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Repression of Hyphal Proteinase Expression by the Mitogen-Activated Protein (MAP) Kinase Phosphatase Cpp1p of *Candida albicans* Is Independent of the MAP Kinase Cek1p

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Cpp1p is a putative mitogen-activated protein (MAP) kinase phosphatase that suppresses *Candida albicans* hyphal formation at 25°C through its probable substrate, the Cek1p filamentation MAP kinase. Here we report that expression of the serum-induced genes *SAP4-6* and *HYR1* increased several fold in hyphal forms of a *cpp1*/*cpp1* null mutant, while the rate and extent of hyphal development up to 5 h were normal. Therefore, we provide evidence that Cpp1p represses hyphal gene expression by acting through a Cek1p-independent mechanism. *SAP4-6* and *HYR1* transcripts were undetectable in a null mutant of another key regulator of filamentation, Efg1p; thus, Efg1p and Cpp1p oppose each other during the expression of these genes in hyphal forms.

Deletion of the mitogen-activated protein (MAP) kinase phosphatase Cpp1p of the human opportunistic pathogen *Candida albicans* was shown to derepress hyphal formation under noninducing conditions (2), and virulence of a *cpp1*/*cpp1* mutant strain was reduced in murine models of candidiasis (2, 5). Hyphal development of *C. albicans* is controlled by at least two regulatory cascades, both of which involve phosphorylation of downstream components. There is a predominant pathway that functions in response to a variety of stimuli, including serum, which involves the kinase Tpk2p and the basic helix-loop-helix transcription factor Efg1p (for enhanced filamentous growth) (10, 14, 17). A second pathway that operates via a nutritionally regulated MAP kinase cascade (Cst20p-Hst7p-Cek1p) phosphorylates and activates the transcription factor Cph1p (6, 7, 9). The development of a yeast cell to a multicellular mycelium that consists of hyphal forms is an important step during pathogenesis, because nonfilamentous strains of *C. albicans* are avirulent (8, 10).

It was suggested that Cpp1p acts to block the yeast-to-hyphal transition under noninducing conditions through inactivation of the MAP kinase Cek1p (2), because previous studies showed that the morphological phenotypes of the *cpp1*/*cpp1* mutation during late stages of hyphal development (4 days) were reversed by additional deletion of the *CEK1* gene (2). In this study, we show that under inducing conditions at 37°C, Cpp1p is a potent suppressor of serum-induced hyphal gene expression. Surprisingly, this activity does not require the MAP kinase Cek1p.

Cpp1p is a suppressor of Cek1p-independent hyphal gene expression. The downstream target genes of Cpp1p-dependent signal transduction were investigated. We determined the mRNA expression level of the type 4, 5, and 6 isogenes (*SAP4-6*) of the secreted aspartyl proteinase (a putative virulence factor [12]) and the *HYR1* gene (coding for a nonessential cell wall component [1]) during hyphal formation. The *C. albicans* strains used for this study are listed in Table 1. For the induction of hyphal development, cells were grown for 48 to 72 h in YPD (1% yeast exract, 2% Bactopeptone, 2% glucose) and diluted into CLICKS RPMI-1640 medium supplemented with 10% selected fetal calf serum to a final density of 20×10^6 cells/ml. Total RNA was extracted from yeast and hyphal forms, and Northern blots were carried out as published previously (16). For detection of the type 4, 5, and 6 isogenes of secreted aspartic proteinase *SAP4-6* mRNA, we labeled (by random priming) *Bgl*II restriction fragments derived from the cloned coding regions of *SAP4-6*. For the detection of *HYR1* mRNA, we used a cloned *HYR1* PCR product spanning the coding region as a template for probe synthesis. *ACT1* hybridization was used to control for equal sample loading as described previously (3). Plasmids for probe synthesis for the

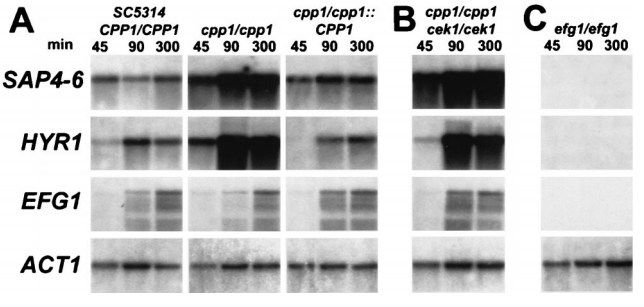

FIG. 1. Northern blot analysis of *SAP4-6*, *HYR1*, *EFG1*, and *ACT1* gene expression of the *C. albicans* wild-type strain SC5314 (*CPP1*/*CPP1*) and the *cpp1*/*cpp1* and *cpp1*/*cpp1*::*CPP1* (A), *cpp1*/*cpp1 cek1*/*cek1* (B), and *efg1*/*efg1* (C) isogenic mutant strains. The genotype and the duration (minutes) of serum-induced hyphal formation prior to RNA extraction are delineated at the top of each column, respectively. The origins of probes used for hybridization are given on the left-hand side (*SAP4-6*, *HYR1*, *EFG1*, and *ACT1*).

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

Strain	Genotype or description	Reference
SC5314	Prototrophic	4
29-1-7+	*cpp1*::*hisG*/*cpp1*::*hisG-URA3-hisG*	2
29RI-10	*cpp1*::*hisG*/*cpp1*::*hisG*::*URA3-CPP1*	2
C5-22b	*cpp1*::*hisG*/*cpp1*::*hisG cek1*::*hisG*/*cek1*::*hisG-URA3-hisG*	2
HLC52	*efg1*::*hisG*/*efg1*::*hisG-URA3-hisG*	10

detection of *EFG1* and *ACT1* mRNA were kindly provided by J. Ernst, Düsseldorf, Germany. Residual radioactivity was stripped from the membranes prior to hybridization with a different probe (16). Autoradiograms were quantitated with a Fuji BAS3000 phosphorimager and accessory software.

As shown in Fig. 1A, the *C. albicans* wild-type strain SC5314 and the *cpp1*/*cpp1*::*CPP1* control strain (integrated chromosomal copy of *CPP1*) were induced to similar levels of *SAP4-6* and *HYR1* expression after initiation of hyphal formation. However, hypha-forming cells of the *cpp1*/*cpp1* mutant expressed approximately 10-fold more *SAP4-6* and *HYR1* mRNA (Fig. 1A). Because the *cpp1*/*cpp1* phenotype of derepressed filamentous growth under noninducing conditions at room temperature was reverted by deletion of the MAP kinase gene *CEK1* (2), we measured the expression of *SAP4-6* and *HYR1* in the *cpp1*/*cpp1 cek1*/*cek1* double mutant under inducing conditions as detailed above and compared it to that of the *cpp1*/*cpp1* single mutant and the isogenic control strains. The overexpression of *SAP4-6* and *HYR1* observed in the *cpp1*/*cpp1* mutant also occurred in the *cpp1*/*cpp1 cek1*/*cek1* double mutant (Fig. 1B). Hence, derepressed *SAP4-6* and *HYR1* gene expression in the *cpp1*/*cpp1* mutant does not require functional Cek1p MAP kinase activity.

Since hyphal formation at room temperature was derepressed in the *cpp1*/*cpp1* mutant (2), the increase in hyphal gene expression might simply reflect derepressed hyphal formation that occurred at 37°C as well. Therefore, serum-induced hyphal development was quantitated by using a Zeiss axiophot microscope equipped with a video device. As illustrated in Fig. 2A, we observed similar percentages of germ tubes emerging from blastoconidia of the SC5314 wild-type strain, the *cpp1*/*cpp1*::*CPP1* control strain, and the *cpp1*/*cpp1* and *cpp1*/*cpp1 cek1*/*cek1* mutant strains (79.8% ± 5.8%, 93.4% ± 2.6%, 90.3% ± 3.9%, and 90.2% ± 3.1% at 60 min; mean percentage of germ tube-positive blastoconidia ± standard error of three independent experiments with at least 200 cells/time point). The length of the filaments at 4 h was measured on calibrated video images with MetaView software (Universal Imaging Corporation, West Chester, Pa.). The SC5314 wild-type strain, the *cpp1*/*cpp1*::*CPP1* control strain, and the *cpp1*/*cpp1* mutant produced filaments of similar length (51.38 ± 1.8, 55.8 ± 1.7, and 49.9 ± 1.2 μm, respectively), whereas the filaments of the *cpp1*/*cpp1 cek1*/*cek1* double mutant strain extended slightly further to 60.6 ± 1.2 μm (Fig. 2B; mean hyphal length ± standard error of three independent experiments with $n > 80$ hyphal forms; $P < 0.05$ for *cpp1*/*cpp1 cek1*/*cek1* versus all other samples). Chlamydospore formation (15) on cornmeal agar was not affected by deletion of *CPP1* (data not shown). We concluded that the rate and extent of hyphal development of *C. albicans* at 37°C in vitro are not controlled by Cpp1p. Therefore, the derepressed hyphal expression of *SAP4-6* and *HYR1* of the *cpp1*/*cpp1* and the *cpp1*/*cpp1 cek1*/*cek1* mutants is not the result of hyperfilamentation of these mutants at 37°C. In fact, this result suggests that Cpp1p acts on a separate signal transduction pathway that specifically regulates expression of *SAP4-6* and *HYR1*, but not the extent of hyphal formation at 37°C.

Efg1p regulates *SAP4-6* and *HYR1* gene expression. Efg1p is a crucial transcription factor for both hyphal formation (10, 17) and hyphal gene expression (13) of *C. albicans*. Therefore, we hybridized mRNA of the *cpp1*/*cpp1* mutant grown under inducing conditions with an *EFG1* probe in order to examine whether the expression of this transcription factor gene is controlled by Cpp1p. The pattern of *EFG1* mRNA expression was the same for wild-type and *cpp1*/*cpp1* mutant cells (Fig. 1A), which argues against transcriptional regulation of *EFG1* by a Cpp1p-dependent mechanism. However, the *efg1*/*efg1* mutant (10) did not express any detectable amount of *SAP4-6* or *HYR1* mRNA up to 4 h under inducing conditions (Fig. 1C). These results show that Efg1p is required for *SAP4-6* and *HYR1*

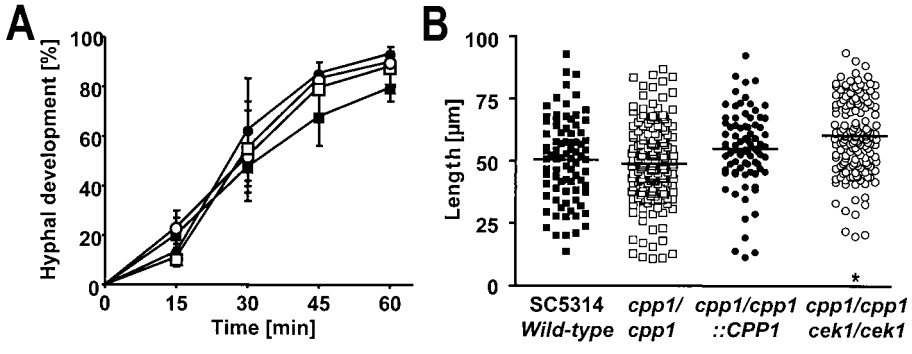

FIG. 2. Percentage of germ tube-positive blastoconidia after 15, 30, 45, and 60 min (A) and extent of filaments (micrometers) after 4 h of serum-induced hyphal formation (B). ■, SC5314 wild-type (*CPP1*/*CPP1*); □, *cpp1*/*cpp1*; ●, *cpp1*/*cpp1*::*CPP1*; ○, *cpp1*/*cpp1 cek1*/*cek1*. (A) Illustrated values are the mean percentage of germ tube-positive blastoconidia ± standard error of three independent experiments with at least 200 cells/time point (B) Each scatter represents cumulative data of hyphal length from three independent experiments with $n > 80$ hyphal forms each. Horizontal bars represent mean hyphal length. *, $P < 0.05$.

expression. In the host, part of the virulence defect of the *efg1*/*efg1* mutant (10) may be attributed to the loss of expression of the *SAP4-6* genes. Since these targets of Efg1p signals are derepressed in the *cpp1*/*cpp1* and *cpp1*/*cpp1 cek1*/*cek1* mutants, we conclude that Cpp1p interacts with the signaling pathway that acts through Efg1p.

The hyperexpression of the *SAP4-6* isogenes and *HYR1* in the double *cpp1*/*cpp1 cek1*/*cek1* mutant (Fig. 1), independent from the percentage and extent of hyphal formation (Fig. 2), indicates a minor role of Cek1p for the activation of these genes in and the generation of hyphal forms at 37°C. Inhibition of the *SAP4-6* isogenes and *HYR1*—mediated by a yet-to-be-identified Cpp1p target in its unphosphorylated form—is removed in *cpp1*/*cpp1* null mutants. The absence of the phosphatase would permit inappropriate release of an inactive sequestered transcription factor, because the regulatory component now is phosphorylated. Thus similar to the *Saccharomyces cerevisiae* MAP kinase-specific phosphatase Msg5p (11), Cpp1p seems to have more than one substrate. The observation that Efg1p and Cpp1p act in opposition to each other on *SAP4-6* and *HYR1* expression suggests that both regulatory proteins affect a common signal transduction pathway. The *C. albicans* Efg1p transcription factor contains a single potential site for protein kinase A phosphorylation at T^{206} (sequence IRPRVT^{206}TT), which was reported previously to be essential in hyphal development and chlamydospore formation (15). Accordingly, Efg1p function during hyphal formation is presumably regulated by the balanced activity of kinase(s) and phosphatase(s), part of which may be the result of Cpp1p-dependent negative signals.

In summary, the MAP kinase phosphatase Cpp1p of *C. albicans* regulates morphological changes and transcription of hyphal genes by two mechanisms that are either MAP kinase Cek1p dependent (cell morphology at room temperature) or independent (gene transcription at 37°C), respectively. Thus, Cpp1p emerges as an important negative regulator of Cek1p-dependent morphological development and Efg1p-dependent gene expression in *C. albicans*.

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REFERENCES

1. **Bailey, D. A., P. J. F. Feldmann, M. Bovey, N. A. R. Gow, and A. J. P. Brown.** 1996. The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. J. Bacteriol. **178:**5353–5360.
2. **Csank, C., M. Constantin, S. Meloche, K. Schröppel, M. Röllinghoff, D. Dignard, D. Y. Thomas, and M. Whiteway.** 1997. Derepressed hyphal growth and reduced virulence in a VH1 family related protein phosphatase mutant of *Candida albicans*. Mol. Biol. Cell **8:**2539–2547.
3. **Delbrück, S., and J. F. Ernst.** 1993. Morphogenesis-independent regulation of actin transcript levels in the pathogenic yeast *Candida albicans*. Mol. Microbiol. **10:**859–866.
4. **Gillum, A. M., E. Y. Tsay, and D. R. Kirsch.** 1984. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli* pyrF mutations. Mol. Gen. Genet. **198:**179–182.
5. **Guhad, F. A., C. Csank, H. E. Jensen, D. Y. Thomas, M. Whiteway, and J. Hau.** 1998. Reduced pathogenicity of a *Candida albicans* MAP kinase phosphatase (CPP1) mutant in the murine mastitis model. APMIS **106:**1049–1055.
6. **Köhler, J. R., and G. R. Fink.** 1996. *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. Proc. Natl. Acad. Sci. USA **93:**13223–13228.
7. **Leberer, E., D. Harcus, I. D. Broadbent, K. L. Clark, D. Dignard, K. Ziegelbauer, A. Schmidt, N. A. Gow, A. J. Brown, and D. Y. Thomas.** 1996. Signal transduction through homologs of the ste20p and ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. Proc. Natl. Acad. Sci. USA **93:**13217–13222.
8. **Leberer, E., K. Ziegelbauer, A. Schmidt, D. Harcus, D. Dignard, J. Ash, L. Johnson, and D. Y. Thomas.** 1997. Virulence and hyphal formation of *Candida albicans* require the Ste20p-like protein kinase CaCla4p. Curr. Biol. **7:**539–546.
9. **Liu, H., J. R. Köhler, and G. R. Fink.** 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science **266:**1723–1726.
10. **Lo, H. J., J. R. Köhler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.
11. **Navarro-García, F., M. Sánchez, J. Pla, and C. Nombela.** 1995. Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. Mol. Cell. Biol. **15:**2197–2206.
12. **Sanglard, D., B. Hube, M. Monod, F. C. Odds, and N. A. R. Gow.** 1997. A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. Infect. Immun. **65:** 3539–3546.
13. **Sharkey, L. L., M. D. McNemar, S. M. Saporito-Irwin, P. S. Sypherd, and W. A. Fonzi.** 1999. *HWP1* functions in the morphological development of *Candida albicans* downstream of *EFG1*, *TUP1*, and *RBF1*. J. Bacteriol. **181:** 5273–5279.
14. **Sonneborn, A., D. P. Bockmuhl, M. Gerads, K. Kurpanek, D. Sanglard, and J. F. Ernst.** 2000. Protein kinase A encoded by TPK2 regulates dimorphism of *Candida albicans*. Mol. Microbiol. **35:**386–396.
15. **Sonneborn, A., D. P. Bockmühl, and J. F. Ernst.** 1999. Chlamydospore formation in *Candida albicans* requires the Efg1p morphogenetic regulator. Infect. Immun. **67:**5514–5517.
16. **Srikantha, T., B. Morrow, K. Schröppel, and D. R. Soll.** 1995. The frequency of integrative transformation at phase-specific genes of *Candida albicans* correlates with their transcriptional state. Mol. Gen. Genet. **246:**342–352.
17. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. **16:**1982–1991.

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