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# Identification and characterization of a highly conserved calcineurin binding protein, CBP1/calcipressin, in *Cryptococcus neoformans*

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**Calcineurin is the conserved target of the immunosuppressants cyclosporin A and FK506. Using the yeast two-hybrid system, we identified a novel calcineurin binding protein, CBP1, from the pathogenic fungus *Cryptococcus neoformans*. We show that CBP1 binds to calcineurin *in vitro* and *in vivo*, and FKBP12–FK506 inhibits CBP1 binding to calcineurin. *Cryptococcus neoformans cbp1* mutant strains exhibit modest defects in growth under stress conditions and virulence, similar to but less severe than the phenotypes of calcineurin mutants. *Saccharomyces cerevisiae* mutants lacking the CBP1 homolog RCN1 are, like calcineurin mutants, sensitive to lithium cation stress. CBP1 shares a central peptide sequence motif, SPPxSPP, with related proteins in *S.cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Caenorhabditis elegans* and humans, and peptides containing this motif altered calcineurin activity *in vitro*. Interestingly, the human CBP1 homolog DSCR1 is encoded by the Down's syndrome candidate region interval on chromosome 21, is highly expressed in the heart and central nervous system, and may play a role in calcineurin functions in heart development, neurite extension and memory.**

*Keywords*: calcineurin/*Cryptococcus neoformans*/Down's syndrome/pathogenic fungi/protein phosphatase

## Introduction

Calcineurin is a $Ca^{2+}$/calmodulin-activated serine/threonine-specific phosphatase that is structurally and functionally conserved from yeast to man (reviewed in Hemenway and Heitman, 1999). The enzyme is a heterodimer composed of a catalytic A subunit and a regulatory B subunit that has four EF hands and is structurally related to calmodulin. Calcineurin is a critical calcium sensor in cells. When $Ca^{2+}$ levels are low, calcineurin is inactive because a C-terminal autoinhibitory domain is docked in the active site. When intracellular $Ca^{2+}$ levels increase, $Ca^{2+}$/calmodulin binds to a C-terminal calmodulin binding site on the calcineurin A subunit, causing conformational changes that dislodge the autoinhibitory domain to result in enzyme activation (Hashimoto *et al*., 1990). Introduction of a point mutation into the autoinhibitory domain or proteolytic cleavage of the autoinhibitory domain results in constitutive activation of calcineurin (Hubbard and Klee, 1989; Fruman *et al*., 1995).

The immunosuppressive drugs cyclosporin A (CsA) and FK506 inhibit calcineurin in association with highly conserved binding proteins, cyclophilin A and FKBP12, which are conserved from yeast to humans (Liu *et al*., 1991; Foor *et al*., 1992; Breuder *et al*., 1994; Odom *et al*., 1997; Cruz *et al*., 1999). CsA and FK506 inhibit the immune system by blocking signal transduction in the antigen response pathway of T-lymphocytes. Normally, antigen presentation to the T-cell receptor results in increased intracellular $Ca^{2+}$ levels, and calcineurin is activated and regulates nuclear import of the transcription factor NF-AT (Shaw *et al*., 1995; Loh *et al*., 1996; Shibasaki *et al*., 1996; Wesselborg *et al*., 1996). In the yeast *Saccharomyces cerevisiae*, calcineurin is also a critical $Ca^{2+}$ sensor and regulates nuclear import of a transcription factor, Tcn1/Crz1, which is distantly related to NF-AT and regulates the expression of genes whose products regulate cell wall biosynthesis and cation transport, including *FKS2*, *PMR1* and *PMC1* (Matheos *et al*., 1997; Stathopoulos and Cyert, 1997; Stathopoulos-Gerontides *et al*., 1999).

Activated calcineurin is a heterotrimer composed of the catalytic A subunit, the regulatory B subunit and calmodulin. In addition, calcineurin forms stable interactions with several other proteins. For example, calcineurin–NF-AT and calcineurin–Tcn1 complexes have been detected using affinity chromatography or the two-hybrid system (Wesselborg *et al*., 1996; Matheos *et al*., 1997). Calcineurin also physically interacts with the AKAP79 scaffold protein in a macromolecular complex that also contains cAMP-dependent protein kinase (Coghlan *et al*., 1995; Klauck *et al*., 1996). Finally, recent studies have identified a novel inhibitor of calcineurin, called Cabin 1 or Cain, which interacts with and inhibits calcineurin in a phosphorylation-dependent manner in mammalian cells (Lai *et al*., 1998; Sun *et al*., 1998).

Calcineurin is the conserved target for both the immunosuppressive and the antifungal actions of CsA and FK506 (Odom *et al*., 1997; Cardenas *et al*., 1998, 1999; Cruz *et al*., 2000). In the yeast *S.cerevisiae*, calcineurin regulates cation homeostasis, cell wall biosynthesis and responses to pheromone (Cyert and Thorner, 1992; Nakamura *et al*., 1993; Cunningham and Fink, 1994, 1996; Hemenway *et al*., 1995; Mazur *et al*., 1995; Moser *et al*., 1996). In the fission yeast *Schizosaccharomyces pombe*, calcineurin is required for growth at low temperature and for mating (Yoshida *et al*., 1994; Plochocka-Zulinska *et al*., 1995). Calcineurin



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is essential for cell cycle progression in *Aspergillus nidulans* (Rasmussen *et al*., 1994), and regulates hyphal elongation and is also essential for growth in *Neurospora crassa* (Prokisch *et al*., 1997; Kothe and Free, 1998). Although calcineurin is highly conserved from fungi to man, fungal homologs of the endogenous mammalian calcineurin inhibitor Cabin 1/Cain have not yet been identified.

In the studies described here we have identified a novel calcineurin binding protein, CBP1, from the pathogenic fungus *Cryptococcus neoformans*. *Cryptococcus neoformans* is an important opportunistic pathogen in immunocompromised patients, and previous studies have revealed that calcineurin is required for cation homeostasis, growth at 37°C and virulence of this pathogen (Odom *et al*., 1997; Cruz *et al*., 2000). Here a two-hybrid screen was conducted using the calcineurin A catalytic subunit to screen a *C.neoformans* two-hybrid library and identify the CBP1 protein. We show that calcineurin and CBP1 form a stable complex *in vivo* and *in vitro* and that FKBP12–FK506 inhibits CBP1 binding to calcineurin. The *CBP1* gene was disrupted by transformation and homologous recombination. In contrast to calcineurin mutant strains, *cbp1* mutants were viable at 37°C. The *cbp1* mutant strain did exhibit a modest virulence defect, similar to but not as severe as calcineurin mutants, which are avirulent. Interestingly, CBP1 shares a small central region of homology with related proteins in *S.cerevisiae* and man, and is the most broadly conserved of all known calcineurin binding proteins with the exception of calmodulin. *Saccharomyces cerevisiae* mutants lacking the CBP1 homolog YKL159c were viable and sensitive to cation stress, similar to calcineurin mutants. Expression of the *C.neoformans* CBP1 protein in *S.cerevisiae* restored cation resistance in calcineurin mutant strains. In summary, our studies identify a novel, conserved calcineurin binding protein that regulates calcineurin signaling cascades *in vivo*. The presumptive human CBP1 homolog DSCR1 is the first gene contained in the Down's syndrome candidate region. Both calcineurin and DSCR1 are highly expressed in the central nervous system (CNS) and heart, and could have shared functions that are perturbed in patients with Down's syndrome (Fuentes *et al*., 1995; Miyazaki *et al*., 1996).

## Results

### *Identification of the calcineurin binding protein CBP1*

A two-hybrid screen was conducted to identify proteins that interact with calcineurin. The *C.neoformans* calcineurin A subunit was fused to the GAL4 DNA binding domain (GAL4-BD) and co-expressed in the yeast two-hybrid reporter strain PJ69-4A with a *C.neoformans* two-hybrid library consisting of cDNA fused to the GAL4 activation domain (GAL4-AD). A truncated form of calcineurin A lacking the C-terminal autoinhibitory domain was employed to allow binding to the calcineurin active site in the absence of $Ca^{2+}$/calmodulin. From a screen of 5 million transformants, 24 $Ade^{+}$ isolates were obtained (Figure 1A). All 24 isolates were also $His^{+}$ and expressed β-galactosidase activity (Figure 1A), indicating that all three GAL reporter genes are expressed. The library plasmids were rescued in *Escherichia coli*, and all 24 activated the two-hybrid reporter genes when re-introduced with a plasmid expressing the GAL4–calcineurin fusion protein but not with a control vector (Figure 1A). Restriction mapping and sequencing revealed all isolates contained an identical ~750 bp insert corresponding to a novel gene, which we named *CBP1* for calcineurin binding protein 1.

In two-hybrid assays, the *C.neoformans* CBP1 protein interacted with GAL4 fused to the *C.neoformans*, *S.cerevisiae* or murine calcineurin A catalytic subunits. The calcineurin inhibitor FK506 blocked CBP1–calcineurin interactions in the two-hybrid system (Figure 1A), and the effects of FK506 were dependent upon endogenous FKBP12 in the two-hybrid host strain (Figure 1B). The *C.neoformans* CBP1 protein also activated reporter gene expression when co-expressed with GAL4-BD–calcineurin B fusion protein in the two-hybrid assay, although the level of activation was reduced compared with CBP1 binding to calcineurin A, or calcineurin A binding to calcineurin B (Figure 1C, upper panel). CBP1 failed to interact with calcineurin A in a two-hybrid host strain lacking calcineurin B (Figure 1C, lower panel). Finally, a series of truncated forms of the CBP1 protein was assessed. This analysis revealed that residues 1–166 were sufficient for binding, whereas truncated proteins consisting of residues 1–111 or 116–249 failed to interact, although by western blotting we were unable to determine whether these proteins were stably expressed (Figure 1D and data not shown). These observations suggest that CBP1 interacts with both the calcineurin A and B subunits and may compete with FKBP12–FK506 binding to the calcineurin AB interface (Cardenas *et al*., 1995; Griffith *et al*., 1995; Kissinger *et al*., 1995).

### *Isolation of the CBP1 gene: CBP1 is conserved in yeasts, flies, worms and humans*

The portion of the *CBP1* gene obtained in the two-hybrid screen was used as a probe in Southern blot analysis and to clone genomic and cDNA clones corresponding to the *CBP1* locus. Sequence analysis and comparison of cDNA and genomic clones revealed that the *CBP1* gene encodes a 249 amino acid protein (Figure 2) and that the *CBP1* gene contains a single intron. Moreover, this analysis revealed that, in the isolates obtained, the entire CBP1 protein was fused to the GAL4-AD.

Standard Blast searches with the CBP1 sequence (DDBJ/EMBL/GenBank accession No. AF230799) failed to identify any proteins with marked identity to the entire CBP1 protein. A nested BLAST search revealed a limited region of homology, spanning amino acids 125–160, shared between CBP1 and related proteins in *S.cerevisiae*, *S.pombe*, *Drosophila melanogaster*, *Caenorhabditis elegans*, hamster and humans, which are largely of unknown function (Figure 2). Interestingly, this region contains two conserved serine residues in the sequence SPPxSPP (Figure 2). These observations suggest that the *C.neoformans* CBP1 protein is a member of a family of broadly conserved calcineurin binding proteins.

### *CBP1 binds calcineurin in vitro and in vivo*

We next sought to confirm that CBP1 interacts directly with calcineurin. A glutathione *S*-transferase (GST)–CBP1

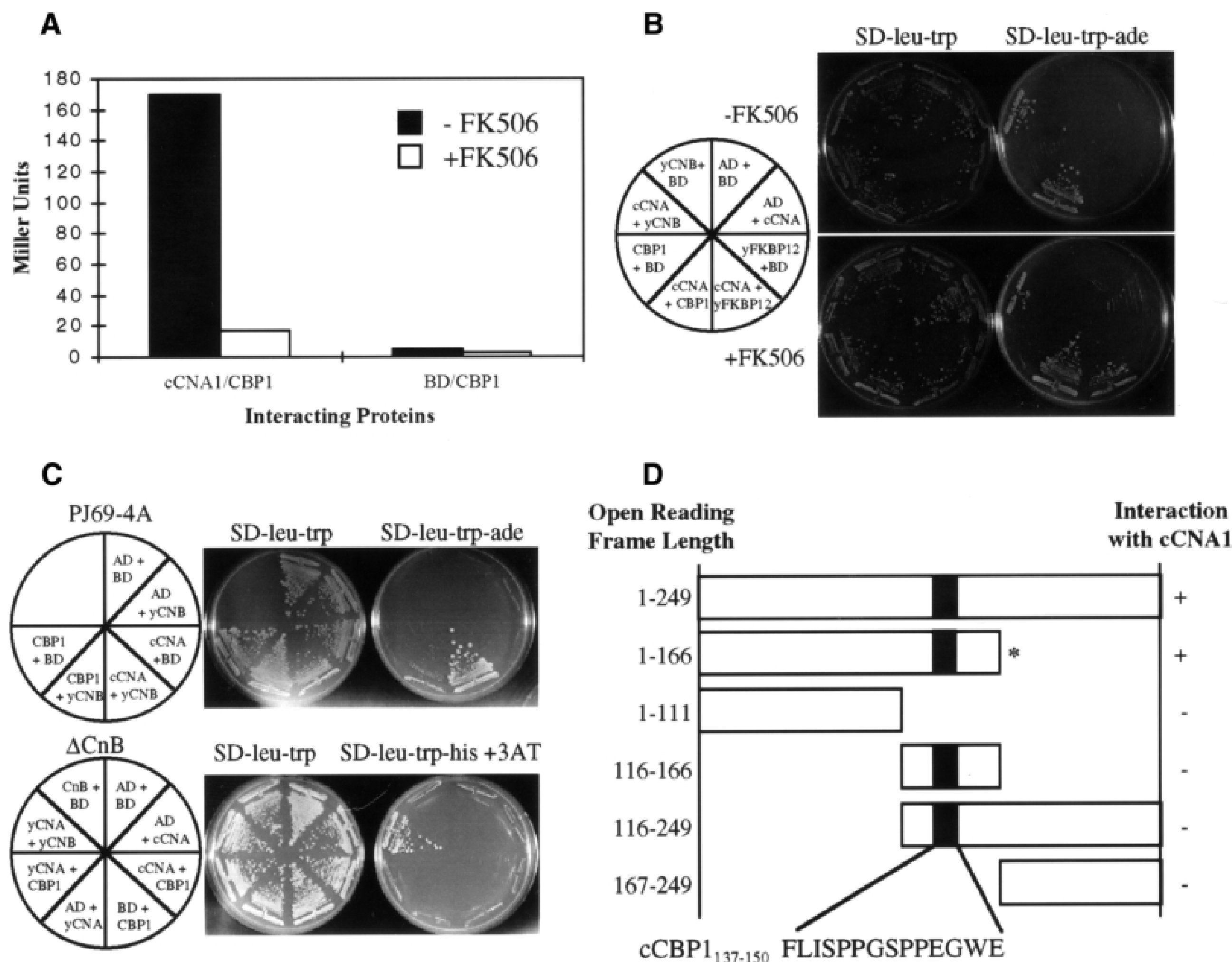


**Fig. 1.** Calcineurin and CBP1 interact in the two-hybrid assay. (**A**) The *C.neoformans* calcineurin A protein (cCNA1) specifically interacts with CBP1 and this interaction is inhibited by FK506. β-galactosidase assays were conducted in the presence and absence of 1 μg/ml FK506 as indicated. (**B**) FK506 inhibition of calcineurin–CBP1 binding requires FKBP12. An *S.cerevisiae* strain lacking FKBP12 (*fpr1* strain SMY87-4) was transformed with plasmids expressing the GAL4 activation domain (AD) or DNA binding domain (BD) fused to the indicated proteins. To detect expression of the *GAL-ADE2* reporter gene, cells were grown on synthetic medium minus leucine, tryptophan and adenine (SD-leu-trp-ade) for 7 days at 30°C. 'y' denotes *S.cerevisiae* proteins and 'c' denotes *C.neoformans* proteins. FK506 stimulated FKBP12 binding to calcineurin A as expected. FK506 did not inhibit CBP1 binding to calcineurin in these cells lacking endogenous FKBP12. Calcineurin A (cCNA1) binding to calcineurin B (yCNB) was not affected by FK506. (**C**) *Saccharomyces cerevisiae* calcineurin B interacts with CBP1 and is required for CBP1 binding to calcineurin A. Isogenic *S.cerevisiae* strains expressing (PJ69-4A) or lacking calcineurin B (ΔCnB, SMY3) were transformed with plasmids expressing the GAL4 AD or BD fused to the indicated proteins. Cells were grown on medium lacking adenine (SD-leu-trp-ade) to detect expression of the *GAL-ADE2* reporter gene, and on medium lacking histidine (SD-leu-trp-his + 5 mM 3-AT) to detect expression of the *GAL-HIS3* reporter gene. (**D**) Two-hybrid analysis of the binding of truncated forms of CBP1 to calcineurin A. The black box represents the highly conserved region of CBP1. Fragment length is indicated in amino acid residues. * indicates the GST–CBP1 fusion protein.

fusion protein was expressed in bacteria, absorbed to glutathione Sepharose beads, and incubated in binding reactions with purified bovine calcineurin. Bound proteins were eluted, fractionated by SDS–PAGE, and the calcineurin A subunit was detected by western blotting. As shown in Figure 3A, GST–CBP1 bound to calcineurin A in the presence or absence of calmodulin. CBP1 binding to calcineurin A was completely inhibited by FKBP12–FK506, and was partially inhibited by EGTA (Figure 3A). No binding of calcineurin A was detected to GST alone (Figure 3A). These findings reveal that divalent cations are required for CBP1 binding to calcineurin and that CBP1 and FKBP12–FK506 compete for binding both *in vitro* and in the yeast two-hybrid system.

To test whether CBP1 also binds calcineurin *in vivo*, the *CBP1* gene was fused to the gene encoding the green fluorescent protein (GFP). The resulting *CBP1–GFP* fusion gene was transformed into *C.neoformans* cells, and the full-length fusion protein was detected by western blot analysis of whole-cell extracts with antisera against GFP (Figure 3B). Next, the CBP1–GFP fusion protein was immunoprecipitated with anti-GFP antibodies, and bound proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and analyzed by overlay blotting with $^{125}$I-labeled calmodulin to detect calcineurin A. As shown in Figure 3B, the CBP1–GFP fusion protein co-immuno-precipitated with the calcineurin A subunit. In comparison, no interaction was detected with GFP alone or CBP1 in

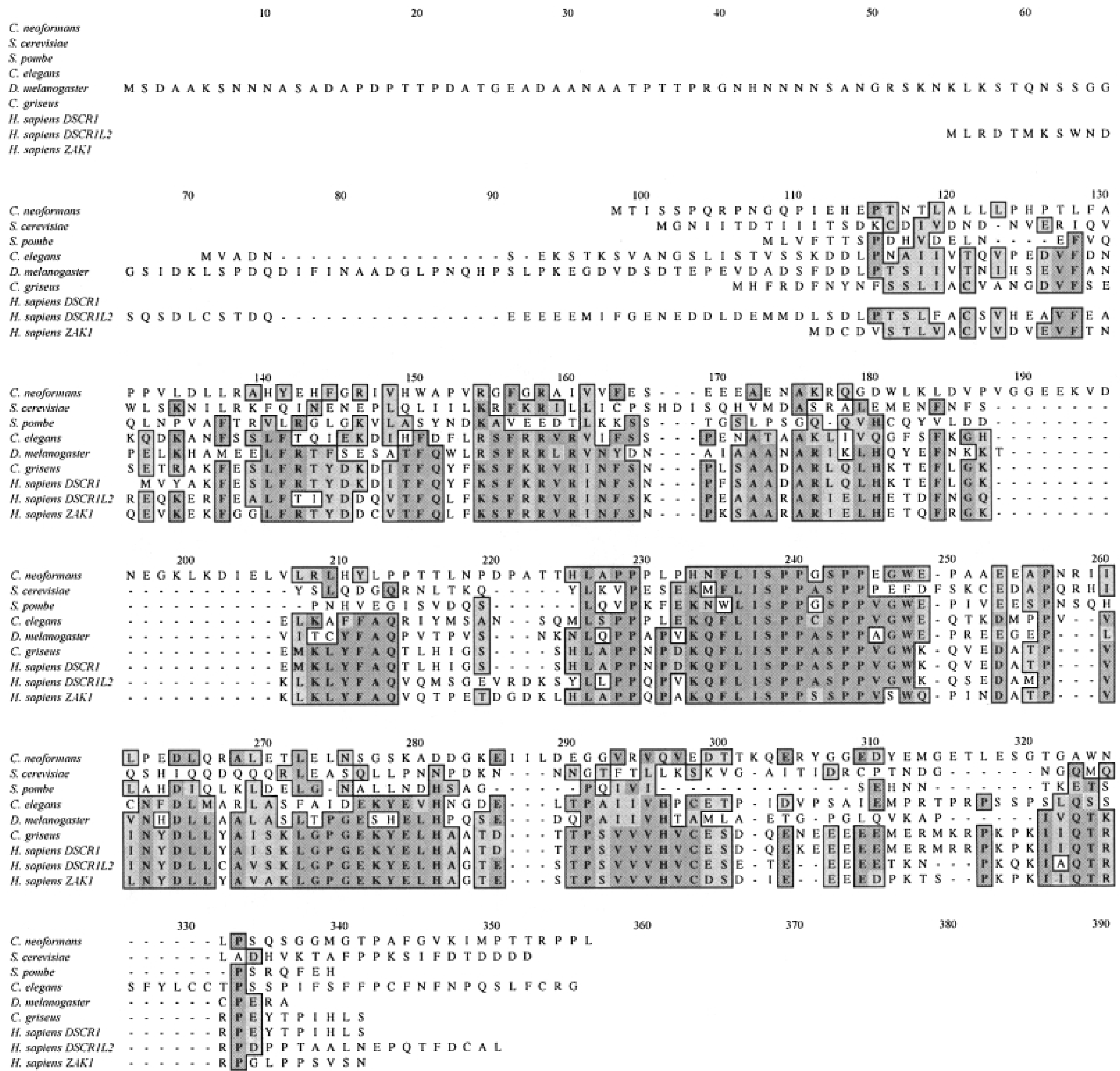

**Fig. 2.** Alignment of *C.neoformans* CBP1 protein homologs. An alignment of the *C.neoformans*, *S.cerevisiae* (DDBJ/EMBL/GenBank accession No. Z28159), *S.pombe* (accession No. Z67757), *C.elegans* (accession No. AF176115), *D.melanogaster* (accession No. AF147700), *Cricetulus griseus* (accession No. U60263) and human (*Homo sapiens*, accession Nos D83407, U28833 and AF176116) CBP1 proteins is depicted. Identical residues are in bold, darkly shaded and boxed; similar residues are lightly shaded and boxed.

which the GFP epitope tag was omitted, there was no co-immunoprecipitating protein with extracts from a *cna1* mutant strain lacking calcineurin A, and no CBP1–calcineurin complex was detected when the anti-GFP antibody was omitted (Figure 3B and data not shown). Thus, CBP1 and calcineurin A also interact *in vivo*.

We tested whether CBP1 binding alters calcineurin enzymatic activity. Synthetic peptides corresponding to the conserved region of CBP1 or the human DSCR1 protein were found to inhibit the activity of bovine calcineurin towards a synthetic phosphopeptide derived from the RII subunit of cAMP-dependent protein kinase (Figure 4A). The magnitude of inhibition (~50%) was less than that of FKBP12–FK506, but was similar to that observed with a peptide corresponding to the auto-inhibitory domain of calcineurin. When calcineurin activity was measured towards the small substrate *p*-nitrophenylphosphate (pNPP), the CBP1 and DSCR1 peptides modestly stimulated activity (1.5- to 2.5-fold), similar to the effects of FKBP12–FK506, as has been previously reported (Liu *et al*., 1991). These findings provide additional evidence that CBP1 interacts with calcineurin and suggest that the conserved region of CBP1 and the CBP1 homolog DSCR1 can alter the activity of calcineurin by interacting with a site distinct from the active site.

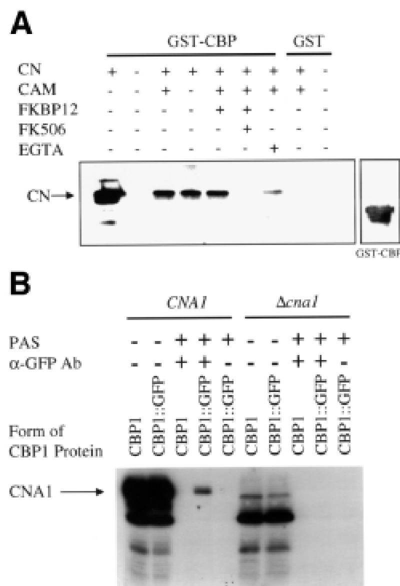

**Fig. 3.** CBP1 binds to calcineurin *in vitro* and *in vivo*. (**A**) Purified GST–CBP fusion protein bound to glutathione agarose was incubated with bovine calcineurin in the presence (+) or absence (–) of calmodulin, FKBP12, FK506 or EGTA for 2 h at 4°C. Reactions were separated on 10% SDS–PAGE and transferred to PVDF membranes. Membranes were incubated with anti-calcineurin (bovine) antibody to detect binding of CBP to calcineurin or anti-GST antibody to detect the GST–CBP fusion protein. The arrow indicates the position of calcineurin. The panel on the right indicates the position of the GST–CBP fusion protein. (**B**) Wild-type strain H99 (*CNA1*) and the isogenic *Δcna1* mutant strain expressing wild-type CBP1 (H99, AO4) or a CBP1–GFP fusion protein (JMC4, JMC6) were grown overnight in rich medium, cells were mechanically disrupted, and immunoprecipitation experiments were conducted with total cell extracts for 1 h at 4°C in the presence (+) and absence (–) of anti-GFP antisera (α-GFP Ab). Proteins bound to the antibody were subsequently precipitated with protein A–Sepharose (PAS) and separated on 12% SDS–PAGE. The calcineurin A protein (CNA1) was detected by incubating the western blots with [$^{125}$I]calmodulin. The first two lanes of the right and left panels are total extract controls that were not incubated with the anti-GFP antisera.

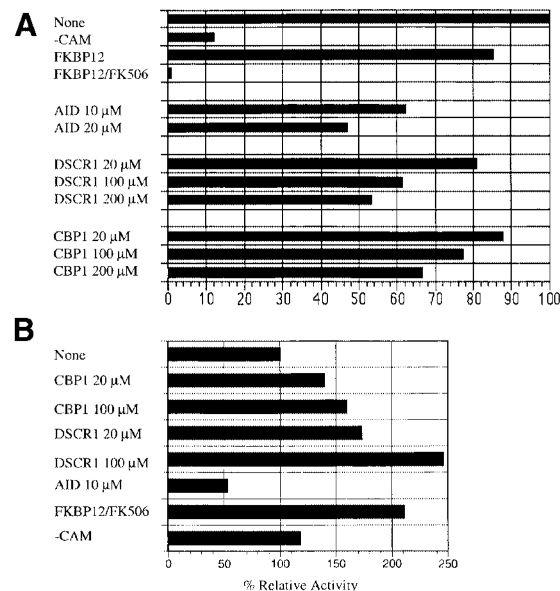

**Fig. 4.** Effect of CBP1 peptides on calcineurin phosphatase activity. (**A**) Calcineurin phosphatase activity was determined by measurement of radiolabel released from [$^{32}$P]phospho-RII peptide at 30°C in the presence or absence (–CAM) of calmodulin, and with the addition of FKBP12, FKBP12–FK506, autoinhibitory peptide (AID), or of the DSCR1 or CBP1 peptides at 20, 100 or 200 μM. (**B**) Calcineurin phosphatase activity was determined by spectrophotometric determination at 405 nm of the reaction product released from pNPP at 25°C. Values shown are % activities relative to uninhibited calcineurin control reactions. Each sample was performed in duplicate.

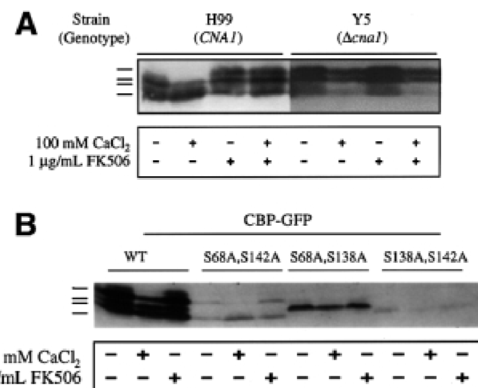

**Fig. 5.** CBP1 electrophoretic mobility is regulated by calcineurin. (**A**) Strains H99 (*CNA1*) and Y5/AO4 (*Δcna1*) expressing a CBP1–GFP fusion protein were incubated for 2 h in the presence (+) or absence (–) of 100 mM $CaCl_2$ or 1 μg/ml FK506. Total proteins were extracted, separated by 9% SDS–PAGE, transferred to nitrocellulose, and probed with anti-GFP antibody. (**B**) Strains expressing CBP1–GFP fusion proteins, either wild-type (CBP1) or mutant forms (CBP1$^{S68A,S142A}$, CBP1$^{S68A,S138A}$, CBP1$^{S138A,S142A}$), were incubated for 2 h in the presence (+) or absence (–) of 100 mM $CaCl_2$ or 1 μg/ml FK506. Total proteins were extracted, separated by SDS–PAGE, transferred to nitrocellulose, and probed with anti-GFP antibody.

### *Calcineurin regulates the electrophoretic mobility of CBP1*

In western blot analysis, the CBP1–GFP fusion protein migrated as up to four distinct species with differing electrophoretic mobilities (Figure 5A). When cells were exposed to 100 mM extracellular $Ca^{2+}$, the band with the slowest mobility disappeared and the relative abundance of the faster migrating species increased (Figure 5A). In contrast, when cells were exposed to the calcineurin inhibitor FK506, the relative abundance of the slowest migrating species increased, and FK506 blocked the effects of $Ca^{2+}$ ions on CBP1 mobility (Figure 5A). To address further whether the effects of $Ca^{2+}$ ions and FK506 were mediated via calcineurin, a similar analysis was conducted in a *cna1* mutant strain lacking calcineurin A (Figure 5A). In this case, in control extracts the slowest migrating form of CBP1 was more abundant than in cells expressing calcineurin. Exposure to $Ca^{2+}$ ions resulted in a decreased level of the faster migrating forms of CBP1. FK506 had no effect on CBP1 mobility in cells lacking calcineurin and did not block the effects of $Ca^{2+}$. Finally, a series of mutant CBP1–GFP fusion proteins was generated in which the conserved serine residues in the FLISPPxSPP motif were replaced with alanines. In these cases, only one or two forms of the CBP1–GFP fusion protein were observed, and there was little or no effect of FK506 or $Ca^{2+}$ (Figure 5B). In two cases protein stability may also be reduced. Taken together, these observations suggest that CBP1 may be a phosphoprotein and could be a substrate

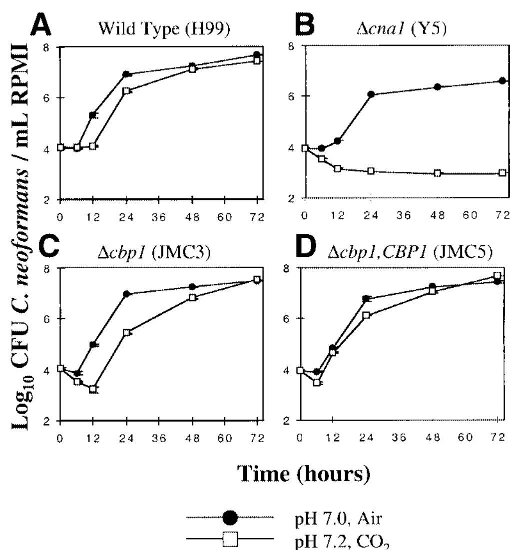


**Fig. 6.** *cpb1* mutant strains are modestly sensitive to 5% $CO_2$ and alkaline pH. The wild-type *C.neoformans* strain H99 (Wild Type) (**A**), the isogenic *Δcna1* mutant strain (Y5/AO4) (**B**), *Δcbp1* mutant strain (JMC3) (**C**) and the *Δcbp1* mutant strain transformed with the *CBP1* gene (JMC5) (**D**) were grown in triplicate in liquid RPMI medium at 30°C for 0–72 h in either an atmosphere of air at pH 7.0 (circles) or at 5% $CO_2$ at pH 7.2 (squares) and the number of surviving colony-forming units (CFU) was measured by dilution and plating on YPD medium and the number of surviving colonies was averaged for triplicate cultures.

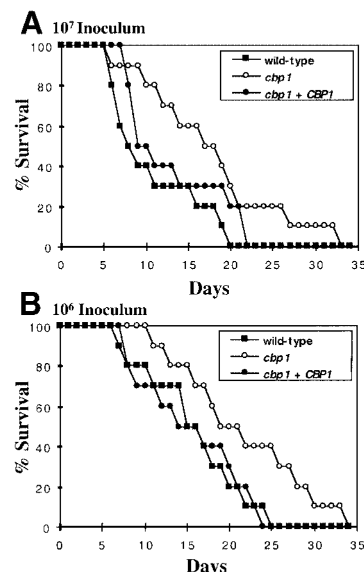


**Fig. 7.** CBP1 is required for full virulence of *C.neoformans*. Mice (10 each) were injected in the lateral tail vein with either $10^7$ (**A**) or $10^6$ (**B**) cells of the wild-type strain H99 (filled squares), the isogenic *cbp1::ADE2* mutant strain (JMC3, open circles) or the *Δcbp1* mutant strain transformed with the *CBP1* gene (JMC5, filled circles). Survival was monitored and plotted with respect to time. *P*-values were generated using the Wilcoxon test. In (A), *cbp1* (JMC3) versus *CBP1* (H99) $P < 0.05$, *cbp1* (JMC3) versus *cbp1* + *CBP1* (JMC5) $p > 0.05$. In (B), *cbp1* (JMC3) versus *CBP1* (H99) $P = 0.05$, *cbp1* (JMC3) versus *cbp1* + *CBP1* (JMC5) $p > 0.05$.

of both the $Ca^{2+}$-activated phosphatase calcineurin and possibly also a $Ca^{2+}$/calmodulin-dependent kinase.

### *Isolation and characterization of a C.neoformans cbp1 mutant strain*

To disrupt the *C.neoformans CBP1* gene, the CBP1 open reading frame from start to stop codon was replaced with the *ADE2* gene and the *cbp1Δ:ADE2* allele was transformed into the *ade2* mutant strain M049 by biolistic transformation. Genomic DNA was isolated and analyzed by PCR and Southern blotting to identify isolates in which the *CBP1* gene was replaced by the *cbp1Δ::ADE2* allele by homologous recombination with no tandem or ectopic integrations. By Southern blotting, the CBP1 open reading frame was present in the *CBP1* wild-type strain M049 and not in the *cbp1Δ* strain (not shown). Hybridization with the 3′ region of the *CBP1* gene confirmed a single copy of the *ADE2* gene was inserted. A *cbp1* + *CBP1* reconstituted strain was generated in which the *CBP1–GFP* gene was introduced into the *cbp1Δ* mutant. By northern blotting, the *CBP1* gene was expressed in the wild-type strain H99 and in the *cbp1* + *CBP1* reconstituted strain, and was not expressed in the *cbp1Δ::ADE2* mutant (not shown).

We next tested whether either the *cbp1* mutant strain or the *cbp1* + *CBP1* reconstituted strain that overexpresses the *CBP1* protein exhibited mutant phenotypes similar to isogenic *cna1* mutants lacking calcineurin A. In contrast to *cna1* mutants lacking calcineurin, both the *cbp1* mutant and the *cbp1* + *CBP1* strain grew normally at 37 and 39°C (not shown) and were not sensitive to 50 mM LiCl or 1.5 M NaCl (not shown). The *cbp1* mutant strain did exhibit a modest growth defect at pH 7.2/5% carbon dioxide (Figure 6C), which was complemented in the *cbp1* + *CBP1* reconstituted strain (Figure 6D), but this defect was less severe than in the isogenic calcineurin mutant (Figure 6B).

Calcineurin mutant strains are avirulent in both a rabbit model of cryptococcal meningitis and in a murine systemic infection model (Odom *et al.*, 1997; Cruz *et al.*, 2000). In contrast, persistence of the *cbp1Δ* mutant strain in the cerebrospinal fluid (CSF) of infected immunosuppressed rabbits was similar to the isogenic CBP1 wild-type strain, indicating that CBP1 is not required for survival in the CNS of this animal model (not shown), which is different from calcineurin mutants (Odom *et al.*, 1997). However, in the murine model, virulence of the *cbp1* mutant strain was modestly reduced compared with the wild-type strain. At an inoculum size of $10^7$, 50% of mice infected with wild-type strain H99 survived to day 8 post-infection, compared with the *cbp1* mutant in which 90% of animals were alive on days 8 and 9 and 50% survived until day 18 (Figure 7A). There was 100% mortality with the wild-type strain by day 20, whereas 100% mortality was delayed until day 33 with the *cbp1* mutant (Figure 7A). Similar results were observed at an inoculum size of $10^6$ cells (Figure 7B). Virulence of the *cbp1* + *CBP1* reconstituted strain was similar to the *CBP1* wild type in both animal models (Figure 7 and data not shown). In comparison, the isogenic *cna1* calcineurin A mutant was completely avirulent in this murine model (Cruz *et al.*, 2000). Taken together, these findings indicate that the CBP1 protein plays a relative but not absolute role in some calcineurin functions in *C.neoformans*, possibly because it is redundant with other factors.

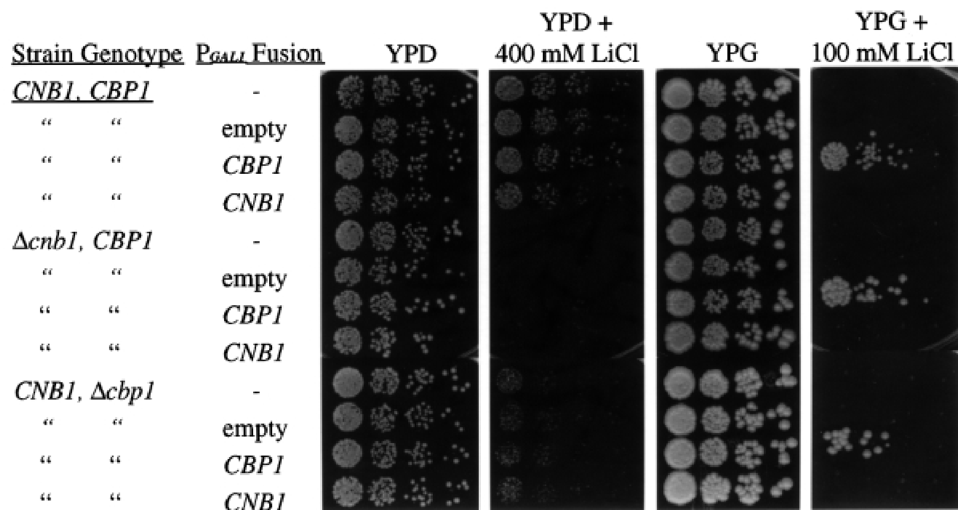


**Fig. 8.** CBP1 functions in cation homeostasis in *S.cerevisiae*. The *C.neoformans CBP1* or *S.cerevisiae CNB1* genes were expressed from the *S.cerevisiae GAL1* promoter on a 2μ plasmid. These plasmids were introduced into either wild-type (*CNB1*, *CBP1*), *Δcnb1* or *Δcbp1* mutant strains of *S.cerevisiae*. Strains with no plasmids (–) or harboring an empty vector (empty) were included as controls. Cells were grown in selective medium overnight and then serially diluted (1250, 250, 50 and 10 cells/spot from left to right) onto rich medium containing either glucose (YPD) or galactose (YPG) supplemented with 100 or 400 mM LiCl. Plates were incubated for 2 days (YPD, YPD + 400 mM LiCl) or 6 days (YPG, YPG + 100 mM LiCl) at 30°C.

### *Analysis of S.cerevisiae CBP1 homolog YKL159c/RCN1*

We next analyzed the functions of the *S.cerevisiae* CBP1-related protein YKL159c/RCN1. The *YKL159c* gene was replaced from start to stop codon with dominant selectable markers conferring G418 or hygromycin resistance by PCR-mediated gene disruption. *ykl159c* mutant strains were viable and had no growth defect on rich or minimal medium or at different temperatures (not shown). By western blotting, the calcineurin B subunit was stably expressed in *ykl159c* mutants, and thus CBP1 is not required for expression or stability of the CNB1 protein (not shown).

Several observations suggest that the presumptive CBP1 homolog YKL159c/RCN1 participates in some calcineurin functions *in vivo*. First, similar to *S.cerevisiae* calcineurin mutants, strains lacking YKL159c were moderately sensitive to $Li^+$ cations (Figure 8). However, while growth of calcineurin mutants was completely blocked by 100 mM LiCl, growth of the *cbp1* mutants was partially inhibited at 200 mM LiCl and more severely inhibited at 400 mM LiCl (Figure 8 and data not shown). Secondly, overexpression of the *C.neoformans* CBP1 protein in wild-type cells or a *cnb1* mutant lacking calcineurin B conferred cation resistance on medium containing galactose (inducing) but not glucose (repressing). In contrast, calcineurin B overexpression did not restore $Li^+$ resistance in *cbp1* mutant cells (Figure 8 and data not shown). These findings suggest that CBP1 may function downstream of calcineurin.

The yeast YKL159c/RCN1 protein does not play a role in all calcineurin functions in yeast. Mutants lacking YKL159c have no defect in response to or recovery from α-factor, whereas calcineurin mutations prevent recovery (not shown). Calcineurin mutations are synthetically lethal with *vph6* or *fks1* mutations (because calcineurin is required for cell wall biosynthesis or cation homeostasis in such mutants), whereas *ykl159c vph6* and *ykl159c fks1* double mutants were viable (not shown). Thus, in both *S.cerevisiae* and *C.neoformans*, CBP1 plays a role in some but not all calcineurin functions.

## Discussion

We have identified a novel and conserved calcineurin binding protein, CBP1, from the pathogenic fungus *C.neoformans*. CBP1 interacts with both the calcineurin A and B subunits in the yeast two-hybrid assay, the calcineurin B subunit is required for formation of the calcineurin A–CBP1 complex, and FKBP12–FK506 inhibited CBP1 binding to calcineurin in the two-hybrid system. The interaction between CBP1 and calcineurin was confirmed by *in vitro* binding assays with purified proteins, revealing that CBP1 and calcineurin directly interact; calmodulin is not required for CBP1 binding to calcineurin. FKBP12–FK506 inhibited CBP1 binding to calcineurin *in vitro*, suggesting that CBP1 may bind to the interface between the calcineurin A and B subunits, which is known to be the case for FKBP12–FK506. This interpretation is consistent with the finding that CBP1 peptides activate calcineurin activity with the small substrate PNPP, and modestly inhibit activity towards larger phosphopeptide substrates. Finally, we showed that CBP1 and calcineurin can be co-immunoprecipitated from *C.neoformans* cell extracts, suggesting that CBP1 and calcineurin interact *in vivo*.

Our observations on the electrophoretic mobility of the CBP1 protein suggest that it could be a phosphoprotein substrate of calcineurin. Exposure of cells to FK506 or $Ca^{2+}$ altered the mobility of CBP1, and site-directed mutagenesis of several conserved serine residues in the CBP1 protein resulted in a reduction or disappearance of altered mobility states of the protein. Because $Ca^{2+}$ increased the mobility of CBP1 in cells expressing

calcineurin, but had the opposite effect in calcineurin mutant cells, CBP1 could be a substrate of both calcineurin and a calcium/calmodulin regulated kinase. These findings may be analogous to other recent studies. For example, the Cot-1 serine/threonine kinase that regulates hyphal elongation in *N.crassa* is found in a physical complex with calcineurin (Gorovits *et al*., 1999). Similarly, the AKAP79 scaffolding protein forms complexes with both calcineurin and the cAMP-dependent protein kinase in mammalian cells (Coghlan *et al*., 1995; Klauck *et al*., 1996; Kashishian *et al*., 1998). Thus, both kinases and phosphatases may be present in common physical complexes with regulatory targets such as CBP1.

To determine the functions of CBP1, the *CBP1* gene was disrupted by homologous recombination in *C.neoformans*. The *cbp1* mutant strain was viable. In contrast to an isogenic calcineurin mutant, the *cbp1* mutant was viable at 37°C and was not cation stress sensitive. The *cbp1* mutant was modestly sensitive to other stress conditions that are toxic to calcineurin mutants (alkaline pH, $CO_2$) and also exhibited a modest virulence defect in mice. Thus, CBP1 functions in some calcineurin-regulated pathways but not others, possibly because calcineurin has several targets, CBP1 is redundant with other factors, or CBP1 regulates only some calcineurin functions.

A small central region of the *C.neoformans* CBP1 protein shares identity with related proteins in *S.cerevisiae*, *S.pombe*, *D.melanogaster*, *C.elegans* and humans that have recently been identified by others and termed calcipressins (K.Cunningham and F.McKeon, personal communication; Rothermel *et al*., 2000). This conserved region contains the peptide sequence FLISPPxSPP, which could be involved in phosphorylation and calcineurin binding. This conserved peptide sequence is not present in the calcineurin binding sites of other calcineurin substrates or regulators, including NF-AT, Crz1/Tcn1, Cabin 1/Cain or AKAP79. We have disrupted the gene encoding the CBP1 homolog in *S.cerevisiae* (YKL159c). Mutants lacking the yeast CBP1 homolog are, like calcineurin mutants, cation sensitive. Overexpression of the *C.neoformans* CBP1 protein in wild-type or a *cnb1* mutant strain conferred cation resistance, suggesting that CBP1 may function downstream of calcineurin when overexpressed. These observations reveal that the functions of CBP1 and calcineurin are related. CBP1 may be a target of the calcineurin signaling cascade, a regulator of calcineurin signaling functions, or both.

Most interestingly, the human CBP1 homolog DSCR1 is encoded on chromosome 21 and is the first gene in the Down's syndrome candidate region interval (Fuentes *et al*., 1995; Miyazaki *et al*., 1996). Because Down's syndrome results from trisomy for chromosome 21, DSCR1 may be relatively overexpressed in patients with this disorder. The most marked manifestations of Down's syndrome include mental retardation and congenital heart defects. Both DSCR1 and calcineurin are highly expressed in the CNS and heart (Fuentes *et al*., 1995), and calcineurin and NF-AT are now known to play a prominent role in regulating cardiac development and hypertrophy (Molkentin *et al*., 1998; Ranger *et al*., 1998; Sussman *et al*., 1998). Calcineurin also regulates CNS functions, including neurite extension and both long-term memory and long-term potentiation (Ferreira *et al*., 1993; Mulkey *et al*., 1994; Chang *et al*., 1995; Mansuy *et al*., 1998; Winder *et al*., 1998). The role of calcineurin in neurite extension may be analogous to the known roles of calcium and calcineurin in regulating hyphal elongation in fungi (Jackson and Heath, 1993; Prokisch *et al*., 1997). These findings suggest an intriguing link between calcineurin, CBP1/DSCR1 and cell morphogenesis in both fungi and man.

In summary, our studies identify CBP1 as a highly conserved and ubiquitous calcineurin binding protein. CBP1 represents the first calcineurin binding protein, other than calmodulin, that is conserved from yeast and fungi to man. Our studies highlight the potential of molecular genetic studies in pathogenic fungi to identify conserved elements of signaling cascades, and may provide insights into the multiple roles of calcineurin in immunosuppressive drug action, cardiac and CNS functions, and fungal virulence.

## Materials and methods

### *Yeast strains, media, genetic methods and compounds*

Strains used in this study are listed in Table I. The *S.cerevisiae* strains PJ69-4A, JK9-3da, SMY3 and SMY87-4 used in this study were as described (Heitman *et al*., 1991; Cardenas *et al*., 1994; James *et al*., 1996; Arndt *et al*., 1999). The *C.neoformans* serotype A strain H99 and its derivative M049 were as described (Perfect *et al*., 1993). Both *S.cerevisiae* and *C.neoformans* strains were grown in complete (YPD) and minimal (SD) media as described (Sherman, 1991). Regeneration medium for biolistic transformation was prepared as described (Toffaletti *et al*., 1993). V8 mating-starvation medium was prepared as described (Odom *et al*., 1997). Immunosuppressant-containing medium was as described (Heitman *et al*., 1993). YPD was supplemented with salts as indicated. FK506 was from Fujisawa.

### *Yeast two-hybrid system*

Primers were designed to hybridize to regions of the *C.neoformans CNA1* gene such that the autoinhibitory domain of the calcineurin A protein was omitted from the C-terminus of the protein. Using *C.neoformans* cDNA as a template, forward primer 5′-GCTTCCCCGGGCACTCAGACC (1420) and reverse primer 5′-GGCTCTGCAGCATTAAGAGGG (1422) were used to PCR amplify the 1.8 kb 5′ region of the *CNA1* cDNA using standard PCR conditions. Primers 1420 and 1422 contain *Sma*I and *Pst*I restriction sites, respectively. The PCR product was digested with both *Sma*I and *Pst*I, gel purified, and ligated in-frame with the GAL4-BD in the two-hybrid vector pGBT9 to yield plasmid pJMM119.

The *S.cerevisiae* reporter strain PJ69-4A (James *et al*., 1996) was cotransformed with plasmid pJMM119 and the *C.neoformans* two-hybrid library (Cruz *et al*., 1999) or with other control plasmids (Table II) by a high efficiency lithium acetate/heat shock method (Gietz *et al*., 1995). Double transformants were isolated on medium lacking tryptophan and leucine (to select for the introduced plasmids) and also lacking either adenine, or lacking histidine and containing 10 mM 3-aminotriazole to assay reporter gene expression. β-galactosidase assays were performed as described (Cardenas *et al*., 1994).

*Cryptococcus neoformans* cDNA library plasmids were rescued from strain PJ69-4A by transforming miniprep yeast DNA into *E.coli* strain HB101 and selecting for leucine prototrophy on M9 medium containing ampicillin. Three size classes of plasmids were identified. All plasmids were retransformed into strain PJ69-4A harboring plasmid pJMM119 to confirm the interaction. Only one class retained the ability to interact with calcineurin A. Several independent plasmids from this class were sequenced, revealing that all encoded the same protein, CBP1.

Protein truncations were generated by PCR amplification of the *CBP1* gene using the two-hybrid library clone as a template and standard PCR conditions. Primer sequences were: 5′-GGTTGTCGGCTGCAGGTAG-TGTAA (1915), 5′-GCAACGTCGACTGGAAACGC (1916), 5′-GCC-GCGTCGACCATATCCC (1917), 5′-GGAATTCGCTGCAGCGTC-GAC (1918), 5′-GTTTCCACTGCAGGTTGCTAATCCTCA (1919) and 5′-CCACCGTCGACTCAACCCCGACCC (1920). PCR products were digested with *Sal*I and *Pst*I, and ligated into *Sal*I and *Pst*I-digested plasmid pGAD424.

**Table I.** Strain list

| *S.cerevisiae* strains | Genotype | Reference |
|---|---|---|
| PJ69-4A | *MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ* | James *et al*. (1996) |
| SMY87-4 | PJ69-4A *fpr1Δ::hisG* | Arndt *et al*. (1999) |
| Y190 | *MAT***a** *trp1-901 his3 leu2-3,112 ura3-52 ade2 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3* | Harper *et al*. (1993) |
| SMY3 | Y190 *TOR1-3 cnb1::ADE2* | Cardenas *et al*. (1994) |
| JK9-3da | *MAT***a** *leu2-3,112 ura3-52 trp1 his4 rme1 HML***a** | Heitman *et al*. (1991) |
| SCY78 | JK9-3da *ykl159cΔ::G418* | this study |
| *C.neoformans* strains | Genotype (derived from strain) | Reference |
| H99 | | Perfect *et al*. (1993) |
| M049 | *ade2* (H99) | Perfect *et al*. (1993) |
| AO4 | *cna1Δ::ADE2 ade2* (M049) | Odom *et al*. (1997) |
| AO10 | *cna1Δ::ADE2 CNA1 ade2* (AO4) | Odom *et al*. (1997) |
| JMC3 | *cbp1Δ::ADE2 ade2* (M049) | this study |
| JMC4 | *CBP1::GFP Hyg$^r$ ade2* (H99) | this study |
| JMC5 | *cbp1Δ::ADE2 CBP1::GFP Hyg$^r$ade2* (JMC3) | this study |
| JMC6 | *cna1Δ::ADE2 CBP1::GFP Hyg$^r$ade2* (AO4) | this study |
| MCC3 | *MATa cna1Δ::ADE2 ade2 ura5* (serotype D, JEC156) | Cruz *et al*. (2000) |
| JMC7 | *CBP1-S68A, S142A::GFP Hyg$^r$ ade2* (H99) | this study |
| JMC8 | *CBP1-S68A, S138A::GFP Hyg$^r$ ade2* (H99) | this study |
| JMC9 | *CBP1-S138A, S142A::GFP Hyg$^r$ ade2* (H99) | this study |

**Table II.** Plasmids

| Plasmid | Description | Reference |
|---|---|---|
| pGAD424 | 2μ *LEU2 GAL4(AD)* | Bartel *et al*. (1993) |
| pGBT9 | 2μ *TRP1 GAL4(BD)* | Bartel *et al*. (1993) |
| pJMM119 | 2μ *TRP1 GAL4(BD)::CnCNA1* | this study |
| pML59 | 2μ *LEU2 GAL4(AD)::ScFKBP12* | Lorenz and Heitman (1995) |
| pYCBA | 2μ *LEU2 GAL4(AD)::ScCNB1* | this study |
| pCH113BD | 2μ *TRP1 GAL4(BD)::ScCMP1* | Cardenas *et al*. (1994) |
| pYDF2BD | 2μ *TRP1 GAL4(BD)::MmCNA* | Cardenas *et al*. (1994) |
| pmCnA | 2μ *TRP1 GAL4(BD)::MmCNA* (His-Glu) | this study |
| pYCBB | 2μ *TRP1 GAL4(BD)::ScCNB1* | this study |
| pJMM131 | 2μ *LEU2 GAL4(AD)::CnCNA1* | this study |
| pJMM160 | *Δcbp1::ADE2* | this study |
| pJMM185 | *Hph CBP1::GFP* | this study |
| pJMM190 | *Hph pGAL7::CBP1* | this study |

### Isolation and characterization of the CBP1 genomic locus

Genomic DNA was isolated from *C.neoformans* serotype A strain H99 as described (Pitkin *et al*., 1996). DNA from the *CBP1* two-hybrid library clone was gel-purified, random-primer labeled, and used to probe digested genomic DNA immobilized on Nytran$^+$ membrane (Shleicher and Schuell) as described by the manufacturer. The *CBP1* locus was mapped and a sub-genomic library of *Pst*I-cleaved genomic DNA was size-selected, ligated into *Pst*I-cleaved/Calf Intestine Alkaline Phosphatase-treated pBluescript SK, and screened by colony hybridization to isolate an 11.0 kb *Pst*I fragment containing the entire *CBP1* gene. One strongly hybridizing clone was isolated, mapped to confirm that it contained the *CBP1* locus, and sequenced. Comparison of genomic sequence with the two-hybrid clone identified the intron–exon borders.

### GST tagging of the CBP1 protein and calcineurin binding assays

A C-terminal truncation of the CBP1 coding region (residues 1–153) was obtained by amplification with primers CGCGTCGGATCCTATCCCCAGCAA (1782) and CTCCAGGAATTCCAGTGCACGTTG (2056) and cloned in-frame into the *Bam*HI–*Eco*RI sites within the polylinker of the *E.coli* gene fusion vector pGEX-2TK (Pharmacia). The resulting GST–CBP1 fusion was expressed in TOP10 cells (Invitrogen).

Calcineurin binding assays with GST–CBP1 were performed using 50 μl of GST or GST–CBP1 bound to glutathione agarose per reaction in the presence or absence of 1 U (50 μg) of calcineurin (Sigma), 1 U (2 μg) of calmodulin (Sigma), 4 mM EGTA, 8 μg of FKBP12 (Sigma) or 20 μM FK506. Reactions were incubated for 1 h at 4°C in reaction buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.2% Triton X-100, 7 mM $CaCl_2$ and phenylmethylsulfonyl fluoride) prior to the addition of GST or GST–CBP1 substrate. Binding reactions were incubated at 4°C for 2 h, followed by two washes in reaction buffer. Samples were separated on 10% SDS–PAGE, transferred to PVDF and immunoblotted with polyclonal anti-calcineurin antibody (Upstate Biotechnology) or monoclonal anti-GST antibody (Santa Cruz Biotechnology) and visualized with ECL (Amersham).

### Calcineurin phosphatase assays

Calcineurin phosphatase assays were performed with [$^{32}$P]RII peptide as previously described (Liu *et al*., 1991; Heitman *et al*., 1993). Peptides were provided by the HHMI peptide synthesis facility at DUMC. Peptide sequences are as follows: [DSCR1 18] KQFLISPPASPPVGWKQV and [CBP1 18] HNFLISPPGSPPEGWEPA. The calcineurin autoinhibitory domain peptide [AID] ITSPEEAKGLDRINERMPPRRDAMP was obtained from Calbiochem. Prior to the addition of substrate, reactions containing calcineurin in the presence or absence of inhibitors were incubated at 30°C for 30 min. Reactions were terminated 20 min after the addition of substrate. Assays using pNPP were performed as described previously (Haddy *et al*., 1992).

#### *In vivo tagging of the CBP1 protein*

The genomic *CBP1* locus was PCR amplified from the cloned 11.0 kb *Pst*I fragment using forward primer 5′-TATTGTTGAGAAGTACTC (2387) and reverse primer 2041 and cloned into pCR2.1-TOPO (Invitrogen) resulting in plasmid pCn3gS. The open reading frame of GFP was amplified from a plasmid template (Del Poeta *et al*., 1999) using forward primer 5′-TATTAAAAGGCCTAAAGGT (2392) and reverse primer 5′-CTGAGGCCTTTTGTACAA (2465), digested with *Stu*I and ligated into *Stu*I-digested pCn3gS plasmid DNA. A clone with GFP in the correct orientation was digested with *Spe*I and *Not*I to release the *CBP1::GFP* gene fusion. This fragment was ligated into *Spe*I- and *Not*I-digested pHYG7-KB1 (Hua *et al*., 2000) to yield plasmid pCn3gS–GFP, which was transformed into *C.neoformans* strains H99, M049, JMC3 and AO4 (Y5) (Odom *et al*., 1997; Cruz *et al*., 2000) by biolistic transformation. Multiple hygromycin B resistant strains were isolated from each transformation event and were single colony purified. Cell extracts were prepared from at least two transformants of each strain (Odom *et al*., 1997), separated on 12% SDS–PAGE, transferred to nitrocellulose, and probed with *Anti-Aquorea victoria* GFP antibody. Subsequently an anti-rabbit IgG secondary antibody was applied and detected with the ECL detection system. All strains harboring the pCn3gS–GFP plasmid expressed a protein of ~60 kDa that cross-reacted with the anti-GFP antibody; those lacking the plasmid had no cross-reacting protein. Highly conserved serine residues including those within the highly conserved SP domain of CBP1 were replaced with alanines by site-directed mutagenesis by PCR overlap.

#### *Gene replacement of the CBP1 open reading frame*

The gene replacement construct was generated as follows. Forward primer 5′-TAGTTCGGAATTCCACAATC (2038) and reverse primer 5′-TGAAGGGATCCTGCGCGCGATATGG (2039) were used to amplify a 920 bp fragment of the promoter and 5′ untranslated region of the *CBP1* gene. The PCR product was digested with *Eco*RI and *Bam*HI and ligated into *Eco*RI- and *Bam*HI-digested pUC119 to yield plasmid pJMM152. The 550 bp 3′ untranslated region and terminator of the *CBP1* gene was amplified using forward primer 5′-TTTATGGTCGACCAG-CGCGCGAGTCT (2040) and reverse primer 5′-GTATGCTTAAGC-TTCTTCTCGG (2041), digested with *Sal*I and *Hin*dIII, and ligated into plasmid pJMM152 digested with *Sal*I and *Hin*dIII, yielding plasmid pJMM154. The *ADE2* genomic locus was amplified from plasmid pCnade2*ΔApa*I (Sudarshan *et al*., 1999) using forward primer 5′-GGG-TACCGCGCGCTTGAACGCC (1938) and reverse primer 5′-CAGGTG-AAGTTGCGCGCGAAACGAAC (1939), digested with *Bss*HII and ligated into plasmid pJMM154, yielding plasmid pJMM160. Approximately 10 µg of circular form pJMM160 plasmid DNA was transformed into the *C.neoformans ade2* strain M049 by biolistic transformation (Toffaletti *et al*., 1993; Sudarshan *et al*., 1999). Transformants were isolated on synthetic regeneration medium lacking adenine. Approximately 530 transformants were isolated and screened by PCR amplification. Primers 5′-GGACGAGAGCAAGCAAGTCCG (1732) and 5′-CAG-TTGAAGAGATGTTGCTGGGG (1612) amplify a 420 bp product from the *CBP1* wild-type locus and no product from the *cbp1Δ::ADE2* allele. Strains lacking the 420 bp PCR product were rescreened by Southern analysis to identify strains in which the *CBP1* gene had been replaced by the *ADE2* gene.

#### *Northern blot analysis*

Total RNA was isolated from *C.neoformans* strain H99 grown in 10 ml of YPD medium at 30°C as described (Chomczynski and Sacchi, 1987). Briefly, cells were centrifuged in a tabletop centrifuge and the cell pellet was frozen at –80°C and lyophilized overnight to complete dryness. The pellet was lysed by vortexing with 2 mm glass beads. To the pulverized cells was added 500 µl of RNA extraction buffer (guanidine thiocyanate, EDTA), 100 µl of 2 M sodium acetate pH 5.2, 200 µl of water-saturated phenol and 200 µl of chloroform, with vortexing after each addition. Cells were incubated on ice for 30 min and subsequently centrifuged at maximum speed in a tabletop centrifuge for 10 min at 4°C. The aqueous phase was transferred to an Eppendorf tube and an equal volume of isopropanol was added. RNA was precipitated on ice for 10 min and then centrifuged at maximum speed in a microfuge for 5 min. The pellet was washed with 70% ethanol, dried, and resuspended in ~100 µl of water. RNA blot analysis was performed as described using Nytran+ membranes (Schleicher and Schuell).

#### *Isolation of S.cerevisiae cbp1 mutant strain*

The *S.cerevisiae YKL159c* gene was disrupted by PCR-mediated gene disruption with the KanMX gene cassette (Wach *et al*., 1994; Lorenz *et al*., 1995). Flanking primers with 40 bp of homology to the 5′ and 3′ regions of the YKL159c and 20 bp of homology to the KanMX cassette used for PCR amplification were: (2825) 5′-AAGCAATAAACCAA-CCGATATATAAAACACAGAACTGCAGCAGCTGAAGCTTCGTA-CGC and (2826) 5′-GCATTTAAGTCTCTTAAGCCAACAAATCGC-CTCGCCATCTGCATAGGCCACTAGTGGATCTG. The *ykl159cΔ::G418* gene disruption PCR product was transformed into yeast strain JK9-3da, G418-resistant transformants were selected, and gene disruption was confirmed by isolation of genomic DNA and PCR analysis with the flanking primer (2827) CCATATTTACTTAGGTCA and primer 2825.

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