INFECTION AND IMMUNITY, July 2006, p. 4366–4369 Vol. 74, No. 7
0019-9567/06/$08.00+0 doi:10.1128/IAI.00142-06

Role of Calcineurin in Stress Resistance, Morphogenesis, and Virulence of a *Candida albicans* Wild-Type Strain

Teresa Bader,[1] Klaus Schröppel,[2] Stefan Bentink,[1] Nina Agabian,[3] Gerwald Köhler,[3] and Joachim Morschhäuser[1]*

Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, D-97070 Würzburg,[1] and Institut für Klinische Mikrobiologie, Immunologie und Hygiene, Universität Erlangen, Wasserturmstraße 3, D-91054 Erlangen,[2] Germany, and Department of Cell and Tissue Biology, University of California, San Francisco, 521 Parnassus, San Francisco, California 94143[3]

Received 27 January 2006/Returned for modification 18 March 2006/Accepted 6 April 2006

By generating a calcineurin mutant of the *Candida albicans* wild-type strain SC5314 with the help of a new recyclable dominant selection marker, we confirmed that calcineurin mediates tolerance to a variety of stress conditions but is not required for the ability of *C. albicans* to switch to filamentous growth in response to hypha-inducing environmental signals. While calcineurin was essential for virulence of *C. albicans* in a mouse model of disseminated candidiasis, deletion of *CMP1* did not significantly affect virulence during vaginal or pulmonary infection, demonstrating that the requirement for calcineurin for a successful infection depends on the host niche.

Calcineurin is a conserved Ca^{2+}/calmodulin-activated, serine/threonine-specific protein phosphatase that regulates a variety of physiological processes in eukaryotic organisms. We and others had recently investigated the requirement of calcineurin for virulence of *Candida albicans*, the major fungal pathogen of humans. *C. albicans* mutants in which the gene encoding the catalytic (*CMP1*/*CNA*) or the regulatory (*CNB1*) subunit of calcineurin was inactivated were hypersensitive to salt, alkaline, and membrane stress and avirulent in a mouse model of disseminated candidiasis (1, 2, 4, 11). All these calcineurin mutants had been constructed using the *URA3* gene as a selection marker for targeted gene inactivation in a *ura3* auxotrophic host strain. However, it has recently become evident that using the *URA3* marker for the generation of *C. albicans* mutants can cause phenotypes that are unrelated to target gene inactivation (13). An alternative approach avoiding all potential problems related to the use of auxotrophic markers and host strains is inactivation of the target gene in a *C. albicans* wild-type strain with the help of a recyclable dominant selection marker (10, 17). In the present work we used this strategy to confirm the role of calcineurin in resistance to various types of stresses and to investigate its importance for the virulence of *C. albicans* in different host niches.

The previously described *MPA*^{R} marker, which confers resistance to mycophenolic acid (MPA), is a mutated derivative of the *C. albicans IMH3* gene encoding IMP dehydrogenase, the target of MPA. When *MPA*^{R} was used in *C. albicans*, sometimes a high proportion of MPA-resistant transformants apparently had substituted the *MPA*^{R} marker for the genomic *IMH3* gene but did not contain the mutagenesis cassette inserted into the target gene locus (16). In contrast, when the *MPA*^{R} marker was used for targeted gene inactivation in the related species *Candida dubliniensis*, virtually all MPA-resistant transformants had specifically integrated the cassette into the target locus, because the sequence divergence between the two species prevented integration of the *C. albicans*-derived *MPA*^{R} marker into the orthologous *C. dubliniensis* IMP dehydrogenase gene *IMD1* (14). These observations suggested that a similar *MPA*^{R} marker derived from *C. dubliniensis* should also improve targeted insertion into the *C. albicans* genome. Therefore, we cloned the *IMD1* gene from *C. dubliniensis* and introduced the same A251T mutation that conferred MPA resistance on IMP dehydrogenase from *C. albicans* (8). The resulting Cd*MPA*^{R} marker was substituted for the *MPA*^{R} marker in the *MPA*^{R} flipper cassette (17) to generate the Cd*MPA*^{R} flipper, which was then inserted between flanking sequences of the *CMP1* gene (Fig. 1A). This deletion cassette was used to transform the *C. albicans* wild-type strain SC5314 to MPA resistance. Southern hybridization analysis demonstrated that 10 out of 17 tested MPA-resistant transformants had specifically inserted the Cd*MPA*^{R} flipper cassette into one of the two *CMP1* alleles. One correct transformant, strain SCCMP1M1 (Fig. 1C, lane 2), was used to excise the Cd*MPA*^{R} flipper by FLP-mediated recombination, generating the MPA-sensitive heterozygous *cmp1* mutant SCCMP1M2 (Fig. 1C, lane 3). After transformation of this strain with the same deletion cassette, integration was successfully targeted to the remaining wild-type *CMP1* allele in the resulting strain SCCMP1M3 (Fig. 1C, lane 4), and subsequent excision of the Cd*MPA*^{R} flipper generated the homozygous *cmp1*-null mutant SCCMP1M4 (Fig. 1C, lane 5). To exclude the possibility that any phenotype of the *cmp1*Δ mutant was caused by a nonspecific mutation, an intact copy of the *CMP1* gene was reintroduced into its original genomic locus. For this purpose, the 5′ *CMP1* flanking region in the deletion construct was replaced by the complete *CMP1* open reading frame (ORF) and upstream sequences (Fig. 1B). The complementation cassette was used to transform the

* Corresponding author. Mailing address: Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, D-97070 Würzburg, Germany. Phone: 49-931-31 21 52. Fax: 49-931-31 25 78. E-mail: joachim.morschhaeuser@mail.uni-wuerzburg.de.

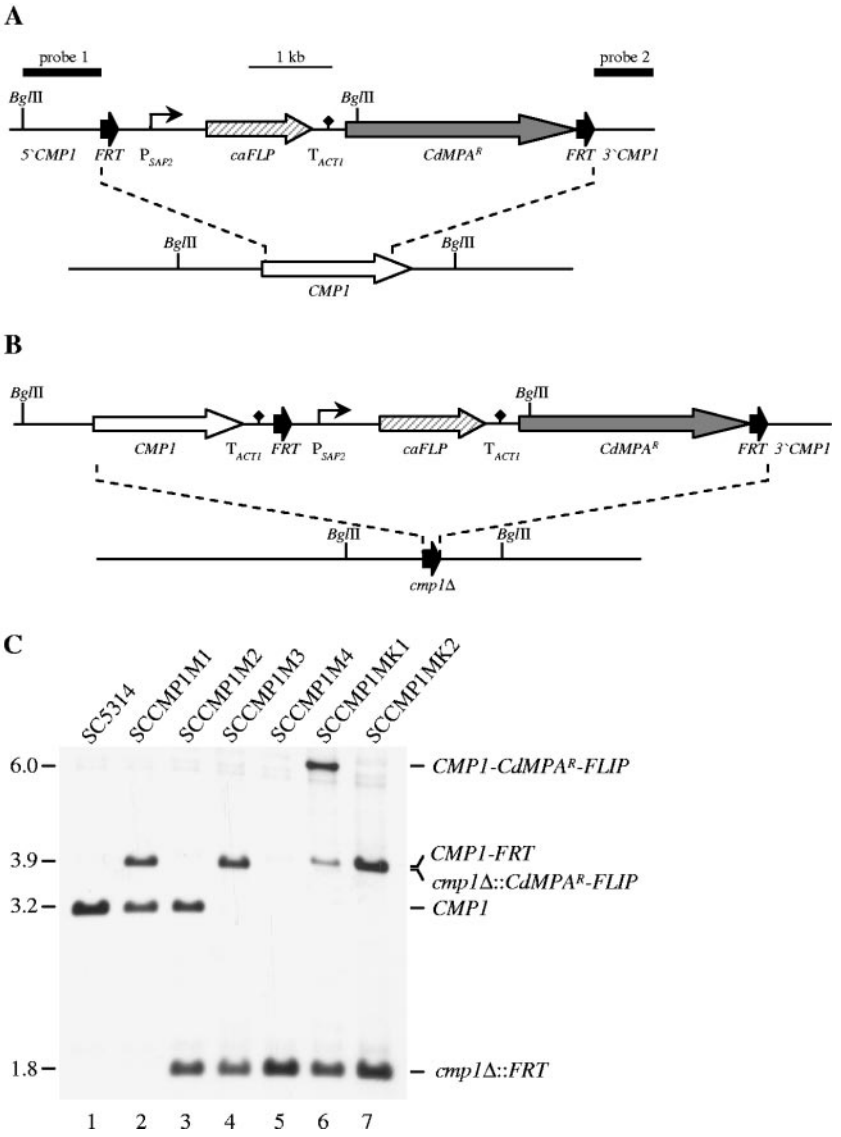

FIG. 1. Construction of the *C. albicans cmp1* deletion mutant and complemented strain. (A) Structure of the *CMP1* deletion cassette (top) and genomic structure of the *CMP1* locus in the parent strain SC5314 (bottom). The *CMP1* coding region is represented by the white arrow, and the upstream and downstream sequences by the solid lines. The direct repeats of the 34-bp *FRT* site (black arrows) bordering the Cd*MPA*^{R} flipper cassette are not drawn to scale. The *SAP2* promoter (P_{SAP2}) is indicated by the bent arrow, the ca*FLP* gene by the hatched arrow, the transcription termination sequence of the *ACT1* gene (T_{ACT1}) by the black diamond, and the Cd*MPA*^{R} marker by the gray arrow. The diagnostic BglII sites are shown, and the DNA fragments used for Southern hybridization analysis of the mutants are indicated by thick bars (probe 1 and probe 2). (B) Structure of the DNA cassette (top) which was used for reintegration of an intact *CMP1* copy (white arrow) into one of the inactivated *cmp1*Δ alleles (bottom). (C) Southern hybridization of BglII-digested genomic DNA of the parent strain SC5314 and mutant derivatives with the *CMP1*-specific probe 1. The sizes of the hybridizing fragments (in kilobases) are given on the left of the blot, and their identities are indicated on the right.

*cmp1*Δ mutant SCCMP1M4, resulting in strain SCCMP1MK1, in which the *CMP1* ORF, together with the Cd*MPA*^{R} flipper, was reinserted into one of the inactivated *cmp1*Δ alleles (Fig. 1C, lane 6). Subsequent excision of the Cd*MPA*^{R} flipper generated the complemented strain SCCMP1MK2 (Fig. 1C, lane 7). This experimental design ensured that the *cmp1*Δ mutant differed from the wild-type parent and the complemented strain only by the deletion of both *CMP1* alleles, but not by the presence or absence of a selection marker.

We first tested the sensitivity of the *cmp1*Δ mutant to various stress conditions using previously described assays (1). Compared with the parental strain SC5314, the *cmp1*Δ mutant SCCMP1M4 exhibited increased susceptibility to elevated salt concentrations (NaCl, LiCl, $MnSO_4$, or $CaCl_2$), sodium dodecyl sulfate, fluconazole, and alkaline pH. Reintroduction of an intact *CMP1* copy into the *cmp1*Δ mutant restored growth to wild-type levels, demonstrating that all the phenotypes of the *cmp1*Δ mutant were caused by inactivation of the *CMP1* gene and not by nonspecific mutations. As reported previously (1), deletion of *CMP1* did not affect the ability of the mutants to

switch to filamentous growth in all solid and liquid hypha-inducing media tested (synthetic low-ammonium dextrose medium, Lee's medium, 10% serum).

To assess whether the previously reported attenuated virulence of *cmp1*Δ mutants constructed in the *ura3* strain CAI4 was due to a position effect of *URA3* or the loss of *CMP1* function, we compared the virulence of the *cmp1*Δ mutant SCCMP1M4, its parental strain SC5314, and the complemented strain SCCMP1MK2 in a mouse model of systemic candidiasis. BALB/c mice (Charles River Breeding Laboratories, Sulzfeld, Germany) were inoculated with 5×10^5 viable cells by intravenous injection and monitored for survival essentially as described previously (1, 12). Kaplan-Meier survival curves were compared using the log rank test (9). A P value of <0.05 was considered significant. Five out of six mice infected with the wild type or the complemented strain died within 18 and 19 days, respectively, whereas all mice infected with the *cmp1*Δ mutant survived beyond day 70 ($P < 0.02$) (Fig. 2A). These results confirmed our previous observation and those of others that calcineurin is essential for the ability of *C. albicans* to cause a systemic infection via the bloodstream (1, 2, 11).

To investigate whether calcineurin is generally required for virulence of *C. albicans* in a mammalian host, we assessed the capacities of the strains to infect other host niches. Infection of the vaginal canal was initiated by inoculating estradiol-treated mice intravaginally with 5.0×10^4 stationary-phase cells in 20 μl of phosphate-buffered saline (6, 15). Vaginal lavage was performed using 100 μl of phosphate-buffered saline at specified time points following infection. The number of *C. albicans* CFU was measured by limiting dilution analysis and calculated by the most-probable-number (MPN) method according to the Poisson distribution (3, 7). The number of negative wells was used to calculate the MPN of live *C. albicans* bacteria in lavage fluids (7). For pulmonary infection, mice treated with Endoxan (200 mg kg of body weight^{-1} given intraperitoneally; Baxter Oncology, Frankfurt, Germany) were anesthetized with 200 mg of Ketavet kg of body weight^{-1} (Pharmacia & Upjohn, Erlangen, Germany), and 2.0×10^5 blastoconidia were administered intranasally (5). The immunosuppressed status of mice was maintained for the duration of the experiment. Generation and comparison of survival curves were performed as mentioned above.

In the pulmonary model of *C. albicans* infection, the *cmp1*Δ mutant exhibited virulence similar to that of the control strains, although killing of animals infected with the *cmp1*Δ mutant was slightly delayed compared with the parental strain SC5314 or the complemented strain SCCMP1MK2 ($P > 0.1$) (Fig. 2B). Similarly, in the mouse model of vaginal candidiasis, no significant differences in the number of CFU grown from the lavage fluids at various times after infection were measured between the *cmp1*Δ mutant and the control strains ($P > 0.5$) (Fig. 2C). These results demonstrate that the requirement of calcineurin for the virulence of *C. albicans* depends on the host niche. Both Sanglard et al. (11) and Blankenship et al. (2) reported that *C. albicans* calcineurin mutants are unable to survive in serum, a phenotype that had not been investigated in our previous study (1). For a direct comparison with the *cnb1* mutants, serum sensitivity tests with our *cmp1*Δ mutants and complemented strains constructed in the wild-type strain SC5314 and from strain CAI4 were performed in the laboratory of

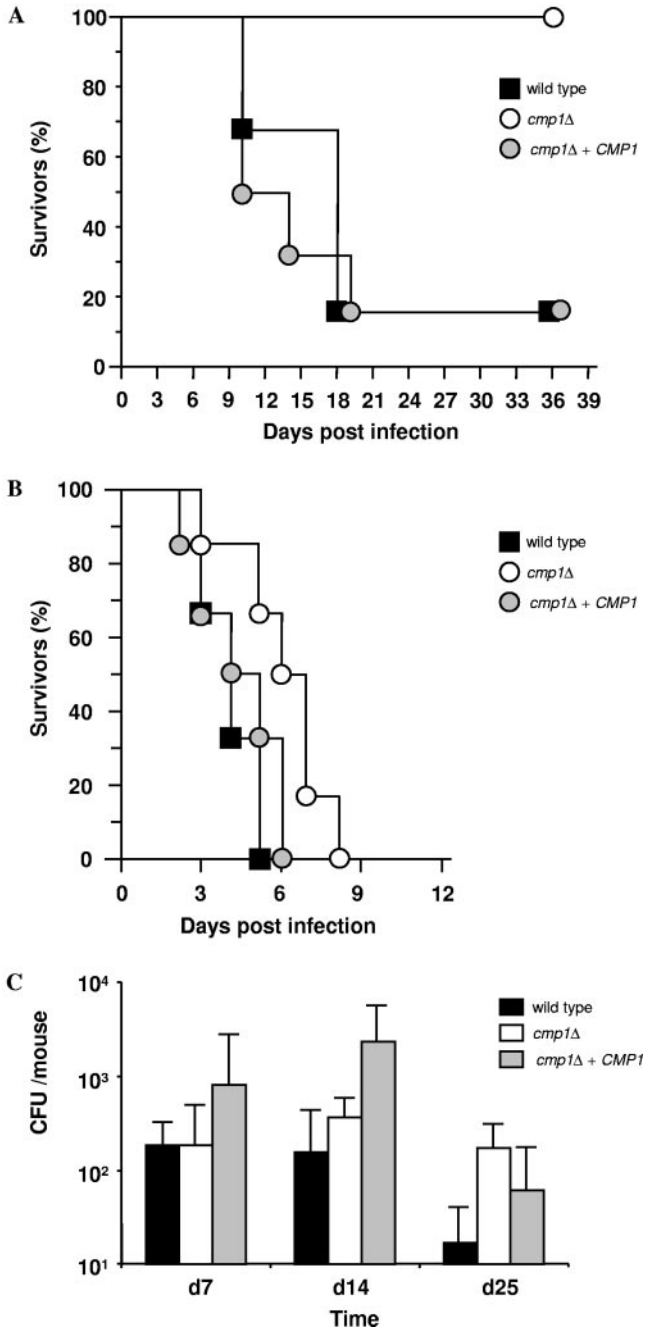

FIG. 2. Virulence of the *C. albicans cmp1*Δ mutant in different infection models. (A and B) Survival of mice after intravenous (A) or intranasal (B) infection with the wild-type parental strain SC5314, the *cmp1*Δ mutant SCCMP1M4, or the reconstituted strain SCCMP1MK2 (*cmp1*Δ plus *CMP1*). (C) Vaginal fungal burden in mice infected intravaginally with the *cmp1*Δ mutant and control strains was measured by determining the CFU in vaginal lavage fluid at the indicated times.

J. Heitman. All mutants were efficiently killed by serum, and reintroduction of a functional *CMP1* copy restored serum resistance (J. Reedy and J. Heitman, personal communication). The serum sensitivity may therefore explain why calcineurin mutants were avirulent after infection via the

bloodstream but retained virulence during vaginal or pulmonary infections.

We thank Jen Reedy and Joe Heitman for performing the serum sensitivity tests with our calcineurin mutants and communicating the results to us. Sequence data for *Candida albicans* were obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida.

This study was supported by a grant from the NRC-HGF Science and Technology Fund (grant 01SF0201/2.2). N.A. and G.K. were supported by NIH grants AI33317 and DE12940, respectively. Sequencing of *Candida albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund.

REFERENCES

1. **Bader, T., B. Bodendorfer, K. Schröppel, and J. Morschhäuser.** 2003. Calcineurin is essential for virulence in *Candida albicans*. Infect. Immun. **71:** 5344–5354.
2. **Blankenship, J. R., F. L. Wormley, M. K. Boyce, W. A. Schell, S. G. Filler, J. R. Perfect, and J. Heitman.** 2003. Calcineurin is essential for *Candida albicans* survival in serum and virulence. Eukaryot. Cell **2:**422–430.
3. **Cochran, W. G.** 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics **6:**105–116.
4. **Cruz, M. C., A. L. Goldstein, J. R. Blankenship, M. Del Poeta, D. Davis, M. E. Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman.** 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. EMBO J. **21:**546–559.
5. **Fallon, K., K. Bausch, J. Noonan, E. Huguenel, and P. Tamburini.** 1997. Role of aspartic proteases in disseminated *Candida albicans* infection in mice. Infect. Immun. **65:**551–556.
6. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1993. *Candida*-specific cell-mediated immunity is demonstrable in mice with experimental vaginal candidiasis. Infect. Immun. **61:**1990–1995.
7. **Garthright, W. E., and R. J. Blodgett.** 2003. FDA's preferred MPN methods for standard, large or unusual tests, with a spreadsheet. Food Microbiol. **20:**439–445.
8. **Köhler, G. A., X. Gong, S. Bentink, S. Theiss, G. M. Pagani, N. Agabian, and L. Hedstrom.** 2005. The functional basis of mycophenolic acid resistance in *Candida albicans* IMP dehydrogenase. J. Biol. Chem. **280:**11295–11302.
9. **Mantel, N., and W. Haenszel.** 1959. Statistical aspects of the analysis of data from retrospective studies of disease. J. Natl. Cancer Inst. **22:**719–748.
10. **Reuß, O., Å. Vik, R. Kolter, and J. Morschhäuser.** 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341:**119–127.
11. **Sanglard, D., F. Ischer, O. Marchetti, J. Entenza, and J. Bille.** 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. Mol. Microbiol. **48:**959–976.
12. **Schweizer, A., S. Rupp, B. N. Taylor, M. Röllinghoff, and K. Schröppel.** 2000. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. Mol. Microbiol. **38:**435–445.
13. **Staab, J. F., and P. Sundstrom.** 2003. *URA3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. Trends Microbiol. **11:**69–73.
14. **Staib, P., G. P. Moran, D. J. Sullivan, D. C. Coleman, and J. Morschhäuser.** 2001. Isogenic strain construction and gene targeting in *Candida dubliniensis*. J. Bacteriol. **183:**2859–2865.
15. **Taylor, B. N., P. Staib, A. Binder, A. Biesemeier, M. Sehnal, M. Röllinghoff, J. Morschhäuser, and K. Schröppel.** 2005. Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. Infect. Immun. **73:**1828–1835.
16. **Wirsching, S., S. Michel, G. Köhler, and J. Morschhäuser.** 2000. Activation of the multiple drug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. J. Bacteriol. **182:**400–404.
17. **Wirsching, S., S. Michel, and J. Morschhäuser.** 2000. Targeted gene disruption in *Candida albicans* wild-type strains: the role of the *MDR1* gene in fluconazole resistance of clinical *Candida albicans* isolates. Mol. Microbiol. **36:**856–865.

Editor: A. Casadevall