mM β-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 (0.1–0.5 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 square papers and dropped into 75 mM phosphoric acid for washing (Roskoski, 1983). PKA specific activity was expressed as pmoles of [γ-^{32}P] incorporated to kemptide/min/mg protein. Since strain *tpk2* Δ/*tpk2* Δ had low activity (Cloutier *et al*., 2003; Souto *et al*., 2006), to ensure accurate measurement of PKA activity in our set of *tpk2* Δ/*tpk2* Δ strains, the specific activity of [γ-^{32}P]ATP was raised to $2–5 \times 10^3$ cpm/pM in the assays. Also, 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. The measured activity was routinely checked for more than 80% inhibition by 20 μM PKI inhibitor.

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Protein determination

Protein concentration was determined with bovine serum albumin as standard (Lowry *et al*., 1951).

Western blot analysis

Bcy1 expression was assessed by Western blot analysis. Proteins from crude extracts were resolved by 10% SDS–PAGE according to Laemmli (1970) and transferred to PVDF membranes by semi-dry electroblotting. The blots were blocked with 5% non-fat dried milk and incubated overnight with anti-*C. albicans* Bcy1 antiserum generated in the laboratory (Zelada *et al*., 1998). Immunological detection was performed using anti-rabbit IgG conjugated to alkaline phosphatase. For Bcy1 expression level analysis loading and transfer were monitored by Ponceau S staining of the membranes. Pre-stained carbonic anhydrase was also included as a transference control.

Stress sensitivity tests

The evaluation of *C. albicans* sensitivity to osmotic shock was performed as detailed below, based on the protocols described by Bahn *et al*. (2003) and Bockmühl *et al*. (2001). For osmotic shock sensitivity testing, cells were grown to the stationary phase in liquid YPD at 30 °C, the OD at 600 nm was adjusted to 0.1 with the same medium, and 5 µl aliquots from the cultures and from 10-fold serial dilutions were spotted onto YPD plates containing 1.5 M NaCl. Sensitivity to heat shock was assessed as follows: cells of wild-type and PKA mutants from the stationary phase were streaked out on YPD plates and were incubated at 50 °C for 30 min, 1 h and 2 h. At these time points, the plates were shifted to 30 °C and growth was analysed after 2 days. To test the susceptibility of *C. albicans* cells to hydrogen peroxide, cells were grown at 30 °C in YPD medium to the specified OD at 600 nm, harvested and washed in PBS. Cell suspensions (1×10^7 cells) were challenged with different concentrations of hydrogen peroxide for 1 h at 30 °C, and viable counts were determined following dilution and plating on YPD plates. Survival percentages were expressed as the means ± standard deviations (SDs) of triplicate samples.

Glycogen content determination

Qualitative assessment of glycogen content was carried out by the iodine/iodide staining method (Toda *et al*., 1985). Quantitative assays of glycogen levels were performed as described by Parrou and Francois (1997).

Results and discussion

Characterization of *C. albicans* heterozygous *TPK* strains

Recent studies have shown that there are clear differences between the stress response of *C. albicans* and that of *S. cerevisiae* (Smith *et al*., 2004). *C. albicans* does not mount a general transcriptional response after exposure to osmotic, heat or oxidative stresses (Enjalbert *et al*., 2003). In *S. cerevisiae*, PKA participation has been established in the response to saline and heat stresses (Norbeck and Blomberg, 2000; Zähringer *et al*., 1998); therefore, it seemed interesting to investigate the involvement of *C. albicans* PKA in the response to a variety of stress conditions. To address this point, we generated heterozygous *TPK1* and *TPK2* mutant strains in a different *BCY1* genetic background. PCR primers were designed to obtain *TPK1::dpl200-URA3-dpl200* and *TPK2::dpl200-URA3-dpl200* deletion constructs (Figure 1) that, following recombination, would precisely replace the coding sequence of the targeted gene with the coding sequence of the marker. The products were used to transform *C. albicans* strains wild-type CAI4 and *BCY1/bcy1* Δ (LG65). This technique allowed us to obtain strains *TPK1/tpk1* Δ (R1U1), *TPK2/tpk2* Δ (R2U2) and *TPK1/tpk1* Δ *BCY1/bcy1* Δ (RG11), respectively.

The new strains grew normally as yeast-like cells in YPD medium at 30 °C (not shown) and they were able to germinate in a variety of liquid and solid inducing media at 37 °C (Table 3). In line with our previous results (Giacometti *et al*., 2006, Cassola *et al*., 2004), those strains devoid of one *BCY1* allele germinated as a mixture of hyphae and pseudohyphae, while the strain lacking both alleles (*tpk2* Δ/*tpk2* Δ *bcy1* Δ/*bcy1* Δ) displayed a mixed population composed of pseudohyphae and ungerminated round cells. PKA-specific activity of the new mutant strains confirmed our previous results showing that *TPK2* accounts for most of the PKA activity of the cell due to the low (poor) expression of the *TPK1* gene (Cloutier *et al*, 2003; Souto *et al*., 2006).

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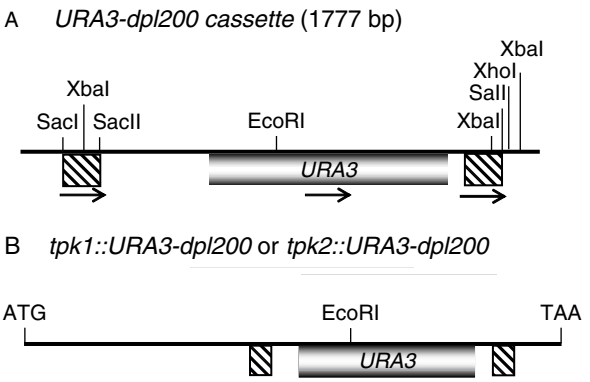

Figure 1. Chromosomal heterozygous deletion of *C. albicans TPK1* and *TPK2*. (A) *URA3-dpl200* cassette from plasmid pDDB57. The *URA3* ORF is shown as a shaded box and repeated sequences as hatched boxes. (B) *TPK* allele, showing the site of insertion of the *URA3-dpl200* cassette after homologue recombination

Tpk1 but not Tpk2 confers osmotolerance to the cell

In *S. cerevisiae* the level of PKA activity strongly affects osmotolerance and osmo-instigated gene expression changes (Norbeck and Blomberg, 2000). Therefore, it seemed relevant to evaluate the response to saline stress of our set of *C. albicans* PKA mutant strains exhibiting different levels of kinase activity and/or with different degrees of regulation (Table 3).

We first evaluated the generation time of wild-type RGI4, *tpk1* Δ/*tpk1* Δ and *tpk2* Δ/*tpk2* Δ mutant strains. As can be seen in Table 4, the doubling times of RGI4 and *tpk2* Δ/*tpk2* Δ strains in YPD medium were similar, while the *tpk1* Δ/*tpk1* Δ mutant showed a slight increase (ca. 17%). During growth in 1 M NaCl, the generation times of RGI4 and *tpk2* Δ/*tpk2* Δ were similar, increasing around 1.6-fold in saline medium. The *tpk1* Δ/*tpk1* Δ mutant displayed the highest-fold increase in generation time (2.8-fold). In view of these findings, we evaluated the survival of these and other PKA mutant strains after being grown under osmotic stress conditions. The results are shown in Figure 2. We found that the *tpk2* Δ/*tpk2* Δ strain, which has a low PKA activity, was even more resistant to saline stress than the wild-type strain. In sharp contrast, all strains devoid of *TPK1*, despite its high level of phosphotransferase activity, had a decreased survival after being subjected to an osmotic stress, this effect being more pronounced in the *tpk1* Δ/*tpk1* Δ mutant. It was also evident that, even in a background of low PKA activity, deletion of one of the two *TPK1* alleles made the cells sensitive to osmotic shock (strain *tpk2* Δ/*tpk2* Δ *TPK1*/*tpk1* Δ). Similar results were obtained after exposing the cells to 1.5 M sorbitol medium (data not shown). These results provided evidence that survival to osmotic stress was specifically mediated by the Tpk1 isoform and not by the more abundant Tpk2 isoform.

The growth impairment caused by the lack of one *BCY1* allele was only observed in a *TPK1* heterozygous background (*TPK1*/*tpk1* Δ *BCY1*/

Table 3. PKA specific activity and germinative phenotype

Strains	PKA specific activity (pMP incorporated/mg/min)		Ratio (minus/plus cAMP)	Germinative phenotype[a]
	Minus cAMP	Plus 10 μM cAMP		
RGI4	120	600	0.2	True hyphae
BCY1/*bcy1* Δ	200	630	0.31	Hyphae/pseudohyphae
TPK1/*tpk1* Δ	100	550	0.18	True hyphae
TPK1/*tpk1* Δ *BCY1*/*bcy1* Δ	180	500	0.36	Hyphae/pseudohyphae
tpk1 Δ/*tpk1* Δ	96	480	0.2	True hyphae
TPK2/*tpk2*Δ	70	380	0.18	True hyphae
*tpk2*Δ/*tpk2*Δ	20	68	0.29	True hyphae
*tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1* Δ	9	35	0.35	True hyphae
*tpk2*Δ/*tpk2*Δ *BCY1*/*bcy1* Δ	40	60	0.66	Hyphae/pseudohyphae
*tpk2*Δ/*tpk2*Δ *bcy1* Δ/*bcy1* Δ	58.5	65	0.9	Pseudohyphae/round cells

[a] Germinative morphology was assessed as described in Materials and methods in minimal medium plus 10 mM GlcNAc and in Spider medium (Shepherd *et al.*, 1980; Liu *et al.*, 1994).

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Table 4. Generation time (h) ± SD ($n = 3$) of *C. albicans* strains used in this study during growth in YPD medium with or without NaCl. The fold change in generation time in 1 M NaCl is given in the last column

Strain	0 M NaCl	1 M NaCl	Fold change in 1 M NaCl
RGI4	2.35 ± 0.2	3.86 ± 0.5	1.6
*tpk1*Δ/*tpk1*Δ	2.75 ± 0.1	7.65 ± 0.4	2.8
*tpk2*Δ/*tpk2*Δ	2.27 ± 0.2	3.99 ± 0.9	1.7

bcy1 Δ). Furthermore, the double *tpk2* Δ/*tpk2* Δ *bcy1* Δ/*bcy1* Δ mutant did not grow under this stress condition, supporting our previous findings showing that this strain is widely affected in its ability to react to changes in the environment (Cassola *et al*., 2004; Giacometti *et al*., 2006).

In a previous paper, Bockmühl *et al*, (2001) attributed overlapping functions to both Tpk isoforms in response to osmotic stress. However, it must be borne in mind that, since they performed their saline stress-challenging experiments with cells pre-grown in a minimal medium, which is already an unfavourable condition, it is likely that this experimental procedure may have masked the differences that we have observed. In parallel tests on the strains prepared by Bockmühl *et al*. (2001) and those prepared independently by us, all strains behaved similarly under our experimental conditions.

PKA regulation is given by multiple factors including cellular localization, cAMP levels and the kinase phosphorylation state, as reported for Tpk1 in *S. cerevisiae* (Portela and Moreno, 2006). In this yeast, a recent phosphorylome study (Ptacek *et al*., 2005) revealed that of all three Tpk isoforms only Tpk1 is phosphorylated by a Ser/Thr protein kinase encoded by *SAT4*. It has been reported that Sat4 is involved in salt tolerance (Mulet *et al*., 1999). The possibility that Tpk1 specificity in *C. albicans* might also be regulated by *C. albicans* homologous Sat4 is an attractive hypothesis. Studies to this end are now in progress in our laboratory.

Thermotolerance survival is mediated by Tpk1 and Bcy1 expression

In *S. cerevisiae*, strains with mutations in the *BCY1* gene compromising its functionality exhibited unrestricted PKA activity and were exquisitely sensitive to heat shock, irrespective of the *TPK* genetic background, suggesting overlapping functions of the three *TPK* genes (Toda *et al*., 1987).

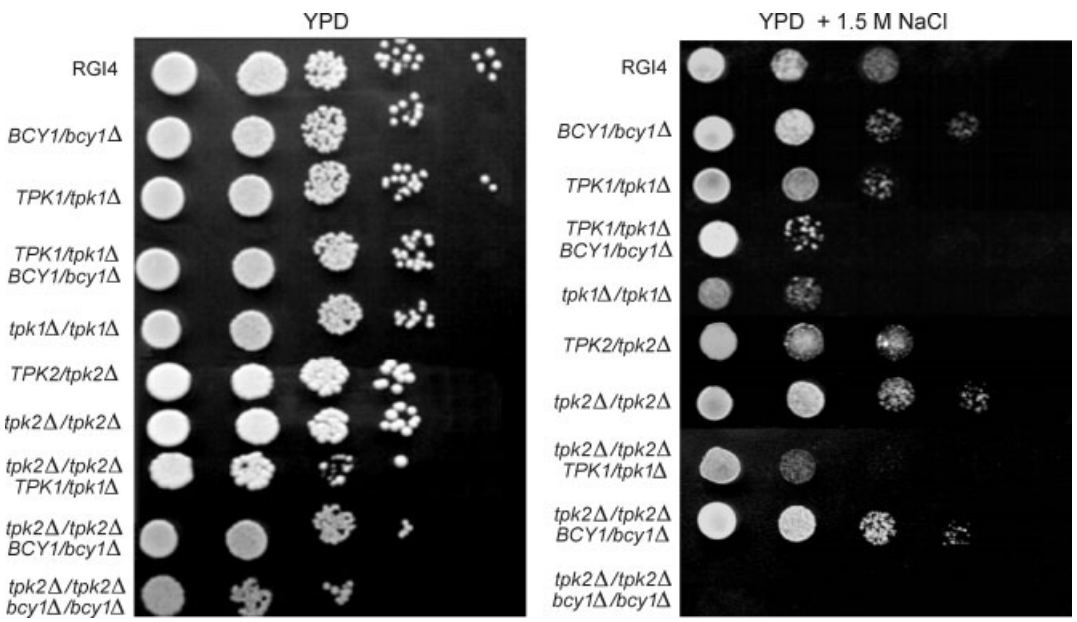

Figure 2. Survival of PKA mutants to osmotic stress. Wild-type and PKA mutant cells were grown in liquid YPD at 30 °C. The OD at 600 nm of the cultures was adjusted to 0.1 using the same medium, and 5 μl aliquots from the cultures and from 10-fold serial dilutions were spotted on YPD containing no addition (left panel) or 1.5 M NaCl (right panel). Results were monitored after 2 days of growth

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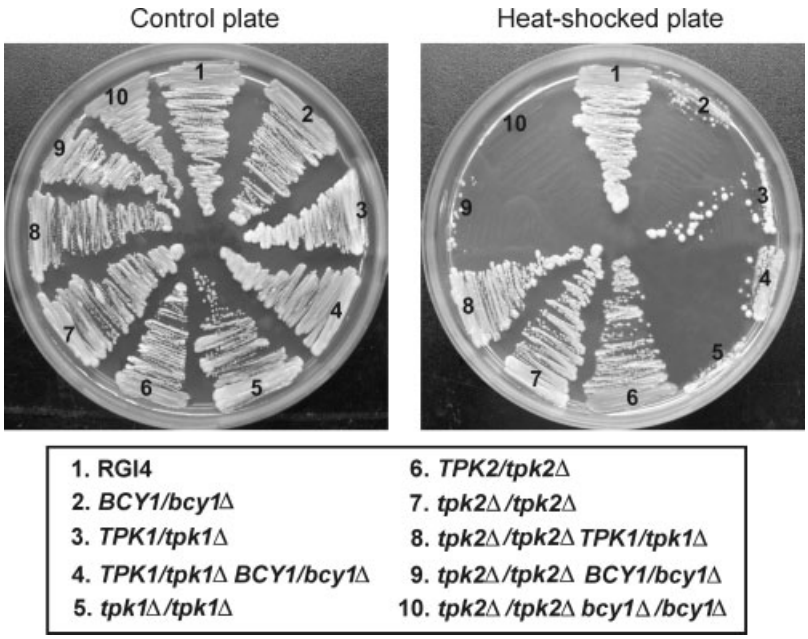

Figure 3. Effect of heat shock on PKA mutants. Yeast cells from the stationary stage were streaked onto YPD plates. The control plate was incubated at 30 °C (left panel); the heat-shocked plate was held at 50 °C for 2 h and then shifted to 30 °C (right panel). Growth was analysed after 2 days

Therefore, it seemed relevant to assess the heat shock response of the whole set of *C. albicans* PKA mutant strains. Yeast cells were pre-incubated at 50 °C for 2 h, and survival was scored in solid YPD grown at 30 °C after 2 days. The results obtained are shown in Figure 3. As can be seen (right plate), heterozygous and homozygous *BCY1* mutants, independently of the *TPK* background, suffered a dramatic loss of viability after the heat pre-treatment, (see streaks 2, 4, 9 and 10). The imbalance between catalytic and regulatory subunits produced by the partial or complete loss of regulation in these mutants could account for the inability to survive a severe heat shock challenge, possibly as a consequence of impairment of the cell structure integrity. Supporting this notion, we have already reported that *BCY1* hetero- and homozygosis leads to abnormal yeast-like shape, which could be associated to alterations of the cytoskeleton, compromising Tpk's subcellullar localization and therefore its physiological functions (Cassola *et al*., 2004; Giacometti *et al*., 2006).

Interestingly, we also found that the deletion of one *BCY1* allele in a wild-type genetic background (streak 2) caused a dramatic sensitivity to heat shock, very probably due to a partial activation of PKA activity (see Figure 5). All mutant strains expressing Tpk2 (*TPK1/tpk1* Δ, *tpk1* Δ/*tpk1* Δ and the double mutant *TPK1*/*tpk1* Δ *BCY1/bcy1* Δ; streaks 3–5) did not tolerate the heat treatment, while mutant cells with *TPK2* heterozygous or homozygous deletions in a different *TPK1* and/or *BCY1* background (streaks 6–10) grew as well as the wild-type strain (streak 1). These results indicated that *TPK1* and *TPK2* may have distinguishing features, suggesting that Tpk2 conferred heat sensitivity to the cell. In the absence of sufficient Tpk1, Tpk2 may interact with inappropriate targets promoting thermal sensitivity. This result could not be attributed to deregulation of phosphotransferase activity, since in the *tpk1* Δ/*tpk1* Δ mutant *BCY1* mRNA levels (Figure 4) and Bcy1 levels (Figure 5) were slightly higher than those detected in the *tpk2* Δ/*tpk2* Δ strain, which tolerated the heat treatment (Figure 3). Furthermore, cAMP dependence of PKA activity in *tpk1* Δ/*tpk1* Δ strain was similar to that of the wild-type strain (see Table 3).

C. albicans, like many other microorganisms, frequently encounters high levels of reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide and hydroxyl radicals, from both endogenous and exogenous sources (Miller

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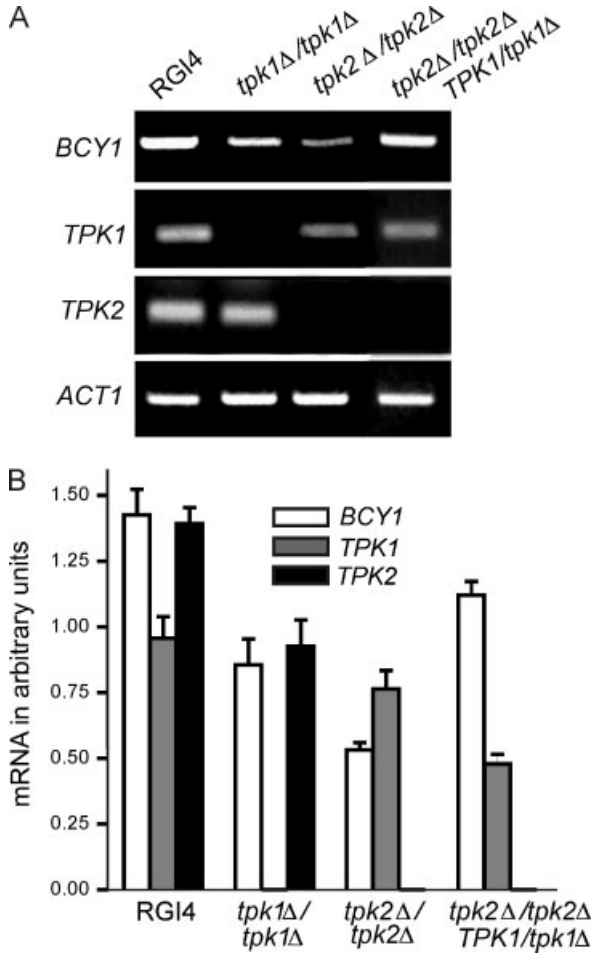

Figure 4. Semi-quantitative RT–PCR analysis of *TPK* and *BCY1* mRNAs. (A) Ethidium bromide-stained agarose gels to visualize *BCY1, TPK1* and *TPK2* transcripts from the stationary phase of wild-type, *tpk1*Δ/*tpk1*Δ, *tpk2*Δ/*tpk2*Δ and *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ mutant strains. (B) RT–PCR data were expressed in arbitrary units and values were normalized to actin (*ACT1*)

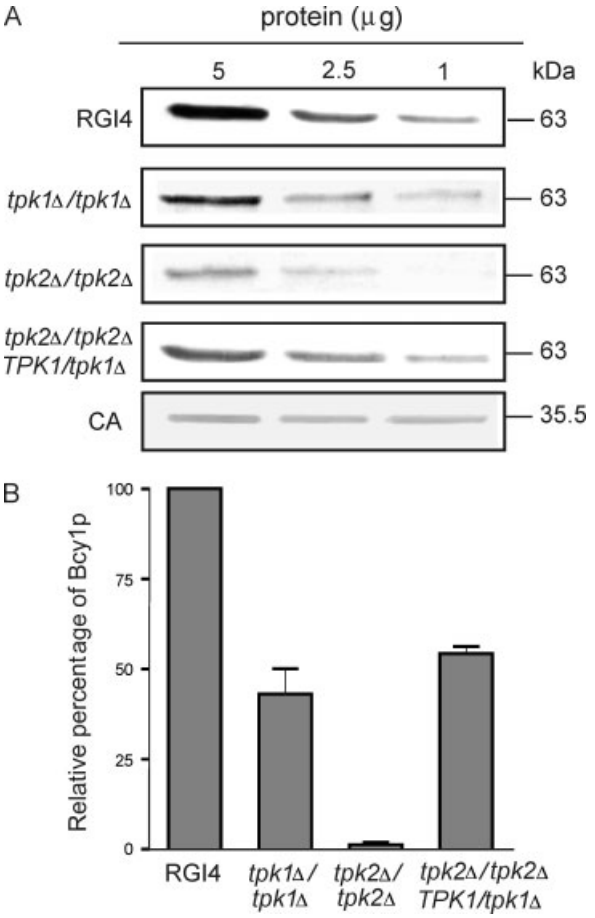

Figure 5. Comparison of Bcy1 levels in the mutant strain *tpk1*Δ/*tpk1*Δ, *tpk2*Δ/*tpk2*Δ and *tpk2*Δ/*tpk2*Δ *TPK1*/*tpk1*Δ with those of the wild-type strain by Western blot analysis. (A) Crude extracts from stationary phase cells were resolved in a 10% SDS–PAGE, transferred to PVDF membranes and developed with anti-*C. albicans* Bcy1 antiserum as described in Materials and methods. The molecular masses of Bcy1 and carbonic anhydrase (CA) are indicated on the right. (B). Densitometry scanning of the blots. Immunoblots were quantified using the GELBASE and SOL (UVP Inc.) programme. To allow comparison of the samples, data in (B) were expressed as a percentage of the immunoreactive blot detected from 1 μg protein of wild-type strain RGI4, arbitrarily set to 100%. Values are means ± SD from six independent experiments

and Britigan, 1997). A recent study provided evidence that *C. albicans* is able to coordinately regulate the oxidative stress response at the global cell population level by releasing protective molecules into the surrounding medium (Westwater *et al*., 2005). While the oxidative stress response has been characterized in some detail at the transcriptional level in *S. cerevisiae*, little is known about the molecular mechanisms responsible for resistance to oxidative stress in *C. albicans*.

Given the results obtained with osmotic and heat shock, it seemed relevant to assess whether PKA was also involved in the response to oxidative stress. Therefore, the whole set of PKA mutant strains was subjected to externally added hydrogen peroxide and the viability of the cultures tested after 1 h of exposure (Figure 6). Yeast cells depleted of Tpk1 were found to be significantly more susceptible to hydrogen peroxide

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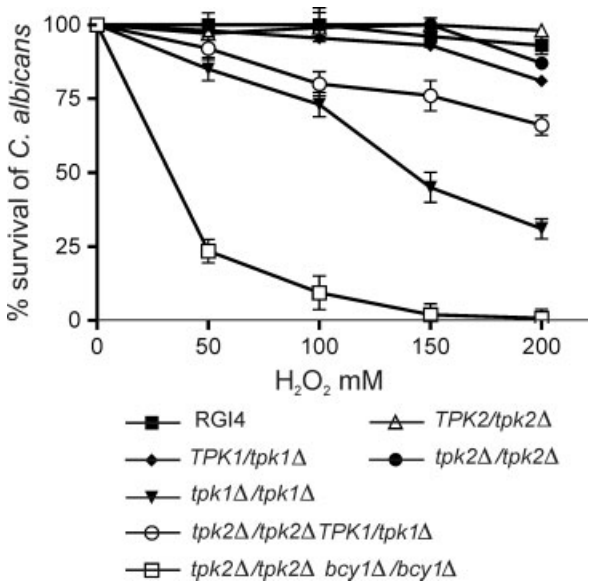

Figure 6. Susceptibility of PKA mutant cells to hydrogen peroxide. Cells from wild-type and PKA mutant strains were grown to stationary phase in YPD medium at 30 °C. Standardized cell suspensions were challenged with different concentrations of hydrogen peroxide for 1 h at 30 °C, and viable counts were determined following dilution and plating on YPD plates. Percentages of survival were expressed as the means ± SD of triplicate samples. A survival rate of >100% reflects the inherent variability associated with the plating process between control and test cultures. $^{**}p < 0.001$ for sample survival compared to sample survival at $OD_{600} = 0.1$ (Student's *t*-test)

than *tpk2* Δ/*tpk2* Δ cells (30% vs. 95% survival at 200 mM H_2O_2, respectively). The survival curve for *tpk2* Δ/*tpk2* Δ *TPK1* /*tpk1* Δ was intermediate between those of *tpk1* Δ/*tpk1* Δ and the wild-type strain.

Similarly to the results obtained after the heat treatment (Figure 3), lack of *BCY1* caused a drastic loss of viability (see mutant *tpk2* Δ/*tpk2* Δ *bcy1* Δ/*bcy1* Δ). However, *BCY1/bcy1* Δ mutants, whatever the *TPK* background, tolerated oxidative stress as well as the wild-type strain (data not shown).

PKA activity in yeast has been claimed to have a strong negative influence on the response to most types of stress and is, in fact, the only good candidate to date for a common denominator for the various yeast stress responses (Siderius and Mager, 1997). However, we show here that in *C. albicans* there is not a direct association between the level of phosphotransferase activity and the ability to survive stress, but rather the significant factor is the quality of catalytic activity provided by the Tpk1 isoform. Further studies are required to address the specific targets of Tpk1 and Tpk2 that elicit the physiological response under stress conditions. Whatever the mechanism, our results show that the two Tpk isoforms play distinct roles in the *C. albicans* stress response.

Tpk1 and Tpk2 have different functions in glycogen accumulation

In *S. cerevisiae*, PKA activity has been shown to be involved in cellular processes other than dimorphism, affecting not only cell growth and maintenance but also carbon storage (Cameron *et al*., 1988; Gimeno *et al*., 1992). We therefore proceeded to analyse the effect of *C. albicans* PKA mutations on the accumulation of glycogen. The mutant devoid of both copies of the Tpk2 isoform accumulated more glycogen than the wild-type. Surprisingly, the opposite effect was obtained in strains lacking one or both *TPK1* alleles (Figure 7A, B). Thus, *TPK1/tpk1* Δ and *tpk1* Δ/*tpk1* Δ strains had noticeably lower levels of glycogen than the wild-type strain. These results strongly suggested that the two isoforms of the catalytic subunit play different roles in carbohydrate metabolism, Tpk2 being more involved in glycogen degradation and Tpk1 in its synthesis. In addition, we found that strain *tpk2* Δ/*tpk2* Δ *BCY1/bcy1* Δ clearly showed a significant reduction in glycogen accumulation compared to its parental *tpk2* Δ/*tpk2* Δ strain. However, in the absence of the two copies of the regulatory subunit (strain *tpk2* Δ/*tpk2* Δ *bcy1* Δ/*bcy1* Δ) no glycogen accumulation was observed, suggesting that the extent of regulation of PKA also affects glycogen content.

It is well known that in higher eukaryotes PKA regulates glycogen metabolism through phosphorylation of a number of enzymes that activate the synthesis or breakdown of this polysaccharide. We found that *C. albicans* PKA mutant strains devoid of one or both *TPK1* alleles were defective in glycogen storage, while strains lacking Tpk2 accumulated more glycogen than the wild-type strain. Thus, PKA isoforms of *C. albicans* had opposite effects on glycogen accumulation. This was surprising since, to our knowledge, there is no other example of opposite roles for

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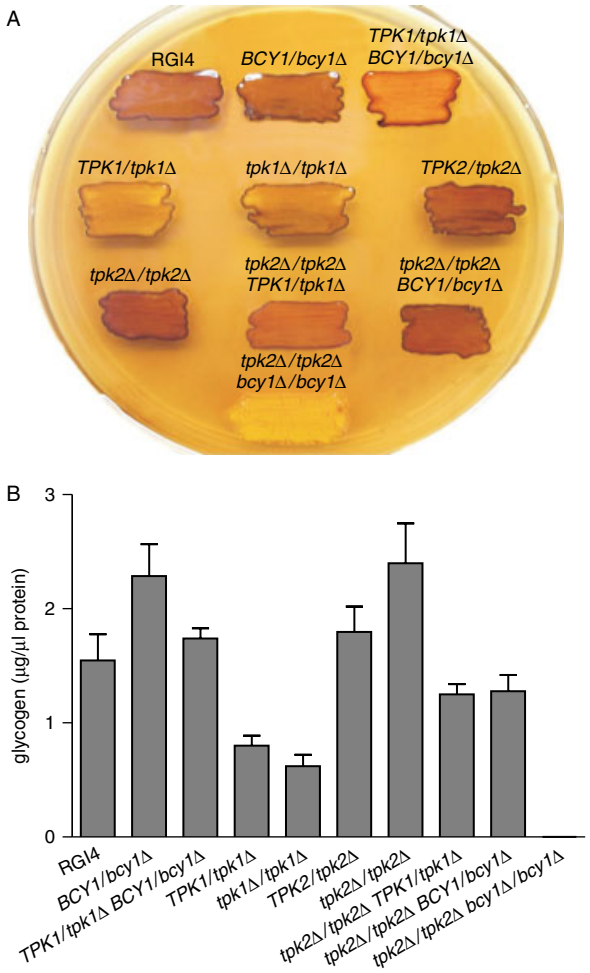

Figure 7. Glycogen accumulation in *C. albicans* PKA mutant strains in solid and liquid YPD medium. (A) *C. albicans* wild-type and PKA mutant strains were streaked out on YPD plates and incubated at 30 °C for 2 days before treatment with an iodine/iodide solution. (B) Liquid cultures were grown in YPD for 2 days and enzymatic determination of glycogen levels was assessed in extracts as described in Materials and methods. Values are means ± SD from three independent experiments

PKA catalytic subunit isoforms in glycogen regulation. How can two isoforms which are thought to be activated by a common second messenger have opposite downstream effects? We conclude that such opposite effects on glycogen accumulation implies different substrate specificity for each Tpk isoform, each one playing a major role in either the synthesis or the degradation of glycogen. Since *C. albicans* does not have a phosphorylase kinase (**http://www.candidagenome.org/**), regulation of glycogen degradation could be due to the direct or indirect phosphorylation and activation of the putative phosphorylase, Gph1. As an alternative hypothesis, direct PKA regulation of glycogen synthesis may be mediated by the phosphorylation of the putative phosphatase inhibitor Ypi1 which, once phosphorylated, may inhibit the Glc7 Ser/Thr phosphatase as described in *S. cerevisiae* (Gimeno *et al.*, 1992; He and Moore, 2005), leading to increased levels of phosphorylated, inactive glycogen synthase. The possibility that a balance between synthesis and degradation of glycogen could also be the result of changes in the proportions of the isoforms seems unlikely, since in PKA mutants the expression pattern of *TPK1* and *TPK2* during vegetative growth was similar to that of wild-type strain (Souto *et al.*, 2006).

In *S. cerevisiae* PKA phosphorylates Sds22, which acts as a positive regulator of Glc7 (Peggie *et al.*, 2002), thus activating glycogen synthase. The functional versatility of Glc7 could be achieved not only by indirect activation or repression through PKA phosphorylation events, but also by the existence of numerous regulatory subunits, forming complexes that target the phosphatase to different subcelullar compartments and/or modulate its enzymatic activity (Bollen, 2001). It is worth mentioning that ORFs highly homologous to *S. cerevisiae* Gph1, Ypi1, Glc7 and Sds22 are present in the *C. albicans* genome (**http://www.candidagenome.org/**). Preliminary experiments from our laboratory showed that all these ORFs were expressed in *C. albicans* wild-type cells (Giacometti *et al.*, unpublished results). Based on our results and those obtained from proteomic analysis in yeast (Ptacek *et al.*, 2005), one could postulate the preferred substrate of each *C. albicans* Tpk isoform in glycogen metabolism. Thus, Tpk2 may be specifically phosphorylating Gph1 and also Ypi1, while Tpk1 may be responsible for the phosphorylation of the positive regulator Sds22 (see Figure 8). However, it cannot be ruled out that *C. albicans* may have developed a different pathway to regulate its carbohydrate reserves that has evolved to fit the environmental niches occupied by this pathogen. Whatever the mechanism, further studies, now in progress in our laboratory, are required to find the specific targets of Tpk1 and Tpk2 that elicit these physiological responses.

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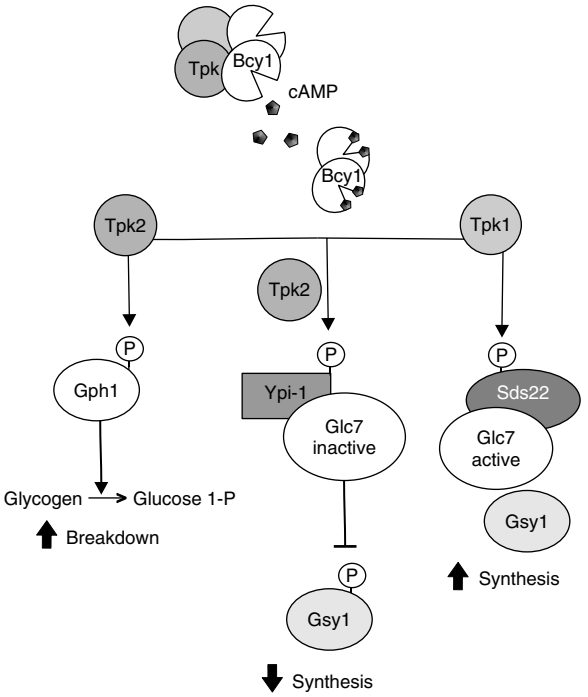

Figure 8. Different substrate specificity of Tpk isoforms could explain opposite effects on glycogen accumulation. A possible model. We propose that Tpk2 is involved at a signalling branch point in dual regulation of the degradation and the inhibition of glycogen synthesis, consisting in the phosphorylation of the glycogen phosphorylase (Gph1), and in the synthesis cascade through the activation of the phosphatase inhibitor Ypi1 (named by analogy to the *S. cerevisiae* orthologue), which inhibits the Ser/Thr phosphoprotein phosphatase Glc7, leading to inactivation of glycogen synthase (Gsy1). We also postulate that the Tpk1 isoform is indirectly related to glycogen synthesis, since phosphorylation of Sds22, a Glc7 positive nuclear regulator, promotes dephosphorylation of Gsy1. The proposal that Tpk2p directly regulates glycogen catabolism may explain why *tpk1*Δ/*tpk1*Δ cells but not *tpk2*Δ/*tpk2*Δ cells are incapable of accumulating the polysaccharide

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