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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*

Monikca Cloutier,[a,1] Rocío Castilla,[b,1] Nathalie Bolduc,[a] Alicia Zelada,[b] Philippe Martineau,[a] Marlène Bouillon,[a] Beatrice B. Magee,[c] Susana Passeron,[b] Luc Giasson,[a] and María L. Cantore[b,*]

[a] *School of Dentistry, GREB Laval University, Sainte-Foy Que., Canada G1K 7P4*

[b] *Cátedra de Microbiología, Facultad de Agronomía, Universidad de Buenos Aires, IBYF-CONICET, Avda. San Martín 4453, 1417 Buenos Aires, Argentina*

[c] *Department of Genetics, Cell Biology and Development, University of Minnesota, 1445 Gortner Avenue, St Paul, MN 55108, USA*

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Abstract

We have cloned the *Candida albicans TPK2* gene encoding a cAMP-dependent protein kinase (PKA) catalytic subunit and generated a *tpk2* homozygous null mutant to assess its ability to germinate in liquid media. *N*-acetylglucosamine (GlcNAc)-induced germ-tube formation was attenuated in the *tpk2* strain and enhanced by compounds that are known to increase the PKA activity in situ. Germination was completely blocked in the presence of the myristoylated derivative of the heat-stable PKA inhibitor (Myr-PKI). These results indicate that *TPK1* acts positively in regulating the morphogenetic transition in *C. albicans* in the absence of the *TPK2* gene. We were able to identify an mRNA from this second form of PKA in both wild-type and *tpk2* null mutant cells. We found that PKA activity measured in the mutant lacking the *TPK2* gene was about 10% of that displayed by the wild-type. The finding that the germinative response of *tpk2* null mutant to serum was severely diminished at low serum concentrations indicates that the level of PKA is an important determinant of filamentous growth at low serum concentrations. The extent of germination attained at higher serum concentrations (5%) was similar in the wild-type and in the *tpk2* null mutant strains suggesting that under these conditions germination was triggered through a PKA-independent pathway.

Keywords: cAMP; cAMP-dependent protein kinase; Morphogenesis; *Candida albicans*

1. Introduction

The dimorphic opportunistic human pathogen *Candida albicans* is able to reversibly switch its mode of growth from a budding yeast (blastospores) to a filamentous form (either hyphae or pseudohyphae) depending on the environmental conditions (Odds, 1988). Dimorphism is a characteristic that *C. albicans* shares with several plant and human pathogenic fungi. Current biochemical and genetic evidences suggest that the ability to alternate between different morphological states is an important virulence and pathogenicity determinant in fungi (Alspaugh et al., 1998; Kronstad, 1997; Liu and Dean, 1997; Lo et al., 1997).

Despite the diversity of signals that can trigger the dimorphic transition, pathways transducing external signals to the cellular machinery are highly conserved in fungi. Two conserved transducing cascades controlling morphogenesis and virulence have been defined: the mitogen-activated protein kinase (MAPK)[2] and the

* Corresponding author. Fax: +54-11-4514-8741.
E-mail address: cantore@mail.agro.uba.ar (M.L. Cantore).
[1] M. Cloutier and R. Castilla contributed equally to this work.

[2] *Abbreviations used:* MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; MyrPKI, myristoylated derivative of the heat-stable PKA inhibitor; GlcNAc, *N*-acetylglucosamine; hCG, human chorionic gonadotropin.

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cAMP/PKA transduction pathways (for a review, see Lengeler et al., 2000).

Molecular and genetic evidence indicate that a MAPK pathway highly related to that of *Saccharomyces cerevisiae* operates in *C. albicans*, and that disruption of any gene in the cascade results in defective filamentous growth under certain conditions (Csank et al., 1998; Leberer et al., 1996; Liu et al., 1994). Involvement of the cAMP-dependent pathway in the yeast-to-mycelial transition has also been well documented (Niimi, 1996; Sabie and Gadd, 1992). Recent reports provided strong genetic evidence on the key role of cAMP in the promotion of hyphal formation in *C. albicans* (Bahn and Sundstrom, 2001; Rocha et al., 2001).

Previous work from our laboratory has shown that the activities of the enzymes related to the cAMP metabolism are modulated during the morphogenetic process (Egidy et al., 1990). We have characterized and purified a PKA from the yeast form of the fungus (Zelada et al., 1998). We have also shown that its in vivo inhibition blocks hyphal growth induced by GlcNAc but not by serum (Castilla et al., 1998). More recently, Sonneborn et al. (2000) and Bockmühl et al. (2001) have shown that two genes (*TPK1* and *TPK2*) code for PKA catalytic subunits in *C. albicans* and that disruption of either gene leads to defective germinative phenotypes under certain inducing conditions. All this biochemical and genetic evidences indicate that PKA activity is an important contributing factor that regulates differentiation of *C. albicans*.

To further investigate the functional role of PKA in *C. albicans* morphogenesis, we have cloned the *TPK2* gene and generated a mutant lacking both alleles of the gene. We present biochemical and molecular evidence showing that the *TPK1* gene is expressed in the wild-type and in the *tpk2* strain. The PKA activity measured in the mutant lacking the *TPK2* gene is about 10% of that displayed by the wild-type. We found that GlcNAc-induced germ-tube formation is attenuated in the *tpk2* strain and that germination is completely blocked in the presence of MyrPKI. Moreover, the poor germinative capability of the *tpk2* null strain is enhanced by compounds that are known to stimulate PKA activity in situ. These results suggest that the catalytic activity of Tpk1p is responsible for maintaining the germinative capability of the *tpk2* null mutant. In this study, we also examined in more detail the involvement of the PKA pathway in serum-induced filamentous growth. We found that germination is sensitive to PKA inhibition only when induced by serum concentrations lower than 2.5%; at higher concentrations the process seems to become independent of the PKA pathway. The possible existence of another role for cAMP, other than binding and activating PKA, is discussed.

2. Materials and methods

2.1. Chemicals

Kemptide (LRRASLG), cAMP, and protease inhibitors were from Sigma Chemical. DE-52 and phosphocellulose paper P-81 were from Whatman. PVDF membranes (Immobilon-P) were from Millipore [γ-^{32}P]ATP and [γ-^{32}P]CTP were from New England Nuclear. Myristoylated PKI (14–24) amide (MyrPKI) was from BIOMOL Research Lab. Glucagon was from Eli Lilly (USA) and human chorionic gonadotropin (hCG) from Laboratorio Elea (Argentina). All other chemicals were of analytical grade.

2.2. Bacterial and fungal manipulations

Escherichia coli strains were grown at 37 °C in LB broth supplemented with 80 μg/ml of ampicillin for plasmid selection or 20 μg/ml of chloramphenicol for fosmid selection. *C. albicans* strains were routinely cultured at 30 °C in YPD (1% yeast extract, 2% peptone, and 2% dextrose). For the growth of Ura$^-$ strains, minimal media were supplemented with uridine (25 μg/ml).

2.3. DNA manipulations and analyses

All DNA manipulations were carried out using standard molecular techniques (Sambrook et al., 1989). Plasmid and fosmid DNA were purified using affinity columns (Qiagen) following manufacturer's recommendations. For sequencing purposes, a set of nested deletions were obtained from the 1.7 kb *Pst*I–*Pst*I and 1.6 kb *Sac*I–*Sac*I fragments (Fig. 1A) using the Double-stranded Nested Deletion Kit (Pharmacia). Sequencing was done using the dideoxy chain termination method (Sanger et al., 1977) with [α-^{32}P]dCTP. Southern hybridization analyses were performed using the Non-radioactive Labelling and Detection Kit (Boehringer Mannheim).

2.4. Isolation of clones encoding the TPK2 gene

Two degenerate oligonucleotides containing inosine [5′-GTA TCG AT(A/C/T) TA(C/T) (A/C)GI GA(C/T) (C/T)TI AA(A/G) CC-3′ and 5′-CA CCG CGG IGC IA(G/A) (G/A)TA (T/C)TC I(T/G)G IGT ICC-3′] were used to amplify a portion of the *TPK2* gene from *C. albicans* strain ATCC 32354. PCR was performed using total genomic DNA and Taq polymerase (35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C). The amplicon was cloned in vector pCR-Script SK(+) (Stratagene) and used as a probe to screen the fosmid library maintained at the University of Minnesota.

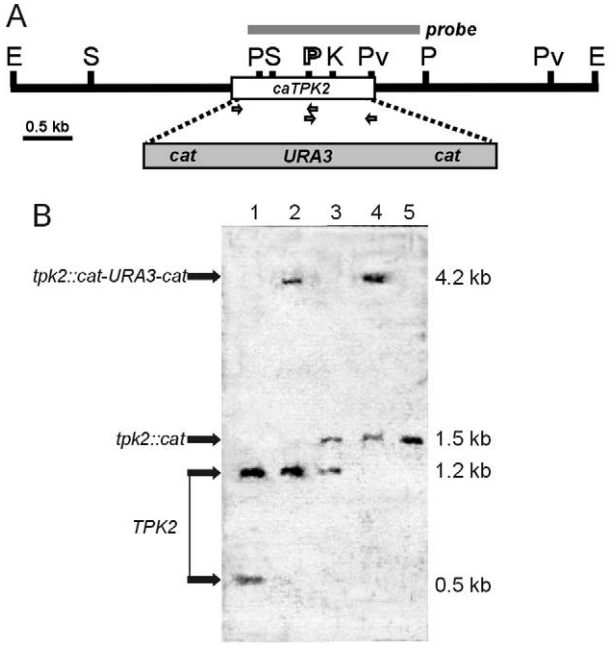

Fig. 1. Deletion of *TPK2* in *C. albicans*. (A) Restriction map of the *TPK2* locus. The thick line represents a *C. albicans* genomic fragment, from fosmid 1D7, containing the *TPK2* locus. The white box indicates the position of the *TPK2* coding sequence. For deleting *TPK2*, pCUC23 was made by replacing the 1.0 kb *Pst*I–*Pvu*II fragment within the coding sequence with a *cat–URA3–cat* cassette (grey box) as illustrated. The short arrows indicate the position of the primers used to confirm deletion of *TPK2* by PCR. The grey line indicates the genomic portion used as a probe in Southern analyses performed to monitor the deletion of *TPK2* alleles. Endonuclease restriction sites are as follows: E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sac*I. The empty "P" indicates that the site is present in the genome of strain CAI4 but not in that of strain 1161 from which the map was originally obtained. (B) Southern blot analysis of *C. albicans* transformants. Total genomic DNA from different transformants was digested with *Pst*I. Lane 1, parental strain CAI4 (*TPK2*/*TPK2 ura3*/*ura3*); lane 2, prototrophic hemizygous mutant (*TPK2*/ Δ*TPK2*::*cat–URA3–cat ura3*/*ura3*); lane 3, auxotrophic hemizygous mutant (*TPK2*/Δ*TPK2*::*cat ura3*/*ura3*); lane 4, prototrophic homozygous null mutant (*TPK2*::*cat–URA3–cat*/ Δ*TPK2*::*cat ura3*/*ura3*); lane 5, auxotrophic homozygous null mutant (*TPK2*::*cat*/Δ*TPK2*::*cat ura3*/*ura3*). The 1.7 kb *Pst*I fragment containing most of the *TPK2* coding sequence was used as a probe (A).

2.5. Deletion of the TPK2 gene

The procedure described by Fonzi and Irwin (1993) was used to generate a *C. albicans tpk2* homozygous null mutant. Plasmid pCUC-23, used to obtain the mutant, was constructed in the following way. First, the 3.0 kb *Eco*RI–*Kpn*I and 2.6 kb *Kpn*I–*Eco*RI fragments from fosmid 1D7 (see Fig. 1A) were subcloned independently in pCRScript (Stratagene), yielding plasmid pMCe and pMC1a, respectively. A 2.3 kb *Pst*I subfragment from pMCe was then inserted into the *Pst*I site of vector pCUC (carrying the *Cat*–URA3–*Cat* cassette), yielding pCUC-27. A 1.7 kb *Pvu*II subfragment of pMC1a was then inserted in pCUC-27, first linearized with *Kpn*I followed by a treatment with the Klenow fragment of DNA polymerase I. This construction, called pCUC-23, was linearized with *Hin*dIII before being used in *C. albicans* transformation experiments. *C. albicans* CAI4 strain was transformed to uracil prototrophy and spontaneous loss of the *Cat*–URA3–*Cat* cassette was identified by 5-fluoroorotic acid resistance. A second round of gene disruption was performed to obtain an homozygous *tpk2* null mutant. Transformation was performed using the Alkali Cation Yeast Transformation Kit (BIO 101) following manufacturer's recommendations.

2.6. RT-PCR analysis

Total cellular RNA was extracted from *C. albicans* yeast cells using the hot acidic phenol method (Ausubel et al., 1994). Contaminating DNA in RNA samples was removed enzymatically using the Message Clean Kit (Gen Hunter). cDNA synthesis was performed using MMLV reverse transcriptase (GibcoBRL) according to manufacturer's recommendations. About 10 ng cDNA (in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTP, 5 U Taq polymerase, and 2 μM of each primer) were used for PCR amplification (35 cycles of 60 s at 94 °C, 60 s at 50 °C, and 60 s at 72 °C). The primer pairs used to verify the deletion of the *TPK2* gene in the homozygous mutant were : 1: 5′-GGCA TATGGTGAATCTTTTAAA-3′ and 4: 5′-CGTCCAT GTAACGGTACTAAC-3′; or 2: 5′-GGGGATCCTAA TTGTTCTTCTG-3′ and 3: 5′-CGGAGAATGTATT ATTGGATAG-3′. The primer pairs used to assess the expression of *TPK1* and *TPK2* genes were: 1: 5′-AGA AGTTCAAGATGTGACTTAT-3′ and 2: 5′-CATCAT CAGAACCACCTTGT-3′ to amplify a 307 bp PCR product of *TPK1* and 3: 5′-GAAGTTAGTACCG TTACATGG-3′ and 4: 5′-CTTCTTGTCAAATCAG CAGTT-3′ to amplify a 270 bp PCR product of *TPK2*.

2.7. Complementation of the tpk2 mutant strain

The wild-type *TPK2* gene was used in a transformation experiment to complement the *TPK2* deletion. A 5.6 kb *Eco*RI fragment containing the wild-type *TPK2* gene (see Fig. 1A) was cloned into vector pCRScript (Stratagene). A 2.9 kb *Bam*HI fragment containing the *C. albicans URA3* gene from pMB7 (Fonzi and Irwin, 1993) was added to that construction. The resulting plasmid, called pPM10, was used to transform strain H2D, one of the *tpk2* homozygous null mutant generated in the course of this work. Ten micrograms of pPM10, linearized with *Hin*dIII were used for transformation.

2.8. Germ tube induction

Germ tube formation experiments were performed essentially as described previously (Castilla et al., 1998) except that the incubation medium described by Shep-

herd et al. (1980) was used. Cells from the stationary phase of growth were washed twice with distilled water and resuspended in the incubation medium to a final density of 2×10^6 cells/ml. Germ-tube inducers, stimulators, and inhibitors were added to the induction media to reach the final concentration indicated in each experiment. Incubations and evaluation of germination were performed as previously described (Castilla et al., 1998). Experiments were conducted in triplicate, and at least 200 cells were scored.

2.9. Preparation of soluble extracts and DEAE column chromatography development

Soluble extracts preparation and DE-52 column chromatography were performed as previously described (Zelada et al., 1998). Briefly, yeast cells were suspended in 10 mM sodium phosphate buffer (pH 6.8) containing 1 mM EGTA, 1 mM EDTA, 10 mM β-mercaptoethanol, 20 μg/ml leupeptin, 20 μg/ml antipain, 20 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 20 mM benzamidine (buffer A). Crude extracts were obtained by breaking the cells in a French press. The lysate was centrifuged at 5000*g* for 15 min to sediment unbroken cells and cellular debris. Crude extracts were centrifuged at 100,000*g* for 45 min and the supernatant obtained was referred to as the S100 fraction. Columns of DE-52 (5 ml) were loaded with the S100 fraction (80 mg protein) and developed with a 50 ml linear gradient from 0 to 300 mM NaCl made in buffer A. Fractions of 0.5 ml were collected and PKA activity was measured in 40 μl aliquots. All operations were performed at 4 °C.

2.10. Assay of PKA activity

Phosphotransferase activity was measured as described previously (Zelada et al., 1998). The standard assay mixture contained, in a final volume of 60 μl, 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.1 mM kemptide, 0.1 mM [γ-^{32}P]ATP (0.1–0.5 Ci/mmol), and 50 μM cAMP (when indicated). After incubation for 10 min at 30 °C, 50 μl aliquots were spotted on square phosphocellulose papers and dropped into 75 mM phosphoric acid for washing as described by Roskoski (1983).

2.11. Protein determination

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.12. Nucleotide sequence accession number

The sequence of *TPK2* has been submitted to GenBank under accession number AF 317473.

3. Results

3.1. Isolation and characterization of TPK2

Two highly conserved amino acid segments (IYRDLKP and GTHEYLAPE, respectively, subdomains VI and VIII) from the conserved regions of PKA catalytic subunits from various microorganisms, were chosen to design two oligonucleotides which served as primers to PCR amplify the corresponding genomic fragment from *C. albicans*. PCR products were resolved by agarose gel electrophoresis and a 146 bp amplicon was cloned in pCR-Script SK(+). The sequence of the amplicon was found to share 83% identity with a region of *S. cerevisiae TPK2* gene (Toda et al., 1987).

The amplicon was then used as a probe to screen a *C. albicans* genomic fosmid library. Three positive clones were recovered (1D7, 9E10, and 15E4), all originating from chromosome 2. Southern analysis revealed that the *C. albicans TPK2* homologue was present on a 5.6 kb *Eco*RI fragment, common to fosmids 1D7 and 15E4. This fragment was subcloned and a restriction map was established (Fig. 1A). The region of this fragment encoding the *C. albicans TPK2* gene was sequenced, revealing an open reading frame of 1326 nucleotides encoding a putative 442 amino acids protein. This sequence was found to be identical to that of the *TPK2* gene recently determined independently by Sonneborn et al. (2000). The Tpk2p deduced sequence shows a high degree of homology in its C-terminal portion (about 300 amino acids) to the three *S. cerevisiae* Tpkp isoforms (Sonneborn et al., 2000; and our data not shown) and also to the recently characterized *CaTPK1* product (Bockmühl et al., 2001). It is worth noting that the *S. cerevisiae* and *C. albicans* Tpk2p isoforms showed the highest homology, revealing glutamine stretches at the N-terminus not present in the other isoforms.

3.2. Chromosomal deletions of TPK2

To further investigate the role of PKA in the control of dimorphism, we performed the deletion of both alleles of *TPK2* in *C. albicans* using the approach described by Fonzi and Irwin (1993). To that end, a *TPK2*::cat–*URA3*–cat deletion construct (pCUC23; Fig. 1A) was generated and used to transform a *ura3 C. albicans* strain to replace the chromosomal *TPK2* gene. As *C. albicans* is a diploid, the second allele of the *TPK2* gene had also to be deleted to generate a null mutant. The genotypes of the hemizygous and homozygous null mutants were confirmed by Southern blot (Fig. 1B).

The absence of *TPK2* mRNA in the *tpk2* homozygous null mutant was confirmed by RT-PCR analysis. Total RNA was extracted from the mutant and from the wild-type strains and reverse transcribed. The cDNA obtained was amplified using two pairs of primers de-

signed from internal portions of the *TPK2* ORF sequence (Fig. 1A). In each case, the expected PCR products (850 and 500 bp) were obtained from parental strain CAI4 but not from the homozygous null mutant (data not shown).

No growth phenotype was observed for the hemizygous or homozygous null mutants when grown in minimal medium or in YEPD.

3.3. Germinative behaviour of the TPK2 mutant

The ability of the *TPK2* homozygous null mutant to undergo the yeast-to-hypha transition was assessed using GlcNAc as germ-tube inducer. As shown in Table 1, germination was reduced in the *tpk2* null mutant when compared to that of the parental strain (30 vs. 65%, respectively). Microscopically, no morphological differences were observed between *tpk2* mutant and parental CAI4-induced cells. Germination of the mutant and parental strains was completely abolished by the presence of MyrPKI, a highly specific PKA inhibitor (Castilla et al., 1998). The results summarized in Table 1, in agreement with those of Sonneborn et al. (2000), show that the *tpk2* mutant is able to germinate in liquid medium thus providing biochemical evidence that residual PKA activity is sustaining germination in the *tpk2* mutant. As stated above, the existence of a second gene for the catalytic subunit of the PKA, named *TPK1*, was recently reported by Bockmühl et al. (2001). Based on the germinative behaviour of *tpk1* mutant in liquid and solid germination inducing media, a redundant role for both *TPK1* and *TPK2* genes was proposed by these authors. Consequently, expression of *TPK1* in the *tpk2* mutant could well account for the results shown in Table 1. To detect its expression, the presence of *TPK1* mRNAs was investigated using qualitative RT-PCR. Based upon the sequence of the *TPK1* and *TPK2* genes, primers were designed such that different size fragments would be RT-PCR amplified from *TPK1* and *TPK2* mRNAs (see Section 2). As can be seen in Fig. 2, amplification from the parental and *tpk2* null mutant strains gave the expected diagnostic fragments, confirming the expression of both *TPK1* and *TPK2* in the parental strain (307 and 270 bp fragments, lanes 1 and 3) and the expression of only *TPK1* in the mutant (307 bp fragment, lanes 2 and 4).

Table 1
Germ tube induction in the *tpk2* mutant

Additions	Germ-tube formation (%)	
	Parental strain CAI4	*tpk2* mutant strain
None	65 ± 5	30 ± 4
MyrPKI (10 μM)	0	0

Note. Yeast cells were induced to germinate as described in Section 2 in the presence of 1 mM GlcNAc. Each value represents the mean ± SD of three separate experiments.

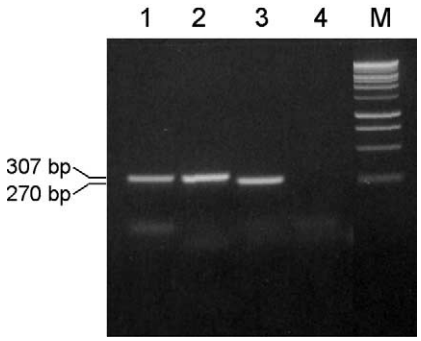

Fig. 2. In vivo expression of TPK1 and TPK2 in wild-type and mutant strains. About 10 ng of cDNA were PCR amplified as described in Section 2 and the products were analyzed by agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed. Wild-type (lanes 1 and 3) and *tpk2* mutant cDNAs (lanes 2 and 4) were PCR amplified with the *TPK1* (lanes 1 and 2) and the *TPK2* (lanes 3 and 4) specific primers.

3.4. Complementation of the TPK2 mutation

The wild-type *TPK2* gene was introduced by transformation into the *C. albicans tpk2* homozygous null mutant (Δ*tpk2*::cat/Δ*tpk2*::cat *ura3*/*ura3*). Microscopically, the transformants obtained were undistinguishable from the null mutant strain or the parental CAI4 strain. Two randomly selected transformants recovered their ability to develop a filamentous morphology with an efficiency similar to that of the parental strain CAI4 when induced with GlcNAc in an experiment similar to that shown in Table 1 (data not shown), indicating that the reduced ability to operate the yeast-to-hypha transition observed in the *tpk2* null mutant strain was a direct consequence of the *TPK2* deletion.

3.5. PKA activity in the tpk2 null mutant

Further evidence of the presence of a catalytically active Tpk1p in *C. albicans* yeast cells was obtained through measurement of PKA activity in S100 fractions derived from both parental and *tpk2* mutant strains. The level of PKA activity detected in the mutant was about 7-fold less than that of the parental strain (3 and 20 mmoles/min/10^{10} cells, respectively). The activity detected was cAMP-dependent and MyrPKI sensitive. To obtain a more accurate measurement of PKA activity, soluble extracts from both strains were partially purified through DE-52 columns. Fig. 3 shows chromatographic profiles obtained from a representative experiment of three performed with extracts obtained from independent cultures. Since columns were loaded with the same amount of protein and identically processed, comparison of the areas under the curves allowed a rough

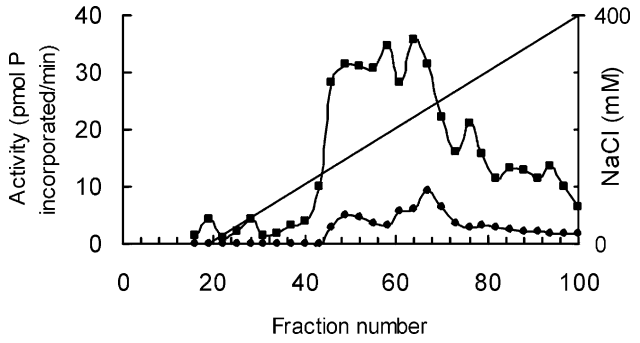

Fig. 3. Elution profiles from the DE-52 columns of PKA activity from parental and *tpk2* mutant strains. The S100 fraction (80 mg protein) from both strains were separately applied onto 5 ml DE-52 columns, washed and developed as described in Section 2. Fractions were assayed for phosphotransferase activity in the presence of 20 μM cAMP. Activity in the mutant strain, (●); activity in the wild-type strain, (■).

estimation of the relative amounts of total PKA activity present in each sample. The results indicate that PKA activity was markedly lower in the mutant, roughly 10% of that of the parental strain. The activity in all fractions was cAMP-dependent and completely inhibited by MyrPKI (not shown).

The results of the above experiments strongly support the notion that the Tpk1p catalytic activity is responsible for the maintenance of the germinative capability of the *tpk2* null mutant strain.

3.6. Activators of PKA enhance germination in the tpk2 mutant

The low level of PKA activity remaining in the *tpk2* mutant could account for its depressed ability to germinate in liquid medium as was previously suggested by Sonneborn et al. (2000).

To verify this hypothesis, we assessed the germinative capability of the *tpk2* null mutant cells in the presence of compounds which are known to stimulate PKA activity in permeabilized cells (Paveto et al., 1991).

Table 2 summarizes the results obtained when glucagon, dbcAMP, or hCG were added to the induction media together with GlcNAc. As can be seen, the basal germination level attained in the presence of suboptimal concentrations of GlcNAc (0.1 mM) was markedly increased by the stimulators in both strains. As expected, germination was blocked by MyrPKI in all cases. These results indicate that a lower PKA activity in the cell correlates with a weaker germinative response to GlcNAc.

3.7. Serum-induced hyphal morphogenesis in the tpk2 null mutant

We have previously observed for *C. albicans* 1001 strain, that MyrPKI was unable to block germination induced by 10% serum (Castilla et al., 1998). Similar results were obtained in this study using the *tpk2* null mutant (not shown). These results are in agreement with the hypothesis of the existence of separate signalling pathways mediating serum and GlcNAc-induced morphogenesis, only the latter involving PKA.

To further investigate the biochemical nature of the mechanisms involved in serum and GlcNAc-induced germination, we analysed the effect of PKA inhibition on germination promoted by low serum concentrations. The results obtained are summarized in Fig. 4. As can be seen, in the CAI4 parental strain, 0.5% serum induced only 20% germination while 2.5 and 5% serum concentrations allowed complete transition to the hyphal form. In the *tpk2* mutant strain, the presence of 0.5% serum had no visible effect while 2.5% serum elicited 50% germination and 5% serum complete germination. These results suggest an interrelation between serum and PKA-mediated signalling pathways which is only revealed at low serum concentrations. The results obtained in the presence of MyrPKI support this hypothesis. In fact, inhibition of PKA activity in the parental strain promoted inhibition of morphogenesis at low serum concentrations (0.5 and 2.5%). In addition, this effect was much more evident in the mutant where 0.5, 2.5 and 5% serum-induced germination was inhibited by MyrPKI. Taking into account that PKA activity is about 10-fold higher in the parental strain compared

Table 2
Effect of stimulators of PKA on the *tpk2* mutant germination

Additions	MyrPKI (10 μM)	Germ-tube formation (%)	
		Parental strain CAI4	*tpk2* mutant strain
None	–	10 ± 3	5 ± 2
Glucagon (10 μM)	–	51 ± 4	42 ± 2
	+	0	0
dbcAMP (0.1 mM)	–	42 ± 2	35 ± 4
	+	0	0
hCG (50 U)	–	35 ± 4	30 ± 4
	+	0	0

Note. Yeast cells were induced to germinate as stated in Section 2 with suboptimal concentrations of GlcNAc (0.1 mM). Activators were added, together with GlcNAc, at the indicated concentrations.

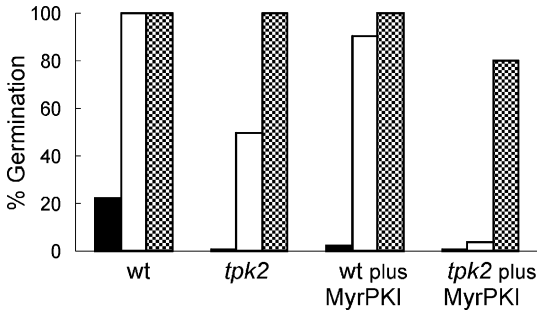

Fig. 4. Effect of MyrPKI on germ-tube formation induced by low serum concentrations in wild-type and *tpk2* cells. Yeast cells were induced to germinate as indicated in Section 2 in the presence of 0.5% (black bars), 2.5% (white bars) and 5% (grey bars) serum in the presence or in the absence of 10 μM MyrPKI. The percentage of germination was scored 2 h after induction; wt, parental strain CAI4.

to that in the *tpk2* null mutant strain, it is possible that the MyrPKI concentration used in the experiments was not sufficient to abolish all PKA activity (from *TPK1* and *TPK2*) in the parental strain. This could account for the maintenance of a serum-induced cAMP-dependent germinative capability. Conversely, it could be that the severely reduced PKA activity in the *tpk2* null mutant was completely inhibited by MyrPKI and thus germination at low serum concentration could not be sustained. We hypothesize that a second, PKA-independent germination pathway plays a role at high serum concentrations thus explaining why MyrPKI failed to block germination at 2.5% serum concentration or higher in wild-type strains.

4. Discussion

Biochemical and genetic studies provided evidence of the central role that PKA plays in regulating the yeast-to-hypha differentiation in *C. albicans* (Bahn and Sundstrom, 2001; Bockmühl et al., 2001; Castilla et al., 1998; Sonneborn et al., 2000). The purpose of the present study was to further investigate the biochemical mechanisms underlying this PKA signaling pathway. To that end, the *C. albicans TPK2* gene coding a PKA catalytic subunit was isolated and characterized. The sequence of the gene predicted a 422 amino acids protein containing all the conserved domains diagnostic for a protein kinase, including the FXXF carboxy terminal sequence characteristic of PKAs. While the work was in progress, Sonneborn et al. (2000) reported the isolation of *CaTPK2* gene and its identification as being the gene encoding the Tpk2p isoform of the PKA catalytic subunit.

To gain insight into the role of PKA in morphogenesis, a *tpk2* homozygous null mutant was generated and its ability to germinate in liquid media was evaluated. No phenotypic differences were observed in the *tpk2* null strain except that GlcNAc-induced morphogenesis in liquid media was impaired compared to that of the parental strain. The fact that germination of the *tpk2* null mutant was completely blocked in the presence of the PKA inhibitor MyrPKI, was indicative of the presence of PKA enzymatic activity in this mutant. This result together with the measurement of PKA activity, provided biochemical confirmation of the existence of a catalytically active, second isoform of PKA as reported recently by Bockmühl et al. (2001). Here, we were able to identify an mRNA from this second form of PKA in both wild-type and *tpk2* null mutant cells.

The PKA activity measured in the *tpk2* mutant was very low in comparison to that of the parental strain indicating that, as was previously found in *S. cerevisiae* (Mazon et al., 1993; Robertson et al., 2000), cells do not compensate for the loss of one of the C isoforms by overexpression of the other. Nevertheless, we cannot ascertain whether the activity displayed by the mutant reflects the physiological contribution of Tpk1p to the total activity of the wild-type since the existence of regulatory interactions between *TPK1* and *TPK2* cannot be ruled out at this point.

A multiplicity of genes encoding PKA catalytic subunits has been described in mammals and fungi (Toda et al., 1987; Uhler et al., 1986). In *S. cerevisiae*, three genes have been identified, *TPK1*, *TPK2*, and *TPK3*, having a functionally redundant role in yeast-like growth and distinct roles in filamentous growth; while *TPK2* acts positively, *TPK1* and *TPK3* act negatively (Pan and Heitman, 1999; Robertson and Fink, 1998). Only two genes, *TPK1* and *TPK2*, encoding PKA isoforms are present in the in *C. albicans* genome (http://www-sequence.stanford.edu/group/candida). According to our results, in spite of the high level of identity shared by *C. albicans* Tpk1p and Tpk2p and their corresponding counterparts in *S. cerevisiae*, specialized functions for each protein with respect to hyphal growth in *C. albicans* are not evident, at least in GlcNAc induced liquid media cultures. In fact, a positive role for the Tpk1p isoform in morphogenetic process is substantiated by the fact that despite its low PKA activity the *tpk2* null mutant is able to germinate. Moreover its germinative capability was substantially improved by in vivo activation of the PKA (Table 2), indicating that the total PKA activity level is critical for triggering germination regardless of the PKA isoform involved. Recently, Bockmühl et al. (2001) established that both *TPK1* and *TPK2* acts positively in both solid and liquid media.

We have previously proposed that serum and GlcNAc act through different pathways to induce the *C. albicans* yeast-to-hypha transition (Castilla et al., 1998). The strongest support for this was the fact that 10% serum was able to overcome the blockade of the GlcNAc-induced morphogenesis promoted by MyrPKI or

by H-89 (Castilla et al., 1998 and our unpublished results). Analysis of the germinative response of the wild-type and *tpk2* null mutant strains to low serum concentrations revealed that PKA is involved in the morphogenetic transition when induced by suboptimal concentrations of the inducer. The germination results obtained at higher serum concentrations could be explained assuming that under these conditions the accumulation of an unidentified serum component is favored which, upon reaching a threshold concentration, triggers germination through a PKA-independent pathway.

Recently, Rocha et al. (2001), have demonstrated that *C. albicans* cells missing both alleles of the adenylyl cyclase gene were unable to germinate under all conditions tested, including liquid and solid serum-containing media. This result suggests that cAMP plays an essential role for transducing environmental signals to both the MAPK and the cAMP/PKA pathways. If according to our proposal, the filament-inducing activity of serum at high concentrations does not rely on PKA activity, then cAMP would act through binding and hence activating targets other than PKA involved in an alternative pathway leading to germination. The fact that the defective hyphal formation of *C. albicans ras1* null mutant could not be suppressed by over expression of either *TPK1* or *TPK2* (Bockmühl et al., 2001) could also be explained through the existence of a cAMP-dependent, PKA-independent pathway involved in the yeast-to-hypha transition. In recent years, evidence of the existence of such target proteins and their importance in cellular signaling in mammals have been presented (Laroche-Joubert et al., 2002). This seems also to be the case in lower eukaryotes. In fact, in *Dictyostelium*, Anjard et al. (2001) have reported that synthesis of cAMP is required for spore differentiation even in the presence of an over-expressed PKA catalytic subunit, suggesting a role for cAMP besides that of being a PKA activator.

Accumulating evidence points to the existence of multiple signaling pathways controlling hyphal growth in *C. albicans* (Ernst, 2000), but the complex interactions between different routes makes it difficult to assess the biochemical mechanisms involved in their regulation. Although the cAMP/PKA signaling pathway clearly appears to play a major role in serum and GlcNAc-induced transition (Bahn and Sundstrom, 2001; Bockmühl et al., 2001; Castilla et al., 1998; Rocha et al., 2001; Sonneborn et al., 2000 and the present work), to date, only Efg1p, a regulator of *C. albicans* morphogenesis, has been identified as a potential PKA target. Its phosphorylation status has been related to hyphal growth (Bockmühl and Ernst, 2001). There is no doubt that searching for the existence of yet unknown cAMP targets as well as for other Tpk1 or Tpk2 substrates, will allow a better understanding of the role of cAMP in the complex network controlling the switch from yeast to filamentous growth in *C. albicans*. These studies are now in progress in our laboratory.

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