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Derepressed Hyphal Growth and Reduced Virulence in a VH1 Family-related Protein Phosphatase Mutant of the Human Pathogen *Candida albicans*

Csilla Csank,*† Constantin Makris,* Sylvain Meloche,* Klaus Schröppel,‡ Martin Röllinghoff,‡ Daniel Dignard,† David Y. Thomas,†§ and Malcolm Whiteway†§‖

*Centre de Recherche, Hôtel-Dieu de Montréal and Department of Pharmacology, University of Montreal, Montreal, Quebec, Canada H2W 1T8; †Eukaryotic Genetics Group, National Research Council of Canada, Biotechnology Research Institute, Montreal, Quebec, Canada H4P 2R2; ‡Institute of Clinical Microbiology and Immunology, University of Erlangen, D-91054 Erlangen, Germany; and §Biology Department, McGill University, Montreal, Quebec, Canada H3A 1B1

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Mitogen-activated protein (MAP) kinases are pivotal components of eukaryotic signaling cascades. Phosphorylation of tyrosine and threonine residues activates MAP kinases, but either dual-specificity or monospecificity phosphatases can inactivate them. The *Candida albicans CPP1* gene, a structural member of the VH1 family of dual- specificity phosphatases, was previously cloned by its ability to block the pheromone response MAP kinase cascade in *Saccharomyces cerevisiae*. Cpp1p inactivated mammalian MAP kinases in vitro and acted as a tyrosine-specific enzyme. In *C. albicans* a MAP kinase cascade can trigger the transition from the budding yeast form to a more invasive filamentous form. Disruption of the *CPP1* gene in *C. albicans* derepressed the yeast to hyphal transition at ambient temperatures, on solid surfaces. A hyphal growth rate defect under physiological conditions in vitro was also observed and could explain a reduction in virulence associated with reduced fungal burden in the kidneys seen in a systemic mouse model. A hyper-hyphal pathway may thus have some detrimental effects on *C. albicans* cells. Disruption of the MAP kinase homologue *CEK1* suppressed the morphological effects of the *CPP1* disruption in *C. albicans*. The results presented here demonstrate the biological importance of a tyrosine phosphatase in cell-fate decisions and virulence in *C. albicans*.

INTRODUCTION

The developmental transition from the predominant yeast form (blastospores) to the hyphal form of the opportunistic pathogen *Candida albicans* is thought to contribute to early steps in invasion of epithelial tissues (reviewed in Fidel and Sobel, 1994); however, both forms can be found in infected human tissues (Odds, 1988). Elucidation of the signaling events that lead to germ tube formation and subsequent hyphal development will help us to understand both the initial steps in the encounter of *C. albicans* cells with host epithelia and the contribution of the yeast and hyphal forms to pathogenesis. Such studies may reveal new targets for antimycotic drugs.

Many environmental factors induce the yeast form of *C. albicans* to form germ tubes which develop into hyphae (Odds, 1988; reviewed in Soll, 1986), suggesting the involvement of multiple signal transduction pathways or multiple inputs into the same pathway. Conditions that mimic those found in the blood, including neutral pH (Buffo *et al.*, 1984) and physiological temperature (Lee *et al.*, 1975; Buffo *et al.*, 1984) induce the yeast to hyphal switch, whereas ambient temperatures or acid pH favor the budding yeast form. In combination with these conditions, serum (Gow and Gooday, 1982), or

‖ Corresponding author.

certain synthetic defined and complex media, allows hyphal development in vitro either in liquid culture (Lee *et al.*, 1975; Buffo *et al.*, 1984) or on solid media (Gow and Gooday, 1982; Liu *et al.*, 1994; Leberer *et al.*, 1996). A conserved protein kinase cascade in *Saccharomyces cerevisiae* drives several cellular responses including pheromone response, yeast to pseudohyphal switching, and agar invasion (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Roberts and Fink, 1994; Herskowitz, 1995). Components of this cascade have recently been shown to stimulate yeast to hyphal switching in *C. albicans* (Liu *et al.*, 1994; Malathi *et al.*, 1994; Clark *et al.*, 1995; Kohler and Fink, 1996; Leberer *et al.*, 1996). The *C. albicans* genes *CST20*, *HST7*, and *CPH1* are homologues of *S. cerevisiae* genes encoding a mitogen-activated protein (MAP) kinase kinase kinase kinase (*STE20*), a MAP kinase kinase (*STE7*), and a transcription factor (*STE12*) involved in this signaling pathway (Liu *et al.*, 1994; Malathi *et al.*, 1994; Clark *et al.*, 1995; Kohler and Fink, 1996; Leberer *et al.*, 1996). In *C. albicans*, these genes function in glucose-independent in vitro hyphal formation on solid surfaces, but not in serum-dependent or in vivo hyphal formation (Liu *et al.*, 1994; Kohler and Fink, 1996; Leberer *et al.*, 1996). These results point toward distinct signaling mechanisms for hyphal development in response to different stimuli, including solid surfaces. Contact sensing at solid surfaces could be involved in stimulating hyphal development as it is in guiding hyphae toward pores (Sherwood *et al.*, 1992). Despite the absence of an effect on hyphal formation in mice, deletion of *CST20* from *C. albicans* results in a minor but significant reduction in virulence in mice (Leberer *et al.*, 1996).

A balance between MAP kinase activation by kinases and their inactivation by phosphatases is likely to be important for decisions which govern developmental processes and virulence in *C. albicans*. We have previously screened a *C. albicans* genomic library in an attempt to find genes which interfere with pheromone-mediated cell cycle arrest in *S. cerevisiae* (Whiteway *et al.*, 1992). One of the genes (*CEK1*) isolated during this screen encodes a *C. albicans* homologue of the *S. cerevisiae* Fus3p and Kss1p MAP kinases involved in pheromone response (Whiteway *et al.*, 1992).This screen also identified a gene called *CPP1* for *Candida* protein phosphatase with similarities to protein tyrosine phosphatases (PTPs) (Whiteway *et al.*, 1993). *CPP1* expression was found, using a series of epistasis experiments, to block the *S. cerevisiae* pheromone response pathway at the level of the Fus3p MAP kinase (Whiteway *et al.*, 1993). We show here that Cpp1p is a member of the VH1 family of dual-specificity phosphatases and is most similar to the *S. cerevisiae* MAP kinase phosphatase, Msg5p, which is involved in adaptation to pheromone (Doi *et al.*, 1994). Although deletion of the mammalian VH1 phosphatase (*MKP1*) gene has no effect on mouse development (Dorfman *et al.*, 1996) and deletion of the two *S. cerevisiae* VH1-phosphatase genes (*MSG5* and *YVH1*) have only subtle effects on *S. cerevisiae* cells (Guan *et al.*, 1992; Doi *et al.*, 1994), deletion of the *CPP1* gene in *C. albicans* derepresses the yeast to hyphal transition at ambient temperatures on solid surfaces under normally noninducing conditions. This result suggests that Cpp1p is required for repression of the yeast to hyphal switch. In addition, under hyphal-stimulating physiological conditions in vitro which inhibit the initial growth of the yeast form, deletion of the *CPP1* gene results in a hyphal growth rate defect. In vivo, this latter effect may be of significance because virulence is greatly reduced in a mouse model for systemic candidiasis when mice are infected with *CPP1*-defective cells.

MATERIALS AND METHODS

DNA Manipulations and Analysis

DNA manipulations were carried out according to standard procedures (Sambrooke *et al.*, 1989). Southern blot analysis was performed with a nonradioactive labeling and detection kit (Boerhinger Mannheim, Canada, Laval, QC) according to the manufacturer's recommendations. Both strands of *C. albicans* sequences in the plasmid M195p4 were sequenced.

Blast similarity searches were done with the GenBank and SwissProt databases. A protein motif alignment was done using Protomat (Henikoff *et al.*, 1995). Alignments of the active site domains of protein sequences were done with the program GeneWorks (IntelliGenetics) using a modified FASTA algorithm as described in the GeneWorks Reference Manual.

Plasmid Constructions

Plasmid pCCY10.2 was constructed by digestion of plasmid M195p4 with *Bgl*II and *Sma*I, generation of blunt ends and religation, leaving the vector YEP352 (Hill *et al.*, 1986) with a 4.5-kb fragment containing the *CPP1* gene and flanking DNA. M195p4, itself, was derived from the original plasmid M153p11 (Whiteway *et al.*, 1992) by partial *Spe*I digestion and religation to the YEP352 *Xba*I site. Plasmid pGALCPP1-M178 expresses an active truncated version of *CPP1* starting at amino acid 178. Synthetic oligonucleotides with 5′ *Eco*RI sites were used to amplify DNA spanning nucleotides 506 to 1886 (nucleotide 1 refers to the first nucleotide of the open reading frame). Polymerase cahin reaction (PCR) products were inserted into the *Eco*RI site of an *S. cerevisiae* centromeric *TRP1*-based shuttle vector pRS314GAL containing the *GAL1 S. cerevisiae* promoter on a *Bam*HI-*Eco*RI fragment modified from the vector pRS314 (Sikorski and Hieter, 1989). The conserved cysteine (residue 516) of the active site region of the *CPP1* polypeptide in pGALCPP1-M178 was changed to a serine residue by site-directed mutagenesis (Kunkel *et al.*, 1987) creating pGALCPP1-M178-C516S. The synthetic oligonucleotide used (5′-GACGTAAAATATTGATTCATTCACAATGTGGAGTATCG3-′) replaces cysteine 516 with a serine, while introducing a *Dra*III site (underlined). Mutants were verified by sequence analysis. pGST-ERK1 was created by subcloning the *Eco*RI fragment encoding hamster *Erk1* from plasmid pCMV/HAPMK (Meloche *et al.*, 1992) into the *Eco*RI site of pGEX-KG (Guan and Dixon, 1991). pGST-CPP1 was created by amplifying nucleotides 767 to 1886 of *CPP1* by PCR using synthetic oligonucleotides with 5′ *Eco*RI sites and then cloning the amplified DNA fragment into pGEX-KT (Hakes and Dixon, 1992). This same fragment, when placed under the control of the *S. cerevisiae GAL1* promoter, interfered with pheromone-induced cell cycle arrest in yeast. For gene disruptions pCCB201 was created in two steps. First, PCR was used to amplify the *CPP1* gene and flanking sequences in pCCY10.2 and to add *Sac*I

Table 1. Yeast strains used in this study

Strain	Relevant genotype	Origin or source
S. cerevisiae		
HC1-4D	*MAT***a** *sst1 sst2 ura3 trp1 leu2 ade-kex2::ura3*	Whiteway et al., 1993
C. albicans		
SC5314	*URA3/URA3 CPP1/CPP1 CEK1/CEK1*	Gillum et al., 1984
CAI4	*ura3 CPP1/CPP1 CEK1/CEK1*	Fonzi and Irwin, 1993
CP29	*ura3/ura3 CPP1/cpp1Δ::hisG-URA3-hisG*	CAI4
CP29-1	*ura3/ura3 CPP1/cpp1Δ::hisG*	CP29
CP29-1-7	*ura3/ura3 cpp1Δ::hisG-URA3-hisG/cpp1Δ::hisG*	CP29-1
CP29-1-7L4	*ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG*	CP29-1-7
CP29-1-7RI	*ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG::CPP1-URA3*	CP29-1-7L4
CP29-1-7CK13	*ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG; CEK1/cek1Δ::hisG-URA3-hisG*	CP29-1-7L4
CP29-1-7CK14	*ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG; cek1Δ::hisG-URA3-hisG/cek1Δ::hisG-URA3-hisG*	CP29-1-7L4

sites using the oligodeoxynucleotide primers O27 (5′-GAACAACCAGGAGAGCTCTTTCCAACTGATTTAATTTG-3′) and O26 (5′-GTTGTCTTTAGTTGGAGCTCCTTATTTTATATAATAGATG-3′) (*Sac*I sites are underlined). After digestion, this 2.3-kb *Sac*I fragment was inserted into the Bluescript KS(+) vector (Stratagene, La Jolla, CA) to yield plasmid pCCB200. Oligodeoxynucleotide primers O24 (5′-GAAGATCTGATATCTATTTTCCCTTGATCTGGATCTG-3′) and O25 (5′-GAAGATCTGTTGTAGCATTTTATATGAAGAAATTCCAATTGGGAG-3′) were then used to delete the PTP-active site region in pCCB200 using divergent PCR while adding *Bgl*II sites (underlined). The amplified fragment was cut with *Bgl*II and joined to a 4-kb *Bam*HI-*Bgl*II *hisG-URA3-hisG* fragment from the plasmid p5921 (Fonzi and Irwin, 1993) to create pCCB201. pCCa2 was constructed by subcloning a 4.5-kb *Pst*I-*Kpn*I fragment from the plasmid pCCY10.2 into pBS-cURA3 (Leberer *et al.*, 1996) containing the *C. albicans URA3* gene.

Construction of plasmids for disruption of the *CEK1* gene (Whiteway *et al.*, 1992) will be presented in greater detail elsewhere. Briefly, the plasmid pMO3 contains an 8-kb *Kpn*I-*Xba*I DNA fragment in the *Kpn*I-*Xba*I sites of the Bluescript KS(+) vector (Stratagene). This insert contains a *C. albicans* genomic DNA fragment in which a 1.2-kb portion of the *CEK1* gene was replaced by a 4-kb *Bam*HI-*Bgl*II *hisG-URA3-hisG* fragment (Fonzi and Irwin, 1993). Deletion of the 1.2-kb portion of the *CEK1* gene was accomplished by reverse PCR with the oligonucleotide primers OT1 and OT2 (positions shown in Figure 8A) which were flanked by *Bgl*II sites for ligation to the *hisG-URA3-hisG* blaster cassette.

S. cerevisiae Pheromone Response

Pheromone response was assessed by transforming plasmids (Rose *et al.*, 1990) into a supersensitive *S. cerevisiae* strain HC1–4D (Whiteway *et al.*, 1993). For examination of growth in the presence of pheromone on solid media, patches of HC1–4D cells transformed with pGALCPP1-M178, pGALCPP1-M178-C516S, and the control vector pRS314GAL were grown on selective solid medium (SC) without tryptophan using 2% galactose as a carbon source (Rose *et al.*, 1990) and were replica plated onto selective medium spread with 5 μg of the yeast mating pheromone α-factor (Sigma, St. Louis, MO).

Biochemical Assays

Recombinant glutathione S-transferase (GST) fusion proteins were produced as follows: Plasmids pGST-CPP1, pGST-ERK1, and pGEX-KT (GST) were expressed in *Escherichia coli*, and the GST fusion proteins or GST were purified as described (Guan and Dixon, 1991). The approximate concentration of recombinant proteins was determined by SDS-PAGE using bovine serum albumin as a protein standard.

The enzymatic activity of Cpp1p was assayed using Erk1 as a substrate. For these experiments, recombinant GST-Erk1 immobilized on glutathione-agarose beads was first activated by incubation with a cytosolic extract of serum-stimulated Rat 1 cells in the presence of 50 μM ATP at 30°C for 30 min (Meloche, 1995). The beads were then washed in phosphatase buffer containing 25 mM HEPES (pH 7.4), 100 mM NaCl, and 1 mM dithiothreitol prior to incubation with GST-Cpp1p at 37°C for 30 min in phosphatase buffer. Myelin basic protein (MBP) phosphotransferase assays were done as described (Meloche, 1995). The tyrosine phosphorylation of Erk1 was evaluated by antiphosphotyrosine immunoblotting using the monoclonal antibody PY20 (ICN). The bands were visualized by chemiluminescence (ECL, Amersham Canada Ltd., Oakville, Ontario) according to the manufacturer's instructions. For phosphoamino acid analysis, Erk1 and Erk2 were immunoprecipitated from ^{32}P-labeled serum-stimulated Rat 1 fibroblasts using the specific Erk1 antiserum SM1 or the specific Erk2 antiserum aIIcp42 and analyzed by two-dimensional phosphoamino acid analysis as described (Meloche, 1995).

Candida Strains and Growth Conditions

All strains are listed in Table 1. To induce germ tube formation in liquid culture, cells were diluted 10-fold from overnight cultures into fresh Spider medium (Liu *et al.*, 1994), Lee's medium (Lee *et al.*, 1975), or 10% fetal bovine serum (Intergen Co., Purchase, NY) and incubated for 3 h at 37°C (Lee *et al.*, 1975).

To induce hyphal growth on solid media, budding *C. albicans* were grown overnight at 30°C with vigorous shaking in YP medium (2% yeast extract, 4% Bacto Peptone) supplemented with either 2% glucose for YPD (Rose *et al.*, 1990) or 2% mannitol for YPM. Twenty-five to 100 cells per plate were then incubated for indicated times at 23°C or 37°C on different media. Solid Spider medium contains 1% nutrient broth, 0.2% K_2HPO_4, 1.4% agar, and 1% of either glucose or mannitol (Liu *et al.*, 1994). Lee's medium is as described (Lee *et al.*, 1975). For serum plates, 10% fetal bovine serum was added to 1.4% agar at 50°C after autoclaving.

To examine agar-penetrating growth beneath colonies, cells were gently scraped off plates with a plastic inoculating loop and plates were then washed several times with sterile water. Photomicroscopy of colonies and invasive growth was done with a Nikon TMS inverted microscope and photographed with Kodak TMAX film.

Nomarski optics was used to photograph germ tubes and to monitor for septa formation and branching of cells scraped from

agar surfaces. A 100× objective and a Leitz Aristoplan microscope were used.

Candida Gene Disruptions

Homologous recombination was used for gene disruptions by a sequential gene disruption strategy using the selectable marker *URA3* flanked by *hisG* direct repeats (Fonzi and Irwin, 1993). The *hisG* repeats facilitate the removal of the *URA3* gene and allow the use of the same selectable marker for disruption of the second allele. This *hisG-URA3-hisG* cassette was used to replace parts of the *CPP1* and *CEK1* genes. For *CPP1* disruptions, the active site region of the *CPP1* gene was deleted to allow a maximum number of defined sequences flanking the disruption cassette and because previous experiments showed that removal of the active site was sufficient to totally inactivate Cpp1p function. pCCB201 was digested with *Sac*I and the isolated fragment was used to transform spheroplasts (Kurtz *et al.*, 1986) of the strain CAI4 (Table 1). Transformants able to grow in the absence of uracil were selected and replacement of the chromosomal gene with the fragment containing a *hisG-URA3-hisG* cassette in place of the active site region was verified by Southern blot analysis using a *Hin*dIII-*Spe*I fragment from the *CPP1* gene as a probe. Transformants with a disruption in the *CPP1* gene were chosen for selection of Ura3$^-$ derivatives on 5-fluoroorotic acid, which kills Ura3$^+$ prototrophs. Ura3$^-$ auxotrophs were examined using Southern blot analysis to identify excisions of the *URA3* repeat leaving behind one copy of *hisG*. These steps were repeated to obtain cells with disruptions in both alleles of *CPP1*. For reintegration of *CPP1* into the genome, the *C. albicans* reintegration plasmid containing the *CPP1* gene and flanking sequences, pCCa2, was linearized with *Nsi*I to target integration to the *Nsi*I site of *cpp1*Δ*::hisG* and transformed into *Ura*$^-$ *C. albicans* containing the double deletion of the *CPP1* gene *cpp1*Δ*ccp1*Δ*::hisG*. Most in vitro studies were done with two completely independent, but phenotypically identical, homozygous mutants: CP29–1-7 (shown in this study) and CP27–1-1.

Disruption of the *CEK1* (GenBank M76585) gene from the *cpp1* null mutant strain was achieved in one step. An 8-kb *Kpn*I-*Not*I insert from the plasmid pMO3 was used to transform CP29–1-7L4 (Table 1) as described above. This fragment contains a 4.0-kb *hisG-URA3-hisG* cassette in place of a 1.2-kb portion of the open reading frame of the *CEK1* gene. Replacement of the chromosomal gene with the exogenously provided fragment was verified by Southern blot analysis using a 3.2-kb *Kpn*I-*Sac*I probe containing the *CEK1* gene and genomic DNA digested with *Spe*I. Of 14 transformants examined using Southern blot analysis, 12 contained a disruption of one allele of *CEK1* and one had both alleles of the *CEK1* gene disrupted (Figure 8A). This transformant was called CP29–1-7CK14 (*cpp1*Δ*cpp1*Δ*/cek1*Δ*cek1*Δ) and was used for subsequent experiments.

Virulence Studies

Inbred female BALB/c mice were obtained from Charles Rivers Breeding Laboratories (Sulzfeld, Germany) and used for infection at 8 to 10 wk of age.

C. albicans in vivo virulence testing and colony-forming unit (CFU) enumeration was done as follows: Strains for infection were routinely grown at 30°C in YPD medium and kept at stationary phase for 48 h prior to infection. Aliquots of approximately 2×10^8 cells were harvested and washed three timed in phosphate-buffered saline, and adjusted to the desired density to be used for in vivo virulence testing. *C. albicans* blastospores were injected i.v. into the tail vein in a final volume of 200 μl. Three to four mice per group were killed 2 and 5 d after infection, and the number of CFUs was quantified using a plate dilution method of homogenized organs on modified Lee's medium agar plates (Soll *et al.*, 1981). Results are expressed as the log CFU per g organ wet weight.

For histological analysis of *C. albicans* cell morphology in vivo, five infected animals were killed 2 d after infection, and the kidneys were solubilized in 20 ml of 10% potassium hydroxide (KOH) solution at 50°C for 3 h. Alkaline-resistant particles, including yeast cells, were sedimented at 1500 × *g*, resuspended in 50 μl of KOH solution containing 5 μg/ml Calcofluor white (Sigma, Deisenhofen, Germany). Slides were directly mounted without further manipulations and screened for *C. albicans* cells with a Zeiss Axiophot microscope under fluorescent light (365-nm filter for excitation and 420-nm filter for emission).

RESULTS

Sequence Analysis of the CPP1 Gene

The *CPP1* gene (GenBank L01038) codes for a 597-amino acid polypeptide with similarity to the dual-specificity (serine/threonine and tyrosine) phosphatases of the Vaccinia VH1 subfamily of PTPs (Figure 1) (Guan *et al.*, 1991; Keyse, 1995; Zolnierowicz and Hemmings, 1996) and maps to *C. albicans* chromosome 1 (Magee, personal communication). A 140-amino acid region of the *CPP1* polypeptide, which contains a core PTP-active site signature sequence (V/I)HCxAGxxR(S/T) (Fischer *et al.*, 1991; Zolnierowicz and Hemmings, 1996), aligns with other members of the VH1 phosphatase family (Figure 1). An active site cysteine residue is required for catalysis by PTPs (Keyse, 1995). Mutation of the equivalent cysteine residue of Cpp1p to a serine, expressed in plasmid pGALCPP1-M178 in *S. cerevisiae*, destroyed its capacity to block pheromone-induced cell cycle arrest (Figure 2), demonstrating that phosphatase activity was required for biological activity.

CPP1 is most similar to two *S. cerevisiae* genes: *MSG5*, which encodes a protein which dephosphorylates the Fus3p MAP kinase (Doi *et al.*, 1994), and *YIL3*, an open reading frame in the databases (SWISS-PROT P40558; YIL003W; putative phosphatase). *CPP1* has 44% and 40% amino acid identity with *MSG5* and *YIL3*, respectively, in the region surrounding the active site (Figure 1B; gray boxes). All three have a cysteine instead of an alanine at position five of the PTP signature sequence. The *CPP1*, *MSG5*, and *YIL3* polypeptides do not contain CH2 domains (Figure 1B), which are domains shared by several mammalian VH1 family phosphatases (many of which dephosphorylate MAP kinases) and the dual- specificity *cdc25* phosphatases (Keyse and Ginsburg, 1993). In addition, Cpp1p contains a putative PEST motif (Rogers *et al.*, 1986) rich in serine (25%) and threonine (15%) residues in its amino terminal domain (Figure 1B), which suggests that Cpp1p may have a short half-life (Rogers *et al.*, 1986).

Biochemical Characterization of Cpp1p Activity

The indications that Cpp1p might act as a MAP kinase phosphatase prompted us to examine the ability of Cpp1p to dephosphorylate and inhibit the enzymatic activity of the mammalian MAP kinase Erk1 in vitro. Cpp1p was found to inhibit the phosphotransferase

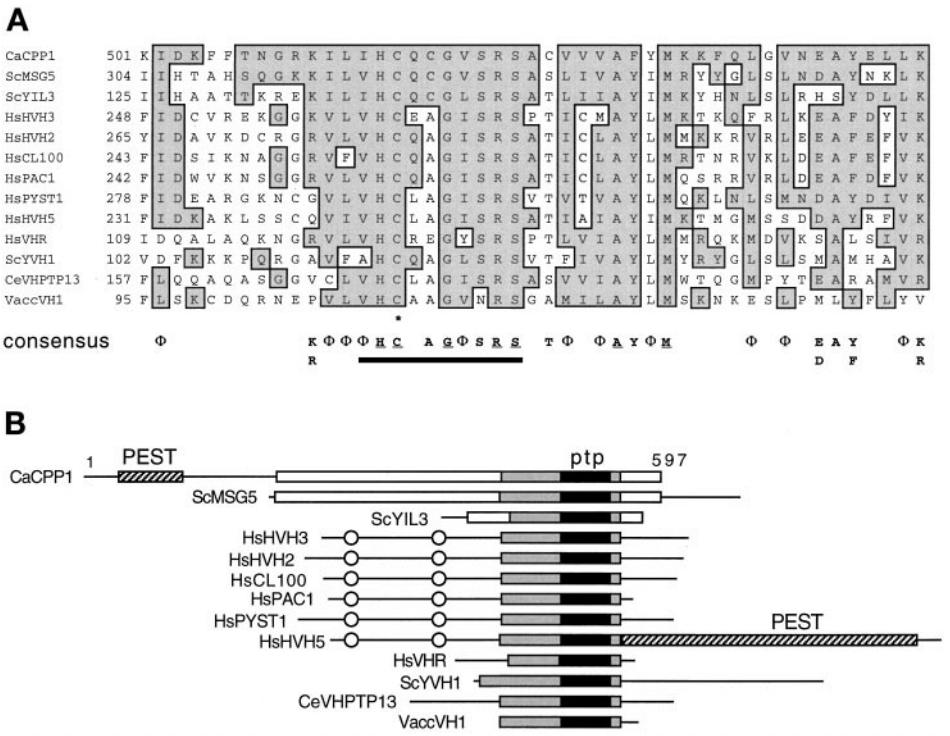

Figure 1. Structural similarity of *CPP1* with VH1 family phosphatases. (A) Similarity of the *CPP1* amino acid sequence to a conserved region surrounding the active-site signature sequence of the VH1 family phosphatases. Aligned sequences are *C. albicans* (Ca) *CPP1*, *S. cerevisiae* (Sc) *MSG5* (Doi *et al.*, 1994), *YIL3* (SWISS-PROT P40558, YIL003W, putative phosphatase), and *YVH1* (Guan *et al.*, 1992), human (Hs) *HVH3* (Kwak and Dixon, 1995), *HVH2* (Guan and Butch, 1995), *CL100* (*MKP1*) (Alessi *et al.*, 1993), *PAC1* (Rohan *et al.*, 1993), *PYST1* (Groom *et al.*, 1996), *HVH5* (Martell *et al.*, 1995), *VHR* (Ishibashi *et al.*, 1992), *Chlamydomonas eugametos* (Ce) *VHPTP13* (Haring *et al.*, 1995), and Vaccinia (Vacc) *VH1* (Guan *et al.*, 1991). Conserved residues (ILVM =Φ; KR; ED; NQ; YF) and identical residues present in the *CPP1* polypeptide and one or more of the other polypeptides are boxed and shaded. A VH1 family consensus is presented below the alignment for residues present in 8 or more of the 13 sequences. Residues identical in all 13 sequences are underlined. The PTP-active site motif is underlined with a black bar and the active site essential cysteine is shown with an asterisk. Numbers at the left of the margin specify the amino acid positions within each polypeptide. (B) Schematic alignment of the *VH1* family phosphatases. Black boxes represent the highly conserved active site region (PTP) aligned in A around which the alignment is centered. The gray boxes represent an extended homology region with lower identities between sequences. White boxes represent additional regions of alignment among *CPP1*, *MSG5*, and *YIL3*. PEST sequences are shown. CH2 domains are circled. Numbers indicate the size of the *CPP1* polypeptide.

activity of Erk1 toward its substrate MBP in a dose-dependent manner (Figure 3A). The inactivation of the enzyme correlated with the dephosphorylation of the phosphotyrosine residue as shown by immunoblotting with an antiphosphotyrosine antibody (Figure 3B). The dephosphorylation of the tyrosine in Erk1 was inhibited by vanadate and Zn^{2+}, which are common inhibitors of tyrosine phosphatases (Walton and Dixon, 1993). Our unpublished observations showed that the Cpp1p-active site mutant Cpp1p^{C516S} could not dephosphorylate Erk1, but was still able to inhibit Erk1 activity. Similarly, site-directed mutants of the equivalent active site cysteine residue of the *Chlamydomonas eugametos VHPTP13* phosphatase or the *S. cerevisiae MSG5* phosphatase retain some ability to inactivate MAP kinases in vitro (Haring *et al.*, 1995).

Interestingly, Cpp1p was able to dephosphorylate phosphotyrosine residues but not phosphoserine or phosphothreonine residues of ^{32}P-labeled activated-Erk1 (and Erk2) as determined by phosphoamino acid analysis (Figure 3C).

Derepression of the Yeast to Hyphal Switch in CPP1 Null Mutants

We constructed null mutants of the *CPP1* gene by sequential gene disruption using a *URA3* gene, flanked by *hisG* repeats, as a selectable marker (Figure 4).

Previous work had shown that null mutants of the *CST20*, *CPH1*, and *HST7* genes encoding *C. albicans* MAP kinase cascade components are defective in normal hyphal outgrowth at 37°C (Liu *et al.*, 1994; Kohler and Fink, 1996; Leberer *et al.*, 1996); this was assessed as the absence of filamentous growth from mature

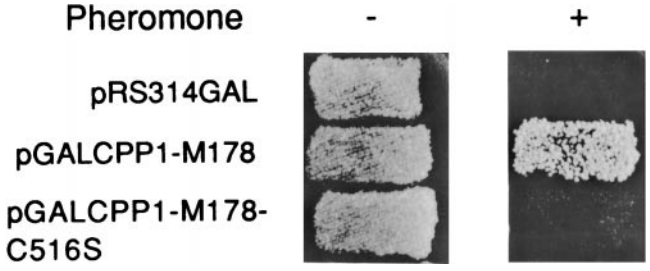

Figure 2. Site-directed mutagenesis of the conserved PTP-active site cysteine stops Cpp1p from interfering with the *S. cerevisiae* pheromone response pathway. Growth of *S. cerevisiae* cells on selective media with or without pheromone is shown. Cells contain the plasmids pRS314GAL (vector), pGALCPP1-M178 containing the extended region of *CPP1* homology with *MSG5*, or pGALCPP1-M178 with the essential PTP-active site cysteine changed to a serine (pGALCPP1-M178-C516S).

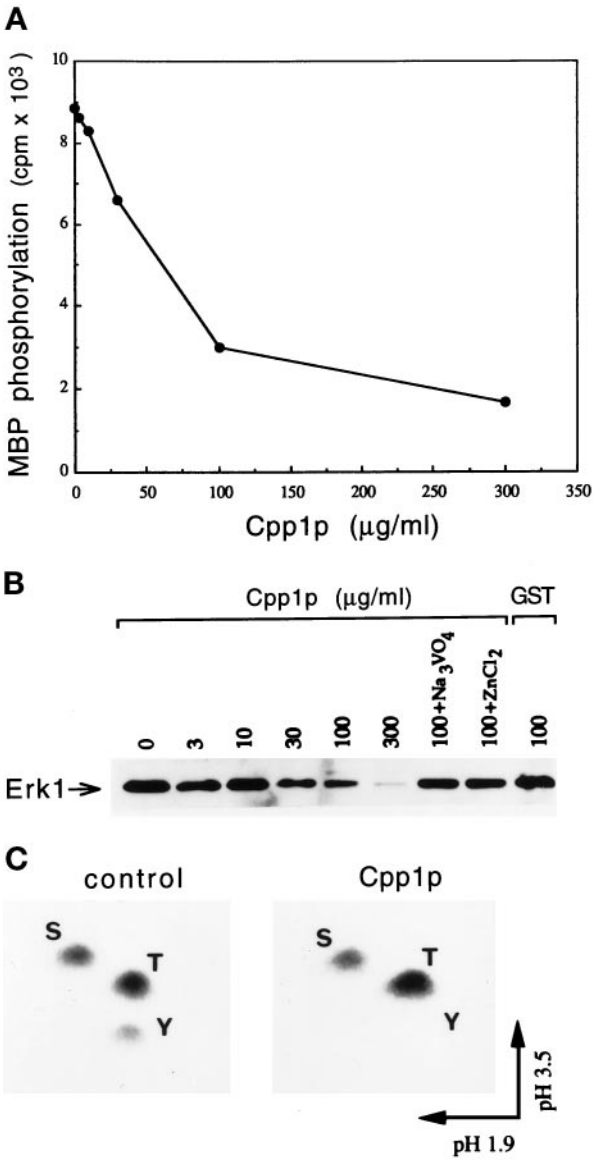

Figure 3. Cpp1p dephosphorylates and inactivates MAP kinase in vitro. (A) Cpp1p inhibits the MBP kinase activity of Erk1. Purified active GST-Erk1 on beads was incubated with indicated concentrations of GST-Cpp1p. Following incubation, the beads were washed and assayed for MBP kinase activity. (B) Cpp1p dephosphorylates Erk1. Purified active GST-Erk1 was incubated in the absence or presence of indicated concentrations of GST-Cpp1p or GST, or with 100 μg/ml GST-Cpp1p in the presence of the phosphatase inhibitors sodium vanadate and zinc chloride. Proteins were resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody. (C) Cpp1p specifically dephosphorylates Erk1 on tyrosine. Erk1 was immunoprecipitated from ^{32}P-labeled serum-stimulated Rat 1 cells and incubated in the absence (control) or presence (Cpp1p) of soluble GST-Cpp1p. Two-dimensional phosphoamino acid analysis of the ^{32}P-labeled Erk1 is shown. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

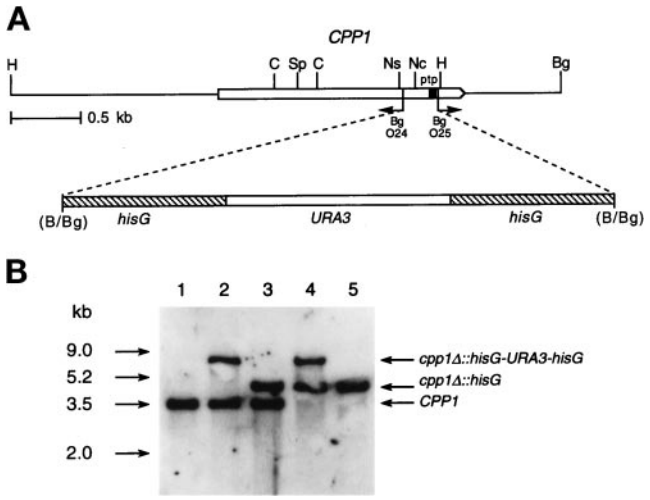

Figure 4. Deletion of *CPP1* in *C. albicans*. (A) Deletion strategy and restriction map of the *CPP1* gene. PCR with the divergent oligodeoxynucleotides O24 and O25 was used to delete the PTP-active site region (ptp) of *CPP1*. A *hisG-URA3-hisG* cassette was then inserted. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Nc, *Nco*I; Ns, *Nsi*I; Sp, *Spe*I. (B) Southern blot analysis of *CPP1* disruptions with a *Hin*dIII-*Spe*I *CPP1* fragment as a probe. Genomic DNA samples were digested with *Hin*dIII from the following strains: lane 1, CAI4 (*ura3/ura3 CPP1/CPP1*); lane 2, CP29 (*ura3/ura3 CPP1/cpp1*Δ*::hisG-URA3-hisG*); lane 3, CP29–1(*ura3/ura3 CPP1/cpp1*Δ*::hisG*): lane 4, CP29–1-7 (*ura3/ura3 cpp1*Δ*::hisG-URA3-hisG/cpp1*Δ*::hisG*), and lane 5, CP29–1-7 L4 (*ura3/ura3 cpp1*Δ*::hisG/cpp1*Δ*::hisG*).

colony borders on solid spider agar in which mannitol, but not glucose, is used as a carbon source. We reasoned that if *C. albicans* Cpp1p has a target in this kinase pathway, it might also modulate hyphal development under these conditions. We examined whether Ura$^+$ *cpp1* null mutants would either derepress filamentous growth under hypha-inhibiting conditions (23°C) or hyperactivate filamentous growth under hypha-inducing conditions (37°C). Under hypha-inhibiting conditions (23°C), *cpp1* null mutants formed hyphae which invaded the agar beneath colonies with either glucose (Figure 5, B and C) or mannitol as carbon sources, and on a wide variety of rich and defined solid media including Lee's medium, YPD, YPM, and 10% serum. Our unpublished observations show that Ura$^-$ *cpp1* null mutants also demonstrate invasive hyphal growth not seen in the Ura$^-$ CAI4 parent. On the other hand, *cpp1* null mutants did not hyperactivate hyphal growth at 37°C on solid Spider medium containing mannitol or glucose (Figure 5A). In liquid culture, *cpp1* null mutants had no effect on germ tube formation at 37°C and little effect was seen at room temperature. Identical phenotypes were obtained with two independent double disruptions, and phenotypes were reversed either by site-directed reintegration of the *CPP1* gene (*cpp1*Δ*/cpp1*Δ*::CPP1-URA3*; Figure 5) or, as our unpublished results show, by high-copy expression of *CPP1* from an *ADH1* promoter. Phenotypes were also reversed by overexpres-

sion of the *CPP1* gene with a deletion of the PEST region (Cpp1pΔPEST), or a deletion of a larger portion of the amino terminus, but were not reversed by overexpression of an active site mutant Cpp1p^{C516S}. This suggests that the mutant phenotypes were the result of the loss of phosphatase activity. Taken together, these results indicate an important role of the Cpp1p phosphatase in repressing the yeast to hyphal transition from stationary *C. albicans* cells in contact with solid surfaces.

A Role of Cpp1p in Radial Mycelial Growth

In contrast to the derepression of hyphal growth under noninducing conditions, we observed, that when compared with controls, *cpp1* null mutants formed a smaller zone of lateral hyphae when grown on carbon sources such as mannitol or raffinose which normally stimulated extensive radial hyphal growth from colony borders. This raised the possibility that absence of Cpp1p was detrimental to extended radial growth of mycelial colonies.

To test this hypothesis, we examined the growth of cells on solid serum media at 37°C (Figure 6). Wild-type *C. albicans* are stimulated on solid serum media to form germ tubes which develop into invasive hyphal colonies (Gow and Gooday, 1982). In addition, serum may provide an in vitro environment more closely related to that of animal hosts. Wild-type cells, *cpp1* null mutants, and *CPP1*-reintegrants resembled each other in the extent of germ tube and hyphal elongation and septum formation up to 8 h after plating at 37°C on serum (Figure 6A; 3 and 8 h). Subsequent growth of the agar-imbedded mycelial colonies was reduced in *cpp1* null mutants when compared with the *CPP1* reintegrants (Figure 6A; 24 and 96 h) or the wild-type strain SC5314 (which our unpublished data demonstrated was phenotypically identical to the *CPP1* reintegrants). In an independent experiment, we counted the number of hyphal tips in a colony (Gow and Gooday, 1982) to estimate the mycelial growth rates of *cpp1* null mutants and wild-type SC5314 strains. Between 6 and 18 h, the rates of growth (μ) were 0.062 h^{-1} and 0.12 h^{-1} for the *cpp1* null mutant and the wild type, respectively. Although the structure of *cpp1* null hyphae appears to be normal, we observed fewer lateral buds on *cpp1* null hyphae than on hyphae from *CPP1* reintegrants (Figure 6B) or the wild-type strain SC5314 (which our unpublished data demonstrated was phenotypically identical to the *CPP1* reintegrants), suggesting that lateral bud formation or accumulation is suppressed in *cpp1* null mutants under these conditions. On the other hand, *cpp1* null mutants were able to make lateral buds from hyphae growing at room temperature. This suggests that a cellular threshold above which hyphal development occurs and below which the yeast form is favored may be lowered in the *cpp1* null mutant strain, and that lowering of this threshold may in addition have some detrimental effects on normal hyphal growth rates.

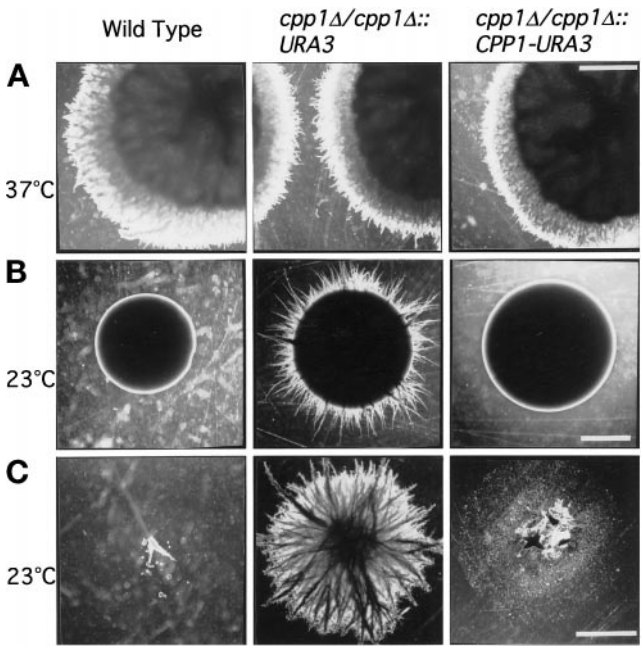

Figure 5. Colonies of *C. albicans* cells grown on solid Spider medium containing glucose. Strain SC5314 (wild type); *cpp1* null mutant CP29–1-7 (*cpp1*Δ/*cpp1*Δ*::URA3*); *cpp1* null mutant into which the *CPP1* gene has been replaced by homologous recombination CP29–1-7RI (*cpp1*Δ/*cpp1*Δ*::CPP1-URA3*). (A) Cells grown for 5 d at 37°C. (B) Cells grown for 8 d at 23°C. (C) Agar-penetrating growth remaining after surface washing of colonies grown for 8 d at 23°C from B (2× objective). Bars, 2 mm.

Virulence Studies

To determine the role of Cpp1p in virulence, mice were injected i.v. with *cpp1* null mutants (*cpp1*Δ/*cpp1*Δ*::URA3*) and *cpp1* null mutants into which the *CPP1* gene had been reintroduced (*cpp1*Δ/*cpp1*Δ*::CPP1-URA3*), which *in vitro* and in mice acted like the wild-type strain. Mice were monitored for survival and for fungal burden of the liver, lungs, and kidneys. Infection with 5×10^5 stationary phase cells resulted in complete mortality by d 14 for strains containing the wild-type *CPP1* gene (Figure 7A). In contrast, 45% of mice injected with an equal inoculum of *cpp1* null mutant cells were still alive after 40 d. We thought it unlikely that the reduction in virulence of *cpp1* null mutants was the result of a generalized growth defect because *cpp1* null mutants in vitro have only a very minor (8%) difference in yeast form growth rates (71 min for the *cpp1*Δ/*cpp1*Δ*::CPP1-URA3* strain used as a control and 65 min for the *cpp1* null mutant strain (*cpp1*Δ/*cpp1*Δ*::URA3*); however, because of the

striking mycelial growth defect seen on serum, we decided to examine the fungal burden of infected tissues.

When *C. albicans* gains access to the blood stream it disseminates to all major organs including the liver, lungs, spleen, brain, and kidneys (Odds, 1988; Anaiffie *et al.*, 1993). To determine whether reduced virulence of mice infected with *cpp1* null mutants occurred because of decreased levels of tissue infection, we determined the fungal burden of the lungs, liver, and the kidneys in two independent experiments (both shown in Figure 7B). By 2 d, the kidneys were much more heavily infected (100- to 1000-fold greater CFUs) than the liver or lungs in mice infected with the *CPP1* reintegrant as a wild-type control strain (Figure 7B, experiment 1, top graph), consistent with previous reports of the fungal burdens in these tissues 2 d after experimental infections (reviewed by Odds, 1988). On the other hand, the fungal burden of kidneys from mice infected with the *cpp1* null mutant was two to three orders of magnitude lower than the fungal burden of kidneys from mice infected with the reintegrant. The filamentous form, and not the yeast form, of *C. albicans* predominated in kidneys infected with both strains (Figure 7C). Surprisingly, livers and lungs from animals infected with either strain showed no significant difference in fungal burden (Figure 7B) at d 2 (lungs and liver) or d 5 (liver). We did not examine fungal burden in the first few hours after infection during which the lungs and the liver typically have the highest fungal counts of any infected organ (Odds, 1988), but we did see the predicted (Odds, 1988) decline in fungal burden in the liver during the course of infection in mice infected with both strains (Figure 7B, experiment two, lower graph), suggesting that the mutant strain was being cleared normally from these organs. From these studies, we suggest that the kidney-specific reduction in fungal burden accounts for the greater number of mice surviving infections with the *cpp1* null mutant strain.

Chromosomal Disruption of the MAP Kinase Homologue CEK1 Suppresses the cpp1 Null Mutant Phenotypes

To verify our assumption that Cpp1p acts on a MAP kinase affecting hyphal growth in *C. albicans*, we constructed a double mutant (Figure 8A) of *CPP1* and the MAP kinase homologue *CEK1* (Whiteway *et al.*, 1992). *CEK1* is a potential member of the MAP kinase cascade involved in *C. albicans* hyphal growth. Like Cst20p, Hst7p, and Cph1p, Cek1p has *S. cerevisiae* homologues (Fus3p and Kss1p) which function in the pheromone response MAP kinase cascade. We found that deletion of *CEK1* completely suppressed the phenotypes of the *cpp1* null mutants (Figure 8B), strengthening our hypothesis that Cpp1p functions as part of the MAP kinase cascade involved in *C. albicans* hyphal growth. *Cpp1* null mutant phenotypes, including: derepressed hyphal development at ambient temperature; reduced growth rates of serum-induced mycelia, and the absence of lateral buds on serum at 37°C, were all reversed by deletion of *CEK1* from the strain (Figure 8B, a–c).

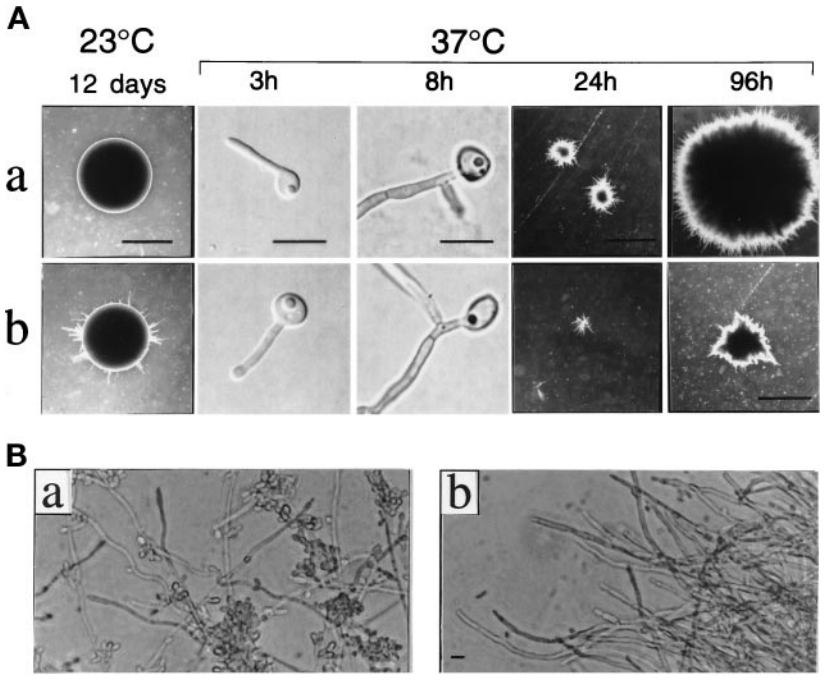

Figure 6. Growth of *C. albicans* colonies on solid 10% serum medium at 23 and 37°C. a, *CPP1* reintegrant strain CP29–1-7RI (*cpp1*Δ/*cpp1*Δ::*CPP1-URA3*) and b, *cpp1* null mutant strain CP29-1-7 (*cpp1*Δ/*cpp1*Δ::*URA3*). (A, 23°C) Yeast-form colonies were grown for 12 d and examined for hyphal outgrowth from colony borders (2× objective; bar, 2 mm). (A, 37°C) Single yeast-form cells spread on agar plates were monitored for germ tube formation and the onset of branching (3 and 8 h, Nomarski optics, 100× objective; bar, 15 μm), and for subsequent formation of invasive hyphal colonies (24 and 96 h; 2× objective; bar, 2 mm). (B) Mycelia from 96-h hyphal colony peripheries grown at 37°C and excised from agar for microscopy (40× objective; bar, 15 μm). Our unpublished observations showed that the wild-type strain SC5314 and the *CPP1* reintegrant gave identical results.

DISCUSSION

Uncovering the genes involved in *C. albicans* pathogenicity is important for the discovery of new therapeutic strategies. Through functional complementation of *S. cerevisiae* genes, *C. albicans* homologues of components of a MAP kinase cascade involved in pheromone response and invasiveness in haploids and pseudohyphal growth in diploids (Gimeno *et al.*, 1992; Liu *et al.*, 1993; Roberts and Fink, 1994; Herskowitz, 1995) have been found which trigger the *C. albicans* yeast to hyphal switch in vitro (Liu *et al.*, 1994; Malathi *et al.*, 1994; Clark *et al.*, 1995; Kohler and Fink, 1996; Leberer *et al.*, 1996). The pheromone response pathway of *S. cerevisiae* is an extensively characterized and genetically tractable system in which to study signal transduction (reviewed in Herskowitz, 1995 and Leberer *et al.*, 1997a). We previously identified *C. albicans* genes that when overexpressed blocked the function of the pheromone-response MAP kinase cascade in *S. cerevisiae* (Whiteway *et al.*, 1992, 1993). Among the genes identified were *CEK1*, a MAP kinase homologue and *CPP1*, a gene with structural similarities to the VH1 family of protein tyrosine phosphatases. The VH1 family contains a growing number of dual- specificity MAP kinase-specific enzymes (reviewed in Keyse, 1995 and Zolnierowicz and Hemmings, 1996). We show here that Cpp1p is a putative MAP kinase phosphatase involved in determining cell-fate decisions in *C. albicans* by blocking the yeast to hyphal transition in the absence of a temperature cue. Genetic evidence in *C. albicans* suggests that Cpp1p mediates this repression by blocking activation of a MAP kinase cascade involved in hyphal growth. The Cpp1p MAP kinase phosphatase, like some components of the MAP kinase pathway that trigger hyphal growth, is involved in *C. albicans* virulence.

The net level and duration of MAP kinase activity in cells depends on the phosphorylation of critical tyrosine and threonine residues on MAP kinases by dual-specificity MAP kinase kinases and on the dephosphorylation of at least one of these residues by protein phosphatases (Keyse, 1995). Both dual- specificity and monospecificity phosphatases have been found to be involved in MAP kinase inactivation (Zhan *et al.*, 1997; Millar *et al.*, 1995), and both types of phosphatases together can coordinate the regulation of a single MAP kinase (Zhan *et al.*, 1997). Several lines of evidence using heterologous systems in vivo or in vitro point to a role of Cpp1p as a MAP kinase phosphatase. First, overproduction of wild-type, but not an active site mutant of Cpp1p, interferes with pheromone-mediated cell-cycle arrest in *S. cerevisiae*. Genetic evidence suggests that Cpp1p acts at the level of the Fus3p (and Kss1p) MAP kinases of this pathway (Whiteway *et al.*, 1993). These MAP kinases convey the signal from the pheromone receptor to a transcription factor and to the cell cycle machinery and, unlike other components of the cascade, require tyrosine phosphorylation for activity (Gartner *et al.*, 1992). Indeed, Cpp1p is most closely related to the *S. cerevisiae* MAP kinase phosphatase, Msg5p, which acts in synergy with two other tyrosine-specific phosphatases, on the MAP kinases of this cascade (Doi *et al.*, 1994; Zhan *et al.*, 1997). Although Cpp1p is a member of the dual-specificity VH1 phosphatases, we found that under the conditions used, Cpp1p inactivated and dephosphorylated phosphotyrosine residues of the mammalian MAP kinases Erk1 and Erk2 in vitro, but did not dephosphorylate their phosphothreonine or phosphoserine residues. This is not entirely surprising in view of the finding that tyrosine can be the preferred in

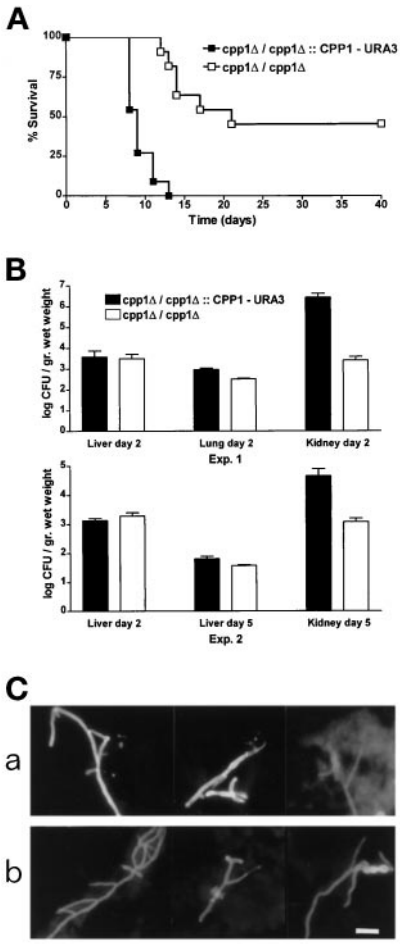

Figure 7. Virulence assays of BALB/c mice infected i.v. with either *cpp1* null mutant (*cpp1*Δ*/cpp1*Δ*::URA3*) or *CPP1* reintegrant (*cpp1*Δ*/cpp1*Δ*::CPP1-URA3*) *C. albicans* strains. (A) Survival curves of mice (n = 11) injected with 5×10^5 cells. (B) Fungal burden of infected tissues. (C) Fluorescence micrographs of Calcofluor white-stained hypha of *C. albicans* purified from KOH-solubilized kidneys 2 d after infection. a, CP29–1-7RI (*cpp1*Δ*/cpp1*Δ*::CPP1-URA3*); b, CP29–1-7 (*cpp1*Δ*/cpp1*Δ*::URA3*). Bar, 15 μm. Our unpublished results showed that survival curves of mice injected with the wild-type strain SC5314 and the *CPP1* reintegrant were identical.

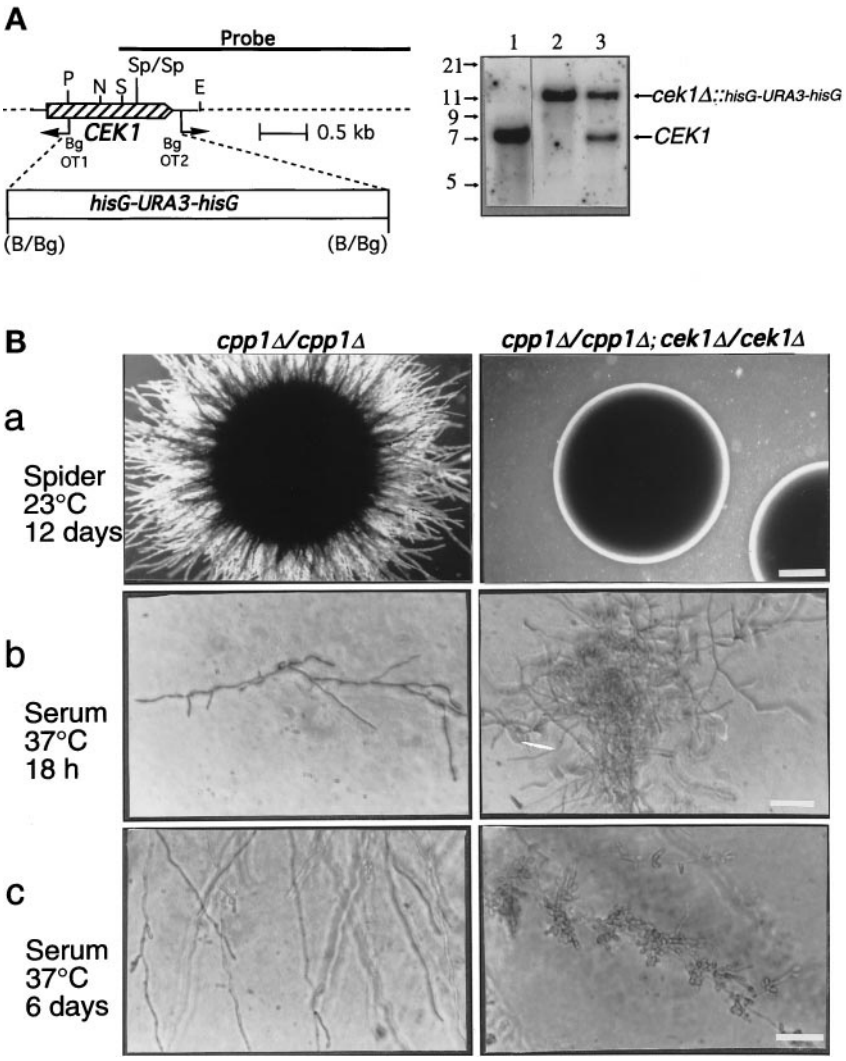

Figure 8. Construction and phenotypic analysis of *cek1/cpp1* double null mutants. (A) Deletion of *CEK1* in *C. albicans*. Left, Deletion strategy and restriction map of the *CEK1* gene. PCR with the divergent oligodeoxynucleotides OT1 and OT2 was used to delete a 1.2-kb fragment of the *CEK1* gene. A 4.0-kb *hisG-URA3-hisG* cassette was then inserted. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; S, *Sac*I; N, *Nsi*I; E, *Eco*RI; and Sp, *Spe*I. Right, Southern blot analysis of *CEK1* disruptions with a 3.2-kb *Kpn*I-*Sac*I fragment containing the *CEK1* gene as a probe. Genomic DNA samples were digested with *Spe*I (absent from the *hisG-URA3-hisG* cassette) from the following strains: lane 1, CP29–1-7 L4 (*ura3/ura3 cpp1Δ::hisG/cpp1Δ::hisG; CEK1/CEK1*); lane 2, CP29-1-7 CK14 (*ura3/ura3cpp1Δ::hisG/cpp1Δ::hisG; cek1Δ::hisG-URA3-hisG/cek1Δ::hisG-URA3-hisG*); and lane 3, *CP29-1-7 CK13 (ura3/ura3cpp1Δ::hisG/cpp1Δ::hisG; CEK1/cek1Δ::hisG-URA3-hisG*). Hybridization of a very small part (between the *Sac*I and *Spe*I sites) of the probe to a 1.4-kb *Spe*I fragment, present only in the wild-type *CEK1* gene, was not detectable in these Southern blots and was not used for diagnostic purposes. (B) Colonies of *cpp1* null mutants (*cpp1Δ/cpp1Δ*) or *cpp1/cek1* double null mutants (*cpp1Δ/cpp1Δ; cek1Δ/cek1Δ*) grown at room temperature (a) on solid Spider medium containing mannitol (2× objective; bar, 1.4 mm) and under physiological conditions (b and c) on solid serum medium (40× objective; bar, 70 μm). In a and b colonies are shown. In c hyphae (and lateral blastospores) from peripheries of mycelial colonies are shown. Our unpublished data demonstrated that the *cpp1/cek1* double null mutant phenotypes resembled those of the wild-type strain SC5314.

vitro substrate of other VH1 phosphatases (Denu *et al.*, 1995; Groom *et al.*, 1996). Because dephosphorylation of either residue of a MAP kinase can result in its inactivation, the tyrosine specificity of Cpp1p is consistent with it having a role in MAP kinase inactivation in *C. albicans*.

Our experiments in heterologous systems suggested to us that Cpp1p functions as a MAP kinase phosphatase in *C. albicans*, and that its physiological target could be the MAP kinase cascade that triggers *C. albicans* hyphal development on solid surfaces. Strains containing deletions of genes encoding the MAP kinase kinase kinase kinase, *CST20*, the MAP kinase kinase, *HST7*, and the transcription factor, *CPH1*, are defective in hyphal growth on solid substrata at physiological temperatures (Liu *et al.*, 1994; Kohler and Fink, 1996; Leberer *et al.*, 1996). Our unpublished results reveal the same phenotype for a strain with a deletion of the *CEK1* MAP kinase gene (Whiteway *et al.*, 1992). *Cpp1* null mutants have an opposite phenotype: they form hyphae from mature colonies on solid substrata at ambient temperatures, and our unpublished observations also demonstrate the same phenotype with cells constitutively overexpressing the Cph1p transcription factor. Both of these strains derepress the yeast to hyphal transition under noninducing conditions and do not hyperactivate invasive hyphal growth at physiological temperatures. However, the strongest evidence that Cpp1p acts on this MAP kinase pathway is that disruption of the *CEK1* MAP kinase gene completely suppresses the phenotypes of the *cpp1* null mutant. These observations support a role of Cpp1p in determining cell fate through the inactivation of a Cek1p MAP kinase cascade, and by inference, through the control of the duration or intensity of MAP kinase response.

The potential importance of controlling the duration of MAP kinase activity for cellular responses is illustrated by the differential roles of transient versus sustained MAP kinase activities in proliferation and differentiation in rat pheochromocytoma PC12 cells (Wu *et al.*, 1994; Fukuda *et al.*, 1995; Marshall, 1995) and in transcriptional induction and cell cycle arrest of *S. cerevisiae* cells exposed to pheromone (Couvé and Hirsch, 1996). In addition, adaptation to pheromone, in the absence of a mating partner, involves shutting off MAP kinase activity to resume the cell cycle (Moore, 1984; Doi *et al.*, 1994). Several phosphatases, including the tyrosine phosphatases Ptp2p and Ptp3p and the dual-specificity phosphatase Msg5p, are involved in this adaptive response (Doi *et al.*, 1994; Zhan *et al.*, 1997). Therefore, by controlling the duration of MAP kinase activity, MAP kinase-specific phosphatases have the potential to determine the end result of a MAP kinase cascade. In addition, MAP kinase phosphatases may also be key modulators of responses by helping to maintain low basal levels of MAP kinase activity (Zhan *et al.*, 1997).

Because of the variety of responses in which components of MAP kinase cascades can function, it is possible that Cpp1p affects the same MAP kinase cascade or other similar MAP kinase cascades in other developmental processes or cellular responses in *C. albicans*. We have observed that although *cpp1* null mutants were able to form germ tubes normally, they had a hyphal growth rate defect and formed few lateral buds under some hyphal-inducing conditions (at physiological rather than ambient temperature). The *cpp1* null mutant hyphae may have a growth defect under these conditions because of the detrimental effects of higher than normal levels of hyphal-inducing cellular activities resulting from the Cek1p MAP kinase cascade being inappropriately hyperactive. Removal of the *CEK1* gene suppresses the hyphal growth rate defect of *cpp1* null mutants, suggesting that this is a plausible situation. Because *cpp1* null mutant hyphae appear to be less capable of differentiating or accumulating new yeast cells, under these same conditions, it is also possible that the development of lateral buds from hyphae could represent an adaptive response to hyphal-inducing stimuli. Once again, this phenotype is suppressed in the *cpp1/cek1* double null mutants. In *cpp1* null mutants the hyphal-inducing signals may remain too high for cells to resume growing in the yeast form. Indeed, overexpression of either Msg5p or Cpp1p in *S. cerevisiae* promotes adaptation and budding growth by shutting off the pheromone response pathway (Doi *et al.*, 1994; Whiteway *et al.*, 1993). Reentry into the budding cell cycle in *C. albicans* could parallel the process of adaptation to pheromone in *S. cerevisiae*.

Tyrosine phosphatases have been shown to play a role in the pathogenicity of the bacterial genus *Yersinia* which includes species responsible for enteric diseases, septicemia, and bubonic plague, and of the viral genus *Orthopoxvirus* which contains the causative agent of smallpox (Bliska *et al.*, 1991; Hakes *et al.*, 1993; reviewed in Ninfa, 1994). In addition, we have found that the Cpp1p MAP kinase phosphatase contributes to the pathogenicity of *C. albicans*. *Cpp1* null mutants demonstrate a dramatic reduction in virulence and, in addition, show reduced fungal load in the kidneys, a typical secondary site of infection. The reduction in virulence may well be attributed to decreased infection of the kidneys, since during experimental candidiasis the kidneys are the most highly infected and abscessed organs in the body (Odds, 1988). What accounts for the reduction in fungal burden in the kidneys? This question is not easily answered in view of the different phenotypes of *cpp1* null mutants in vitro; however, it may simply be that *cpp1* null mutant mycelia have a growth rate defect specifically in the kidneys resembling that seen under physiological conditions in vitro, although one can also envision that the absence of lateral buds in *cpp1* null mutants under physiological conditions could make dissemination from primary sites of infection such as the lungs and liver to secondary sites of infection such as the kidneys more difficult. Other possibilities also exist, such as the *cpp1* null mutant strain being more susceptible to kidney-specific defense mechanisms. Although this study does not answer the question of what the role of the hyphal form of *C. albicans* plays in pathogenicity, our data are consistent with studies that report that *C. albicans* morphological mutant strains that exist in hyphal forms at ambient temperatures are avirulent, as are those mutant strains which are unable to undergo the yeast to hyphal switch (Sobel *et al.*, 1984; Hubbard *et al.*, 1986; Gil *et al.*, 1990; Leberer *et al.*, 1997b). One of the latter class of mutants is a null mutant of a homologue (*CaCLA4*) of the *S. cerevisiae CLA4* gene [a relative of *STE20* (*CST20*)]; although having no growth rate defect, *Cacla4* null mutants cannot make hyphae and are completely avirulent, reinforcing the idea that hyphae are required for virulence.

Because the current state of understanding of *C. albicans* virulence is rudimentary, it is not yet possible to pinpoint the precise molecular basis of Cpp1p-mediated virulence; however, it is through the isolation of genes and the detailed analysis of phenotypes, coupled with virulence studies, that progress can be made in defining some of the elements that define pathogenicity. The Cpp1p phosphatase may indeed serve as a useful target for therapeutics against systemic disease, the most devastating and least treatable form of fungal infection (Odds, 1988), especially in view of its limited structural similarity to mammalian counterparts of this class of enzymes. Most important, the present study demonstrates the involvement of a tyrosine phosphatase in fungal disease and provides a demon-

stration of the important role of a phosphatase of the VH1 family in cell fate decisions.

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