**Yeast**
*Yeast* 2003; **20**: 471–478.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/yea.977

**Research Article**

# cDNA cloning, biochemical and phylogenetic characterization of $\beta$- and $\beta'$-subunits of *Candida albicans* protein kinase CK2

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Received: 15 November 2002
Accepted: 7 December 2002

## Abstract

**We have previously reported that *Candida albicans* protein kinase CK2 is composed of two distinct catalytic ($\alpha$- and $\alpha'$-) and two distinct regulatory ($\beta$- and $\beta'$-) subunits. We report here the isolation of two cDNAs clones, *CaCKB1* and *CaCKB2*, encoding *C. albicans* $\beta$- and $\beta'$-subunits, respectively. The predicted $\beta$- and $\beta'$-proteins have calculated molecular masses of 34 kDa and 31 kDa and show all major features conserved in $\beta$-subunits of other organisms, including the N-terminal autophosphorylation site, the internal acidic region and a potential metal-binding motif. The deduced amino acid sequence of *C. albicans* $\beta$-subunit displays 48% identity with that of *Saccharomyces cerevisiae* and has an unusually long C-terminal acidic region containing a putative autophosphorylation site. *C. albicans* $\beta'$ shows 54% sequence identity with its *S. cerevisiae* homologue. Semi-quantitative RT–PCR analyses indicate that the mRNAs corresponding to both subunits are present in similar amounts in the yeast and hyphal forms of the fungus. To evaluate the biochemical properties of *C. albicans* $\beta$- and $\beta'$-subunits, both proteins were expressed in *Escherichia coli* and purified. Experiments performed *in vitro* indicate that both recombinant subunits reconstitute a fully functional holoenzyme when incubated with stoichiometric amounts of human recombinant $\alpha$-subunit, as judged by their ability to abolish basal phosphorylation of calmodulin by human recombinant $\alpha$-subunit and the reversion of the inhibitory effect by polylysine. In addition, both regulatory subunits can be phosphorylated by human recombinant $\alpha$ subunit. Phylogenetic analysis of $\beta$- and $\beta'$-proteins of *C. albicans* and other organisms shows that the *CKB* gene duplication occurred before the split of the ascomycete and basidiomycete lineages. cDNA sequences of *C. albicans CKB1* (Accession No. AF0599060) and *CKB2* (Accession No. AY172319) have been deposited in the GenBank database.** 

**Keywords:** ***Candida albicans*; protein kinase CK2; regulatory subunit; phylogeny**

## Introduction

Protein kinase CK2 (CK2) is a highly conserved serine/threonine protein kinase ubiquitous among eukaryotic organisms. It has more than 100 known substrates and has been implicated in the regulation of several important cellular processes such as transcription, growth control, cell cycle regulation and morphogenesis (reviewed in Pinna, 1997; Glover, 1998). The mammalian enzyme is composed of two catalytic ($\alpha$- and/or $\alpha'$-) and two identical regulatory subunits ($\beta$-) that associate to form a native active $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ holoenzyme. CK2 activity is stimulated *in vitro* by polybasic peptides such as polyarginine and polylysine, and is inhibited by polyanions, but the mechanism of

regulation of the enzyme *in vivo* remains elusive (Leroy *et al*., 1995). While early studies have established that the $\alpha$-subunit bears the catalytic site of the enzyme, the physiological role of the $\beta$-subunit is still conjectural. For most substrates, the $\beta$-subunit behaves as a general activator of catalytic activity, stimulating it 5–10-fold; however, this is not true for a small subset of potential substrates, of which calmodulin is paradigmatic. For this group of substrates, the $\beta$-subunit acts as an inhibitor of phosphorylation by the catalytic subunit (Meggio *et al*., 1994). In addition, the $\beta$-subunit stabilizes the catalytic subunit against proteolytic degradation and denaturation by heat or urea (Meggio *et al*., 1992a). *In vitro*, the $\beta$-subunit undergoes autophosphorylation but the physiological importance of this process has not been determined.

In the last decade, genes encoding CK2 $\alpha$- and $\beta$-subunits have been cloned in the yeasts *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and, recently, in the filamentous fungus *Neurospora crassa. S. cerevisiae* has two genes (*CKA1* and *CKA2*) coding for the $\alpha$- and $\alpha'$-subunits and two genes (*CKB1* and *CKB2*) encoding $\beta$- and $\beta'$-subunits, respectively (Chen-Wu *et al*., 1988; Padmanabha *et al*., 1990; Reed *et al*., 1994; Bidwai *et al*., 1995), while *N. crassa* has two genes for the $\beta$- and one for the $\alpha$-subunit (Yang *et al*., 2002). Although in the fission yeast *Sz. pombe* only one gene for the catalytic subunit and one for the regulatory subunit have been described (Roussou and Draetta, 1994), a second *CKB* gene was recently found during the sequencing of the *Sz. pombe* genome (Wood *et al*., 2002).

Previously, we have provided biochemical evidence indicating that the *C. albicans* CK2 holoenzyme is a heterotetramer composed of $\alpha$-, $\alpha'$-, $\beta$- and $\beta'$-subunits (Walz *et al*., 1998). Here, we report the cloning of *C. albicans* CK2 $\beta$- (*CaCKB1*) and $\beta'$- (*CaCKB2*) cDNAs as well as their heterologous expression and biochemical characterization. We also present a phylogenetic analysis of *C. albicans* Ckb proteins to determine their evolutionary relationship to Ckb from other organisms.

## Materials and methods

### Organism and culture conditions

*C. albicans* yeast cells (ATCC 32354) were grown to late exponential phase in YPD medium (2% w/v dextrose, 2% w/v peptone, 1% w/v yeast extract) at 28 °C with continuous shaking. Yeast-to-hyphal transition was induced by incubation with 5 mM N-acetyl-glucosamine at 37 °C, essentially as described by Shepherd *et al*. (1980).

### Isolation of *CKB1* and *CKB2* cDNAs

All DNA manipulations were carried out using standard techniques (Sambrook *et al*., 2000). Degenerate primers (5′-TTRTAYGGWTTRATHCATGCH-3′ and 5′-DGTHCCAAARTADGCWCCDTC-3′) were synthesized based on highly conserved regions of the corresponding subunits and used to PCR-amplify CKB sequences from a *C. albicans* cDNA library. A PCR fragment of 270 bp was obtained and, based on its sequence, a new pair of primers was synthesized and used to screen a *C. albicans* λZAP cDNA library (kindly provided by A. J. P. Brown; Swoboda *et al*., 1993) by sib selection (Isola *et al*., 1991). A cDNA clone was isolated corresponding to the *CaCKB1* cDNA. During the course of this work, the partial sequence of a different *CKB* gene was released at the *C. albicans* Genome Project website (**www. sequence. standford. edu/group/candida**). Based on this sequence, we designed appropriate primers to isolate this second *CKB* gene (*CaCKB2*) from the same a *C. albicans* λcDNA library. Both Bluescript cDNAs clones were excised from λZAP, following the manufacturer's instructions (Stratagene).

### RT–PCR analysis

Total cellular RNA was extracted from *C. albicans* yeast cells by the hot acidic phenol method (Ausubel *et al*., 1994). RT–PCR was performed using standard techniques (Sambrook *et al*., 2000). Primers 5′-ATTGTCTCTGCAAGTACC-3′ and 5′-TGCAGAATGTGCCAATAACGC-3′ were used to amplify a 190 bp fragment from *CaCKB1*, while primers 5′-GTATATTATATGGATTGATTCATGC-3′ and 5′-ATACAATTTAACTGATGCCATTC-3′ were used to amplify a 179 bp fragment from *CaCKB2*. Normalization was done amplifying histone H4 cDNA with primers 5′-GCTAAACGTCACAGAAAG-3′ and 5′-GATCCAATGAAGGACGG-3′.

### Site-directed mutagenesis, expression and purification of $\beta$- and $\beta'$-subunits

Since in *C. albicans* the CUG codon codes for serine, it was necessary to mutagenize serine codons present in the *CaCKB1* ($S^{44}$) and *CaCKB2* ($S^{50}$) cDNA sequences from CTG to TCG to allow for a correct expression of the $\beta$-subunits in *E. coli*. A two-stage PCR, using megaprimers (Sarkar and Sommer, 1990), was performed using the excised Bluescript plasmids containing the *CKB* cDNAs as template. The first PCR stage was performed according to standard protocols, using 100 ng template, 25 pmol 3′-specific primer (containing a *Bam*HI restriction site) and an excess (50 pmol) of mutagenic primer. Each cycle consisted of 30 s at 94 °C, 60 s at 55 °C and 30 s at 72 °C. The product of the first PCR stage was used as a megaprimer for the second PCR step, using an excess of 5′-specific primer (containing a *Nde*I restriction site). The final PCR products were digested with *Nde*I and *Bam*HI and cloned into the pET28a-c(+) bacterial expression vector (Novagen). The fidelity of the cloned sequences was checked by automated dideoxy-DNA sequencing. pET28a-c(+) bearing $\beta$ or $\beta'$ sequences were transformed into *E. coli* BL21(DE3) by electroporation. Transformants were grown individually overnight at 37 °C in 3 ml LB medium containing 30 μg/ml kanamycin. The whole culture was then used to inoculate 50 ml fresh LB-kanamycin medium. When the culture reached $OD_{600} = 0.6$, protein expression was induced with a final concentration of 1 mM IPTG. After an additional 3 h incubation period, cells were harvested and used for protein purification. Purification of the expressed N-terminal His-tagged $\beta$-subunits through $Ni^{2+}$ chelating resin and elimination of the His-tag were performed according to the manufacturer's instructions (Novagen).

### Phosphorylation assays

The interaction between $\alpha$- and $\beta$- and/or $\beta'$-subunits was investigated by assessing the ability of $\beta$- or $\beta'$-subunits to inhibit basal phosphorylation of calmodulin by the recombinant human CK2-$\alpha$-subunit (rhCK2-$\alpha$). Calmodulin (10 μM) was phosphorylated for 10 min at 37 °C in a medium containing 50 mM Tris–HCl buffer, pH 7.5, 12 mM $MgCl_2$, 20 μM [$\gamma$-$P^{32}$]ATP (specific activity, 1000–2000 cpm/pmol) and rhCK2-$\alpha$ (100 nM) alone or in combination with equimolar amounts of $\beta$- and/or $\beta'$-subunits. Phosphorylation of $\beta$- and $\beta'$-subunits was performed at 37 °C using rhCK2-$\alpha$ (100 nM) and $\beta$- or $\beta'$-subunits (5 μM) in a 20 μl assay mixture containing 50 mM Tris–HCl buffer, pH 7.5, 12 mM $MgCl_2$, 50 μM [$\gamma$-$^{32}$P]ATP (specific activity 2000 cpm/pmol). The reactions were stopped by boiling after addition of an equal volume of 2× SDS sample buffer (Laemmli, 1970). The samples were analysed by 12% SDS–PAGE. Gels were dried and autoradiographed at −70 °C during 18 h.

### Phylogenetic analysis

*CKB* gene sequences were retrieved by homology searches using the BLAST program (Altschul *et al*., 1990) from the Genbank database (**www.ncbi.nih.gov**) and from the following fungal genome project home pages: *Sz. pombe* (**www.sanger.ac.uk/Projects/S_pombe**); Génolevures Project (**http:// cbi.labri.ubordeaux.fr/Genolevures**); *Phanerochaete chrysosporium* (Joint Genome Institute, **www.jgi.doe.gov/programs/whiterot.htm**) and *Cryptococcus neoformans* (The Institute for Genomic Research; **www.tigr.org**). In some cases, complete sequences had to be assembled from raw partial reads. Deduced Ckb protein sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) with default parameters, and regions that could not be unambiguously aligned were eliminated (alignment available upon request). Phylogenetic analysis was performed using the PHYLIP program package, Version 3.57c (Felsenstein, 1995). Pairwise protein distances were calculated using the Dayhoff PAM001 matrix (PROTDIST program) and trees were constructed using the neighbour-joining method (NEIGHBOR program; Saitou and Nei, 1987). A consensus majority rule tree was constructed with CONSENSE and confidence values were estimated with SEQBOOT using 100 bootstrap replicates of the sequence data. GenBank Accession Nos of annotated sequences used in this paper are: *H. sapiens* Ckb (X16312); *D. melanogaster* Ckb1 (AAA28430); Ckb2 (AAD00080); *Sz. pombe* Ckb1 (X74274); Ckb1′ (AL033385); *N. crassa* Ckb1 (AAM14625); Ckb2 (AAM14626); *A. thaliana* Ckb1 (P40228); Ckb2 (P40229); Ckb3 (O81275); Ckb4 (O80507); *S. cerevisiae* Ckb1 (A56421);

Ckb2 (A54907); *K. thermotolerans* Ckb1 (assembled from AL421646 and AL421647); *P. angusta* Ckb1 (assembled from AL433810 and AL431426).

## Results and Discussion

### Isolation and characterization of *C. albicans CK2* $\beta$- and $\beta'$-subunit cDNAs

Using a PCR-based approach, we have isolated two *CKB* clones from a *C. albicans* cDNA library. The two CK2 $\beta$-subunit cDNAs were named *CaCKB1* and *CaCKB2*, based on amino acid sequence identity with the *S. cerevisiae* Ckb1 and Ckb2 proteins. The 1.2 kb *CaCKB1* cDNA encodes a putative 293-residue polypeptide that has highest identity (48%) with the *S. cerevisiae* $\beta$-subunit encoded by *CKB1* (Bidwai *et al*., 1995). The *CaCKB2* cDNA is also 1.2 kb long and codes for a putative 267-residue polypeptide with highest identity (54%) to the *S. cerevisiae* $\beta'$-subunit encoded by *CKB2* (Reed *et al*., 1994). Alignment of the deduced amino acid sequences of *C. albicans* $\beta$- and $\beta'$-subunits with those from several organisms (Figure 1) shows that both *C. albicans* Ckb proteins contain all major features common to $\beta$-subunits, viz. the N-terminal autophosphorylation site ($S^3$DPEED in CaCkb1 and $T^8$DSSSD in CaCkb2), the region involved in the interaction with the $\alpha$-subunit [GA(F/Y)GTXFP; Boldyreff *et al*., 1996] and the zinc-finger motif implicated in $\beta$-dimerization ($CPX_3C$-$X_{22}$-$CPX_1C$; Chantalat *et al*., 1999). An internal acidic region that binds to polyamines and is involved in regulating the interaction with the $\alpha$-subunit is also found in both *C. albicans* $\beta$-subunits around position 60.

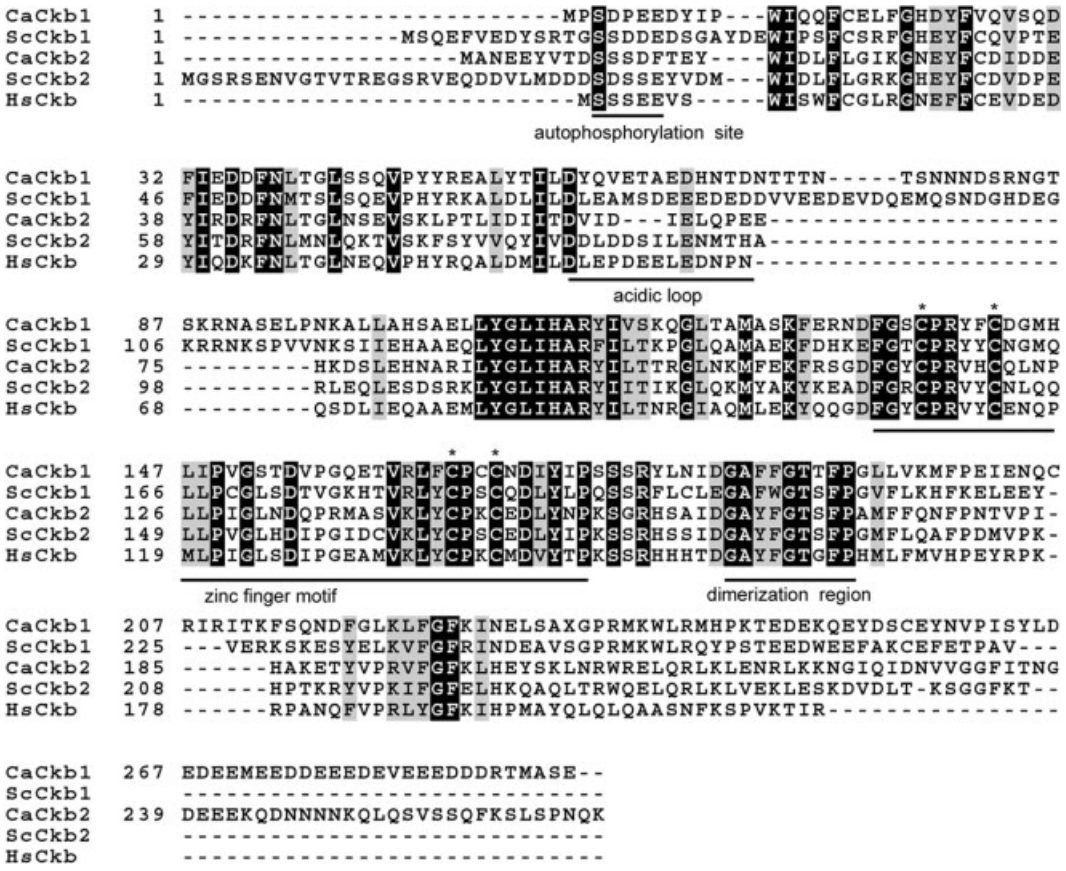


**Figure 1.** Alignment of the deduced amino acid sequence of CK2 $\beta$-subunits from various species. Sequences shown are: *C. albicans* Ckb1 and Ckb2; *S. cerevisiae* Ckb1 and Ckb2 and *H. sapiens* Ckb. Invariant residues are boxed in black and conservative changes are boxed in grey. The acidic loop, the autophosphorylation site, the $\alpha$-dimerization motif and the zinc-finger motif are underlined. Cysteine ligands of the zinc are marked with an asterisk

An unusually long C-terminal tail of strong acidic character is found in CaCkb1, while CaCkb2 has a long C-terminal tail containing several glutamine and asparagine residues. In addition, CaCkb1 has a 26 amino acid insertion between positions 72 and 95, similar to that of *S. cerevisiae* Ckb1 (Figure 1; Bidwai *et al*., 1995).

To assess whether *C. albicans CKB* genes would be differentially expressed during yeast to hyphal transition, RT–PCR analyses were performed on mRNA samples obtained from yeast and mycelial cells using specific primers. The results (not shown) indicate that *CKB1* and *CKB2* mRNAs are present in both morphological forms and that they do not seem to be transcriptionally regulated during the morphogenetic transition.

## Biochemical characterization of recombinant *C. albicans CK2* $\beta$-subunits

To study the biochemical characteristics of *C. albicans* CK2 $\beta$-subunits *in vitro*, His-tagged versions of $\beta$- and $\beta'$-subunits were produced by cloning their cDNAs into vector pET28a for recombinant expression of the proteins in *E. coli*. Purification of the proteins by $Ni^{2+}$ affinity-chromatography and subsequent thrombin cleavage of the His-tag portion yielded highly purified recombinant proteins of apparent molecular masses of 40 and 34 kDa for $\beta$- and $\beta'$-subunits, respectively (Figure 2). These values are slightly higher than those predicted from their deduced primary structures (34 kDa for $\beta$ and 31 kDa for $\beta'$). This apparent difference could be attributed to the intrinsic electrophoretic properties of the proteins or to post-translational modifications in the bacterial cells.

The ability of the recombinant $\beta$-subunits to reconstitute a functional holoenzyme was assessed by testing its ability to inhibit basal phosphorylation of calmodulin by rhCK2-$\alpha$ (Meggio *et al*., 1992b). This inhibitory effect of recombinant $\beta$-subunits on calmodulin phosphorylation can be reversed by polybasic compounds, e.g. polylysine. As can be seen in Figure 3, phosphorylation of calmodulin by rhCK2-$\alpha$ is completely abolished by the addition of recombinant $\beta$-subunits and regained by the addition of polylysine at concentrations that do not affect phosphorylation by rhCK2-$\alpha$ alone.

It has long been known that the CK2 $\beta$-subunit is phosphorylated by $\alpha$- and that this phosphorylation

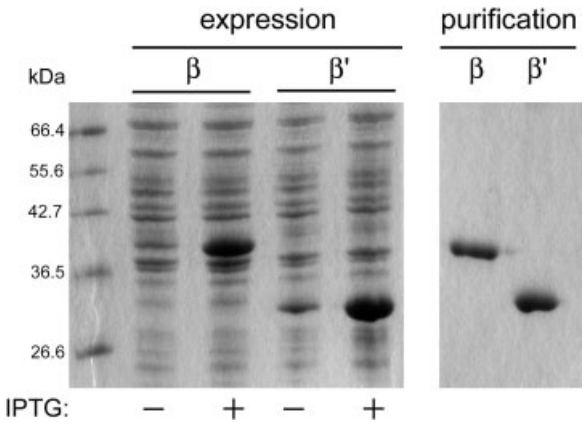


**Figure 2.** Expression and purification of recombinant $\beta$- and $\beta'$-subunits. Left panel: Coomassie blue-stained polyacrylamide gel of protein extracts from *E. coli* strain BL21 (DE3), transformed with pET28 vectors harbouring $\beta$- or $\beta'$-cDNAs, in the absence (–) or presence (+) of 1 mM IPTG. Right panel: Coomassie blue-stained polyacrylamide gel showing recombinant $\beta$- and $\beta'$-proteins purified by $Ni^{2+}$ chromatography. Purified $\beta$- and $\beta'$-proteins have molecular masses of 40 and 34 kDa, respectively

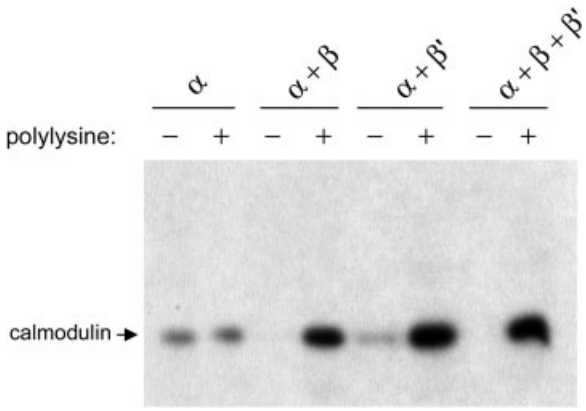


**Figure 3.** Inhibition of calmodulin phosphorylation by $\beta$- and $\beta'$-subunits. Calmodulin (10 μM) was phosphorylated by rhCK2-$\alpha$ (100 nM) alone ($\alpha$) or with equimolar amounts of recombinant *C. albicans* $\beta$ ($\alpha+\beta$) or $\beta'$ ($\alpha+\beta'$) or combined $\beta$- and $\beta'$-subunits ($\alpha+\beta+\beta'$) in the absence (–) or presence of 2 M polylysine for 10 min at 37 °C. Proteins were fractionated by SDS–PAGE and the dried gel was autoradiographed. The figure shows the autoradiogram of one representative experiment

is inhibited by polybasic compounds, either when it occurs within the CK2 heterotetramer or when an excess of recombinant $\beta$-subunit is incubated with the native holoenzyme (Meggio *et al*., 1992b). To evaluate whether the recombinant *C. albicans*

$\beta$-subunits can be phosphorylated, phosphorylation experiments were conducted using rhCK2-$\alpha$ in the absence of substrate. As expected, recombinant $\beta$- and $\beta'$-subunits are very good substrates for rhCK2-$\alpha$ and the autophosphorylation is inhibited by the addition of polylysine (Figure 4).

We conclude that recombinant *C. albicans* $\beta$-subunits behave as the native proteins, inhibiting calmodulin phosphorylation by the rhCK2-$\alpha$, as observed for other CK2 kinases (Meggio *et al*., 1992b) and being phosphorylated by the rhCK2-$\alpha$ as previously demonstrated for the purified native holoenzyme from *C. albicans* (Walz *et al*., 1997).

## Evolutionary analysis of *C. albicans* $\beta$-subunit proteins

To determine the evolutionary relationship of the *C. albicans* Ckb proteins with those of other organisms, we compared its sequence with fungal Ckb amino acid sequences obtained from complete and ongoing large-scale public sequencing projects. From the French Génolevures Project (Souciet *et al*., 2000), we obtained complete sequences of *CKB* genes from the hemiascomycetous yeasts *Kluyveromyces thermotolerans* and *Pichia angusta*, which are most similar to *C. albicans* Ckb1. Although we used only the *P. angusta* and *K. thermotolerans CKB1* sequences, we found partial sequences of *CKB2* genes in these species (Génolevures Project). In addition, we obtained

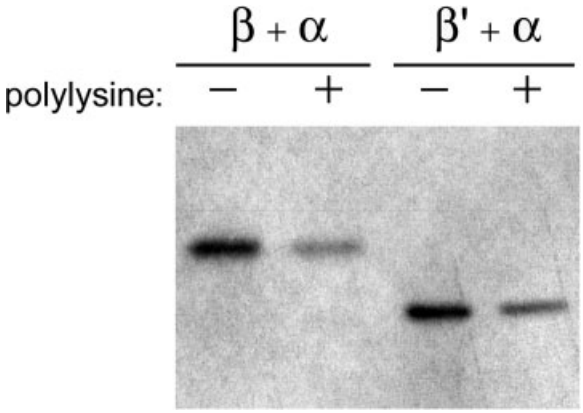


**Figure 4.** Phosphorylation of $\beta$- and $\beta'$-subunits by rhCK2-$\alpha$ subunit. Recombinant *C. albicans* $\beta$- or $\beta'$-subunits (5 μM) were incubated with rhCK2-$\alpha$ (100 nM) in the absence (−) or presence (+) of 2 μM polylysine for 10 min at 37 °C. Proteins were separated by SDS–PAGE and the dried gel was autoradiographed

preliminary *CKB* gene sequences of basidiomycetous fungi from the genome project websites of *Phanerochaete chrysosporium* and *Cryptococcus neoformans* (see Materials and methods). In this paper, we classify these genes as either *CKB1* or *CKB2* according to the similarity of their deduced peptide products to the *S. cerevisiae* Ckb proteins. In our analysis, we also used the recently described Ckb1 and Ckb2 proteins from the filamentous ascomycete *N. crassa* (Yang *et al*., 2002), two Ckb proteins from the archiascomycete *Sz. pombe*; Ckb1 (Roussou and Draetta, 1994) and a second *CKB* gene found during the sequencing of the *Sz. pombe* genome (Wood *et al*., 2002). Since this second *Sz. pombe* Ckb protein is actually more similar to the *S. cerevisiae* and *C. albicans* Ckb1 (see below), we have tentatively named it Ckb1′ in this paper.

The phylogeny of *C. albicans* Ckb proteins was analysed using the PHYLIP package of phylogenetic inference (Felsenstein, 1995), using a distance matrix and the neighbour-joining methods, with branching support evaluated by 100 bootstrap replicates. The tree was constructed using an alignment of amino acid sequences from 14 fungal, three animal and four plant Ckb proteins. In the tree (Figure 5), fungal Ckb proteins fall clearly into two groups, supported by high bootstrap values. In the Ckb1 group, CaCkb1 clusters with the Ckb1 proteins of *P. angusta, S. cerevisiae, K. thermotolerans, N. crassa*, the Ckb1′ of *Sz. pombe* and the Ckb1 proteins from the basidiomycetes *P. chrysosporium* and *C. neoformans*. In the Ckb2 group, CaCkb2 clusters with the Ckb2 proteins of *S. cerevisiae, N. crassa, P. chrysosporium, C. neoformans* and the Ckb1 protein of *Sz. pombe*.

The tree shows that the two *C. albicans CKB* genes did not originate recently, but are rather the result of an ancient gene duplication event that occurred in the fungal lineage. *CKB* gene duplications in plant (as in *Arabidopsis*) and animal (as in *Drosophila*) lineages have clearly occurred independently from the gene duplication in fungi. The *CKB* gene duplication in fungi must have happened before the ascomycete-basidiomycete split, since the basidiomycetes *P. chrysosporium* and *C. neoformans* have two Ckb proteins which cluster tightly with ascomycete Ckb1 and Ckb2 proteins. It has recently been shown that *S. cerevisiae* and some of its close relatives are degenerate

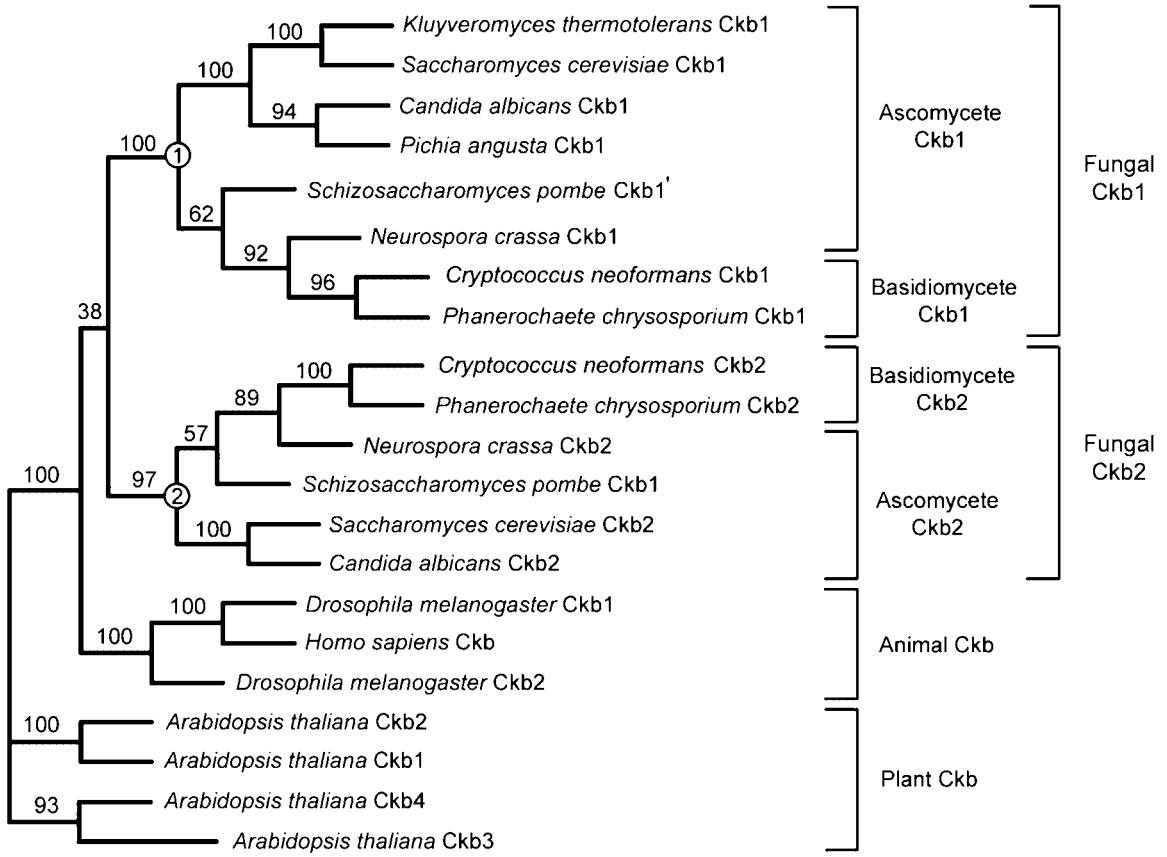


**Figure 5.** Phylogeny of $\beta$-subunit (Ckb) proteins. Consensus unrooted phylogenetic tree of Ckb proteins constructed using the neighbour-joining method as implemented in the PHYLIP program package. Bootstrap values on the branches of the tree represent the number of times out of 100 in which the group of sequences at the right of the branch were present in the consensus tree. Note that both ascomycete and basidiomycete Ckb proteins are arranged into Ckb1 (node 1) and Ckb2 (node 2) subgroups. *C. albicans* Ckb1 = $\beta$-subunit and *C. albicans* Ckb2 = $\beta'$-subunit

tetraploids in relation to other hemiascomycetous yeasts like *C. albicans* (Wolfe and Shields, 1997; Wong *et al*., 2002). Since *S. cerevisiae* possesses only two *CKB* genes like other hemiascomycetes, extra *CKB* gene copies generated after the ancient whole-genome duplication must have been lost from its genome.

## Acknowledgements

We are grateful to Dr Alistair J. P. Brown for providing the *C. albicans* cDNA library and to Dr Olaf G. Issinger for his generous gift of rhCK2-$\alpha$. We are also indebted to Dr Eduardo Passeron for critical reading of the manuscript. Sequence data for *C. albicans* was obtained from the Stanford DNA Sequencing and Technology Center website at **http://www-sequence.standford.edu/group/candida**. Sequencing of *C. albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. *P. chrysosporium* data has been provided freely by the JGI for use in this publication only. Preliminary sequence data from *C. neoformans* was obtained from The Institute for Genomic Research (website at **http://www.tigr.org**). SP is research member from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET). This work was funded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), the Medical Research Council of Canada and the Fonds Emile-Beaulieu.

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