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Functional Characterization of the *MKC1* Gene of *Candida albicans*, Which Encodes a Mitogen-Activated Protein Kinase Homolog Related to Cell Integrity†

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Mitogen-activated protein (MAP) kinases represent a group of serine/threonine protein kinases playing a central role in signal transduction processes in eukaryotic cells. Using a strategy based on the complementation of the thermosensitive autolytic phenotype of *slt2* null mutants, we have isolated a *Candida albicans* homolog of *Saccharomyces cerevisiae* MAP kinase gene *SLT2* (*MPK1*), which is involved in the recently outlined *PKC1*-controlled signalling pathway. The isolated gene, named *MKC1* (MAP kinase from *C. albicans*), coded for a putative protein, Mkc1p, of 58,320 Da that displayed all the characteristic domains of MAP kinases and was 55% identical to *S. cerevisiae* Slt2p (Mpk1p). The *MKC1* gene was deleted in a diploid *Candida* strain, and heterozygous and homozygous strains, in both Ura^+ and Ura^- backgrounds, were obtained to facilitate the analysis of the function of the gene. Deletion of the two alleles of the *MKC1* gene gave rise to viable cells that grew at 28 and 37°C but, nevertheless, displayed a variety of phenotypic traits under more stringent conditions. These included a low growth yield and a loss of viability in cultures grown at 42°C, a high sensitivity to thermal shocks at 55°C, an enhanced susceptibility to caffeine that was osmotically remediable, and the formation of a weak cell wall with a very low resistance to complex lytic enzyme preparations. The analysis of the functions downstream of the *MKC1* gene should contribute to understanding of the connection of growth and morphogenesis in pathogenic fungi.

Signal transduction mechanisms in eukaryotes represent important processes for the regulation of cell functions. Significant progress towards understanding some of the basic processes that mediate cell responses to different stimuli has been made in recent years. Many studies carried out with *Saccharomyces cerevisiae* have documented, for example, the signal transduction cascade activated in response to the mating pheromone, which involves a number of sequentially acting protein kinases (1, 20, 41, 42, 46, 47, 64). More recently, two other signal transduction cascades, namely, the high-osmolarity glycerol response pathway (6) and the *PKC1*-mediated signal transduction pathway (20), have been identified. In each of these cascades, a set of sequentially acting protein kinases appears to be involved in generating the appropriate cellular responses that enable the cell to accommodate to the new physiological situation. Genetic and biochemical analysis has revealed the central role played in these pathways by a special group of serine/threonine kinases, the mitogen-activated protein kinases (MAP kinases), which were first identified in higher eukaryotes. Activation of MAP kinases is dependent on the simultaneous phosphorylation of threonine and tyrosine residues (22, 47) by MAP kinase kinases (MAPKKs), which are themselves phosphorylated by other kinases, the MAPKK kinases (MAPKKKs). The transmission of the signal eventually leads to activation of transcription factors, thus accomplishing the required cellular response.

The aforementioned signal transduction cascades play different roles in yeast cells. Activation of the mating pheromone cascade blocks the cells at the G_1 stage of the mitotic cell cycle and prepares them for mating with a cell of the opposite mating type through the final inhibition of the Cdc28/cyclin complex kinase activity (11, 19). The high-osmolarity glycerol pathway is triggered in response to high external osmolarity and allows the efficient accumulation of intracellular solutes (glycerol in *S. cerevisiae*) that enable the adaptation to growth under low water activity. The stimulus that triggers the third cascade, the *PKC1*-mediated pathway, is unknown, but epistasis experiments indicate that the pathway is based on the sequential participation of the kinase products of the genes *PKC1* (40), *BCK1* (*SLK1*) (12, 39), *MKK1* or *MKK2* (30), and the MAP kinase gene homolog *SLT2* (*MPK1*) (38, 66). The involvement of other elements has been suggested as well (37, 57). Many features of this pathway, especially the gene downstream targets of the Slt2 (Mpk1) protein kinase, remain to be determined. However, the fact that *S. cerevisiae* strains defective in some of these genes display an autolytic phenotype that can be complemented by osmotic stabilization (12, 30, 38, 43, 50, 66) has led to the conclusion that the Pkc1p signal transduction pathway is essential, in this yeast, for the generation of an osmotically stable cell wall during growth, a fundamental requirement for morphogenesis and cell integrity (1, 20). The osmotically remediable autolytic phenotype of *S. cerevisiae* mutants resulting from disruption of any of the kinase genes involved in this cascade is not homogeneous. For example, *pkc1*Δ cells require osmotic stabilization at any temperature to remain viable (50), whereas the mutants deficient in the MAP kinase involved in this cascade, namely *slt2*Δ strains, exhibit thermosensitive autolysis (38, 43) that can be remediated osmotically. This difference implies that Pkc1p may have targets other than the MAPKKK (Bck1p) gene that initiates the activation of the MAP kinase Slt2p.

Signal transduction pathways involving MAP kinase cascades seem to be functionally conserved from yeasts to vertebrates (although with higher complexity in the latter) (33, 47).

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† F. Navarro-García dedicates this work to his parents, F. Navarro-Rodríguez and M. C. García-Huertas.

TABLE 1. Strains used in this study

Microorganism	Strain	Relevant genotype or phenotype	Source
S. cerevisiae	YPH98	**a** *ura3-Δ52 lys2-801*^{amber} *ade2-101*^{ochre} *leu2-Δ1 trp1-Δ1*	63
	YPN98	**a** *ura3-Δ52 lys2-801*^{amber} *ade2-101*^{ochre} *leu2-Δ1 trp1-Δ1 slt2::LEU2*	This work
	YPNA98	**a** *ura3-Δ52 lys2-801*^{amber} *ade2-101*^{ochre} *leu2-Δ1 trp1-Δ1 ADE2 slt2::LEU2*	This work
C. albicans	1001[a]	Wild type	23
	SC5314	Wild type	24
	CAI4	*ura3Δ::imm434/ura3Δ::imm434*	21
	CAI-49[b]	*mkc1Δ::hisG-*Ca*URA3-hisG/MKC1 ura3Δ::imm434/ura3Δ::imm434*	This work
	CM-16	*mkc1Δ::hisG/MKC1 ura3Δ::imm434/ura3Δ::imm434*	This work
	CM-1613[c]	*mkc1Δ::hisG-*Ca*URA3-hisG/mkc1Δ::hisG ura3Δ::imm434/ura3Δ::imm434*	This work
	CM-1613C	*mkc1Δ::hisG/mkc1Δ::hisG ura3Δ::imm434/ura3Δ::imm434*	This work
E. coli	MC1061	*araD139 Δ(ara-leu)7697 Δ(lac)X74 galU galK straA*	R. L. Gourse
	DH5αF′	K-12 $\Delta(lacZYA\text{-}argF)_{u169}$ *supE44 thi-1 recA1 endA1 hsdR17 gyrA relA1* (ϕ80*lacZΔM15*)F′	26

[a] A wild-type strain from the CECT, ATCC 64385.
[b] Strains CAI-45, CAI-41, and CAI-4E have the same relevant genotype as CAI-49.
[c] Strains CM-1610, CM-1615, CM-1620, CM-1622, CM-1624, and CM-1625 have the same relevant genotype as CM-1613.

However, the existence of equivalent signal transduction systems in yeasts other than *S. cerevisiae* is not well documented. Indeed, only in the fission yeast *Schizosaccharomyces pombe* has a MAP kinase pathway been found, and this pathway operates to control mating by this organism (17, 46). Therefore, the identification of similar pathways in other yeast species will be important to establish the universality and functional conservation of these cascades in yeasts. In addition, the analyses of such cascades in other species should help clarify the role and function of the particular cascade. The opportunistic pathogenic yeast *Candida albicans*, which inhabits human gastrointestinal and vaginal tracts as a commensal, represents a particularly interesting system for the identification of signalling pathways in view of its potential virulence (49) as well as its dimorphism. Genetic manipulation of *C. albicans* has been hampered mainly because of its diploidy and lack of sexual cycle (see reference 36), but in the last few years the essential tools for gene isolation and disruption in this species have been developed (9, 10, 21, 28, 35). A *Candida* MAP kinase gene homolog, namely *CEK1*, was recently cloned in *S. cerevisiae* as a multicopy suppressor of pheromone-induced cell cycle arrest (68), but the role of this kinase in *C. albicans* was not investigated. In order to identify *Candida* MAP kinases and address their potential involvement in morphogenesis, we have used *S. cerevisiae* as a basic system for isolation of a *C. albicans* MAP kinase gene homolog of the *S. cerevisiae SLT2* (*MPK1*) gene. The isolated *C. albicans* gene, designated *MKC1*, presumably belongs to the *PKC1*-mediated pathway, representing the first experimental evidence for a similar transduction pathway in this clinically important fungus.

MATERIALS AND METHODS

Strains and growth conditions. Yeast and bacterial strains are listed in Table 1. *C. albicans* 1001, a wild-type strain from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo [CECT]), was used as source of genomic DNA for the construction of the genomic library.

Yeast strains were routinely grown in YED medium (1% yeast extract, 2% glucose) or SD minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose), with shaking at the selected temperature. *C. albicans* Ura$^-$ revertants were selected upon excision of the *C. albicans URA3* gene from integrative transformant strains (see below), on the basis of the resistance of the Ura$^-$ revertants to 5-fluoroorotic acid (5-FOA). The procedure of Fonzi and Irwin (21) was used, but the final concentration of 5-FOA in selective plates was 0.5 mg/ml instead of 1 mg/ml.

Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) broth or Terrific broth (TB) supplemented with 100 μg of ampicillin per ml for plasmid selection. The *E. coli*-yeast shuttle vector YEp352 has been described elsewhere (29).

S. cerevisiae YPN98, an *slt2*::*LEU2* strain, was created by one-step gene replacement at the *SLT2* locus by transformation of strain YPH98 with a 5.85-kb *Xba*I fragment from plasmid YEp352H (66), which contained the *LEU2* gene (obtained as a 4.1-kb *Pst*I fragment from YEp13) inserted at the *Pst*I site in the *SLT2* gene. To minimize the nutritional requirements of the strain used for library screening (see Results) we selected strain YPNA98, a spontaneous Ade$^+$ revertant from YPN98. The *slt2*Δ genotype of the disrupted strains was confirmed by Southern analysis (not shown) as well as by their thermosensitive lytic phenotype. *S. cerevisiae slt2* strains display a thermosensitive autolytic phenotype, detectable in colonies grown in agar with 5-bromo-4-chloro-3-indolylphosphate (BCIP), a dye that stains lysed cells (43). Disruption of the *SLT2* gene resulted in a clear lytic phenotype of the cells incubated at 37°C on BCIP-YED plates, and lysis of these cells in liquid cultures was further confirmed by precise determination of the proportion of lysed cells by flow cytometry (14). This procedure is based on the selective staining with propidium iodide of cells that lose selective permeability as a consequence of cell lysis. More than 95% of cells in cultures of strain YPNA98 lysed at 37°C (30% at 24°C), whereas the proportion of lysed cells in cell cultures of the parental strain, YPH98, was only 2%.

Cell wall digestion assay. Exponentially growing cells were washed with sterile water and diluted with sorbitol (1 M) to 5×10^3 cells per ml in 25 ml of sorbitol. Glusulase (1 μl; DuPont NEN) was added, and the cell suspension was incubated at 50 rpm and 30°C. Samples were taken at different times and plated on YED and YED-plus-sorbitol (1 M) plates.

DNA manipulations and analysis. All DNA manipulations were carried out according to standard procedures (2, 58). Plasmid DNA was isolated from *S. cerevisiae* by a procedure described elsewhere (55). Southern hybridization analysis was carried out with the Nonradioactive Labeling and Detection Kit (Boehringer Mannheim) according to the manufacturer's recommendations, under high-stringency conditions, on positively charged nylon membranes. The *C. albicans* origin of gene *MKC1* was confirmed by two methods: Southern hybridization analysis and PCR amplification of its open reading frame (ORF) with the specific oligonucleotides (based on the determined sequence) 5′-TACGTAAT GGATCAACAAGACGC-3′ and 5′-CCCGGGATAACGTGGTTGTGTG-3′, at 52°C as the annealing temperature, in a Perkin-Elmer Cetus DNA thermal cycler. For the determination of the *MKC1* sequence, a set of nested deletions (about 200 bp uniformly spaced) were constructed on both strands on plasmid pSN6SX with the Exonuclease III Nested Deletion Kit (Pharmacia), with *Sma*I and *Xba*I as nonprotected termini. Plasmid DNA was purified from *E. coli* transformants with Qiagen (Diagen, Hilden, Germany) and sequenced with an automated sequencer (ALF; Pharmacia) according to the method of Sanger et al. (59) with fluoresceinated primers. Sequencing was carried out in the Automatic DNA Sequencing Unit of this university. Sequence comparisons and homologies were carried out with the FASTA algorithm (53).

Genetic transformation procedures. *E. coli* was routinely transformed according to the method of Hanahan (26) except in the case of transformation with the *Candida* genomic library, in which electroporation was used as it gave significantly higher transformation frequencies (approximately 50-fold compared with the classical Hanahan procedure). Briefly, cells of *E. coli* MC1061 growing in LB medium at an optical density at 600 nm (OD_{600}) of 1.0 were collected by low-speed centrifugation ($3,000 \times g$, 5 min), washed twice with sterile cold H_2O and once with 10% (vol/vol) glycerol, and finally resuspended in 1/1,000 of the original culture volume in 10% glycerol. Portions (60 μl) of the cell suspension were incubated with DNA for 1 min at 4°C and subjected to a charge-discharge

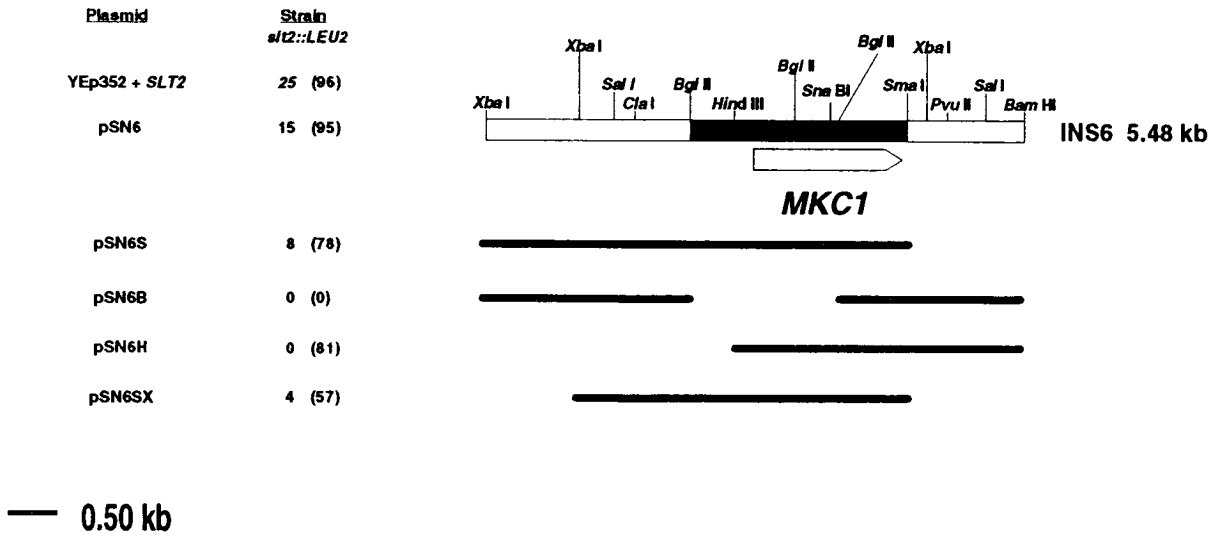

FIG. 1. Restriction map of the *C. albicans MKC1* locus and DNA subfragments complementing the lytic phenotype of *S. cerevisiae* YPNA98 (*slt2*::*LEU2*). Complementation of the lytic phenotype of strain YPNA98 was analyzed by transformation with YEp352 carrying the inserts indicated as solid lines or the whole fragment of DNA cloned. Figures express percentages of transformants able to grow directly at the restrictive temperature (37°C), as well as the percentages of transformants able to grow at the restrictive temperature following 12 h of recovery incubation at the permissive temperature of 24°C (in parentheses), with the total number of transformants obtained at 24°C, in each case, defined as 100%. Data are the mean values of two independent experiments. The solid thick line corresponds to the sequenced region, while the empty arrow indicates the *MKC1* ORF.

pulse (5 ms, 2,400 V, 129 Ω, 0.2-cm [width] cuvette) with an ElectroCell Manipulator 600 (BTX Laboratories, San Diego, Calif.). Cells were recovered with 200 μl of LB broth and immediately plated on LB-ampicillin plates.

S. cerevisiae cells were normally transformed by the lithium acetate protocol (31) or the bicine procedure (15). Electroporation was used for screening strain YPNA98 with the *Candida* library. Cells were recovered at an OD_{600} of 2 to 3, washed with chilled water, and resuspended in 1/1,000 of a volume of 1 M sorbitol, and pools of 400 μl were electroporated (5 ms, 1,450 V, 186 Ω, 0.2-cm [width] cuvette) and plated in selective medium without sorbitol, which resulted in approximately 10^5 transformants per μg of DNA. *C. albicans* was transformed essentially as described previously (28) but with omission of the final incubation step at 30°C prior to being poured onto selective minimal plates.

***Candida* gene disruption.** Disruption of the *MKC1* gene in *C. albicans* was achieved by the procedure described by Fonzi and Irwin (21). Given the diploidy of *C. albicans* strains, the procedure basically consists of three sequential steps intended (i) to disrupt one allele in a Ura$^-$ strain by one-step gene disruption with an appropriate plasmid construct containing the homologous *URA3* gene, (ii) to eliminate the inserted *URA3* in the selected single-disrupted transformants to restore the Ura$^-$ phenotype, and (iii) to disrupt the second allele of the gene by a strategy similar to the first one. In order to develop the appropriate construct, a 5.14-kb *Nru*I-*Sca*I fragment from plasmid pCUB-6 (21), carrying the *hisG*-Ca*URA3*-*hisG* cassette, was blunt ended with the Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates and inserted into the *Sma*I site of pUC19, yielding pUCK20. The aforementioned cassette was now obtained as a 4.02-kb *Bgl*II-*Bam*HI fragment from this plasmid and substituted for the large *Bgl*II fragment of pSN6, thus replacing most of the *MKC1* gene coding region together with the promoter, creating pSN6INT1. Finally, the *Pvu*II-*Pvu*II region containing the *MKC1*::*hisG*-*URA3*-*hisG*::*MKC1* construct from pSN6INT1 was inserted into the large *Pvu*II fragment of pUC19 to obtain pUCINT1, which was used for subsequent disruption experiments. All these fragments are depicted in Fig. 3. The genotypes of all *C. albicans mkc1*Δ strains constructed in this work were checked by Southern blot hybridization with a 0.74-kb *Kpn*I-*Sma*I fragment of plasmid pSN6 as a probe.

Construction of a *C. albicans* genomic DNA library. Genomic DNA was obtained from *C. albicans* 1001 as described elsewhere (61), partially digested with *Sau*3A, and then size fractionated on 0.8% (wt/vol) agarose gels. Fragments corresponding to 5 to 10 kb were eluted and ligated with a *Bam*HI-digested calf intestinal alkaline phosphatase-treated YEp352 for 20 h at 13°C. Ligations were precipitated with ethanol, washed twice with cold ethanol (70% [vol/vol]), and used to transform *E. coli* MC1061 by electroporation (16) (see above). Plasmid DNA (from approximately 2×10^5 independent clones) was isolated directly from the transformants grown on LB-ampicillin plates by alkaline lysis (4) and stored in aliquots at −20°C.

Nucleotide sequence accession number. The sequence of the *C. albicans MKC1* gene has been deposited in the EMBL Data Bank and assigned the EMBL accession number X76708.

RESULTS

***MKC1* is a *C. albicans* functional homolog of the *S. cerevisiae SLT2* gene.** Initial attempts to isolate the functional homolog of the *S. cerevisiae SLT2* gene from *C. albicans* were based on the complementation of the lytic phenotype of *S. cerevisiae* YPN98 (*slt2*::*LEU2 ade2*). Selection of several apparently positive (nonlytic) transformants allowed us to obtain a gene that, after sequencing, was shown to be the *Candida* homolog of the *S. cerevisiae ADE2* gene (data not shown). This indicated that the Ade$^-$ strain was not the optimal one, probably because of its more severe lytic phenotype, which could be alleviated by complementation of the auxotrophic Ade deficiency and thereby give rise to false-positive clones. Therefore, we used *S. cerevisiae* YPNA98 (*slt2*::*LEU2*), an Ade$^+$ revertant from YPN98, for library screening in order to search for a *C. albicans* gene capable of complementing the lytic phenotype. Transformants were grown at 24°C on minimal medium containing BCIP. After 3 days of growth at this permissive temperature, the plates were transferred to 37°C in order to identify nonstained (nonlytic) clones. Approximately 10^5 transformants were screened by this protocol, and 27 candidate transformants were identified by two criteria: limited lysis and enhanced growth compared with those of the strain carrying the control plasmid (YEp352) at 37°C. Of the 27 initially selected transformant clones, two were confirmed to be nonlytic in a second phenotypic screening; only one of them was shown to carry a plasmid that complemented the lytic phenotype of strain YPNA98 upon retransformation. This plasmid, named pSN6, was used for subsequent studies.

Plasmid pSN6 was shown to bear an insert of approximately 5 kb (Fig. 1). A standard restriction and functional analysis allowed us to define a 2.8-kb *Xba*I-*Sma*I internal fragment capable of complementing the autolytic phenotype of *S. cerevisiae* YPNA98 (plasmid pSN6SX; Fig. 1). Plasmid pSN6SX was sequenced (see Materials and Methods) and shown to contain a single ORF encoding 502 amino acids, a putative

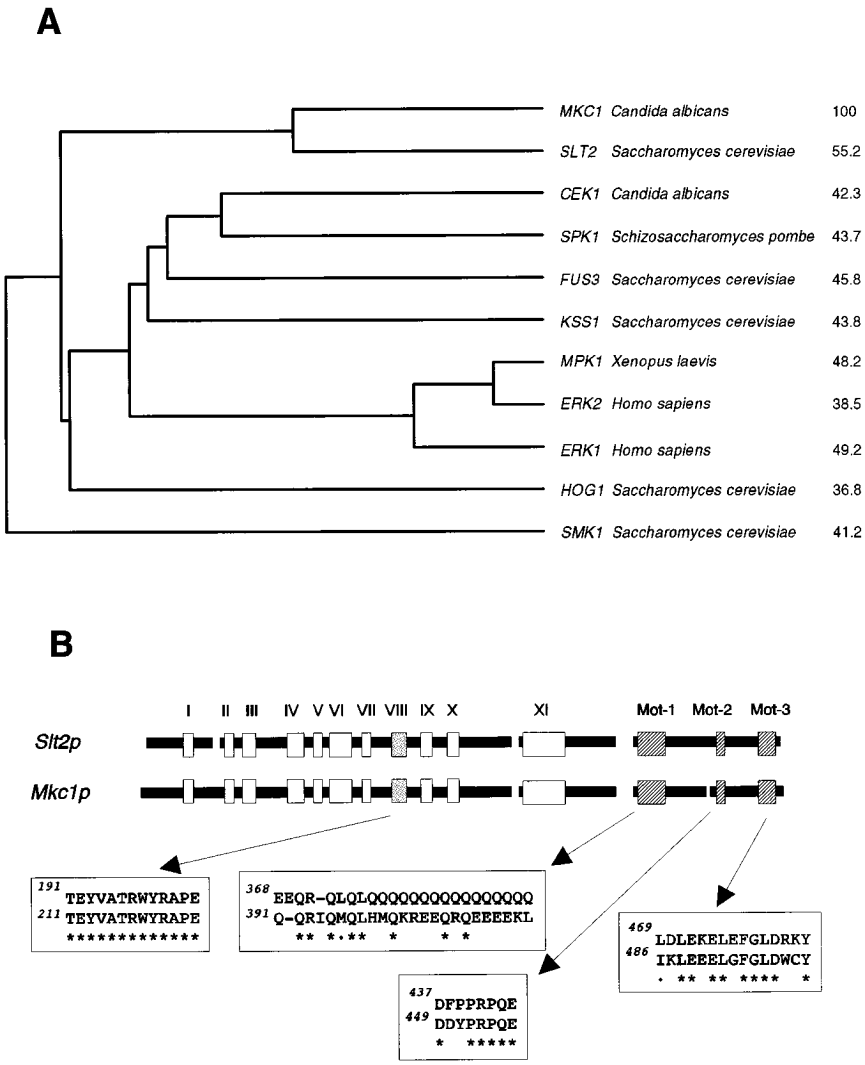

FIG. 2. Comparison of the *C. albicans MKC1* gene product with other MAP kinases on the basis of their amino acid sequences. (A) Dendrogram analysis, based on amino acid sequence alignment and percentage of identity, comparing Mkc1p with other members of the MAP kinase family. Numbers indicate the percentages of identity with Mkc1p. (B) Structural domains of MAP kinases Mkc1p (*C. albicans*) and Slt2p (*S. cerevisiae*). The scheme depicts the positions of all the characteristic serine/threonine kinase subdomains (27) in both proteins and illustrates the homologies of subdomain VIII and three motifs of the nonkinase C-terminal moieties of both proteins. Alignments were done with MACAW PC software.

protein of 58 kDa. The gene was named *MKC1*, for MAP kinase from *C. albicans*. Its more relevant features are described in Fig. 2. Analysis of the ORF implied that it specified a putative MAP kinase homolog, Mkc1p, most similar to Slt2p (Mpk1p) (55.2% identity; Fig. 2A and references 38 and 66). This identity decreased to 42.3% in comparison with *C. albicans* Cek1p (68), 45.8% with *S. cerevisiae* Fus3p (18), 36.8% with *S. cerevisiae* Hog1p (6), 43.7% with *S. pombe* Spk1p (65), and 41.2% with *S. cerevisiae* Smk1p (34). In addition, Mkc1p is 49.2% identical to human ERK1 and 38.5% identical to human ERK2. Other relevant features observed in the MAP kinase Mkc1p were 11 conserved domains characteristic of serine/threonine protein kinases (Fig. 2B). A putative ATP-binding site could be found at Lys-75 (Lys-54 in Slt2p), while the most conserved and characteristic regulatory domain common to all MAP kinases (the kinase subdomain VIII, which includes the Thr-Glu-Tyr phosphorylation motif) was found between residues 209 and 226 (Fig. 2B). A glutamine-rich segment followed by a polyglutamine track (16 residues) is another salient feature of Slt2p (66). A comparable motif, consisting mostly of glutamic acid and glutamine residues, was found in Mkc1p, and two other segments in the C-terminal moieties of the two proteins were very similar (Fig. 2B). The cloned *C. albicans MKC1* gene substituted for *SLT2* function in *S. cerevisiae* and encoded a protein structurally related to known MAP kinases. We concluded that *MKC1* likely specified a MAP kinase, and we undertook the functional characterization of the *MKC1* gene by developing *C. albicans* null mutants.

Deletion of the *MKC1* gene in *C. albicans*. The construction of a homozygous *mkc1*Δ strain was carried out by the sequential steps described in Materials and Methods (Fig. 3). Deletion was carried out in CAI4, a *ura3*Δ strain, with a gene construct that enabled the elimination of all the essential kinase domains in the *MKC1* ORF as well as more than 500 bp of the 5′ upstream region (Fig. 3). Plasmid pUCINT1 was digested with *Pvu*II to generate the appropriate linear fragment for replacement of the above-mentioned essential *MKC1* region upon transformation in CAI4. After the first transfor-

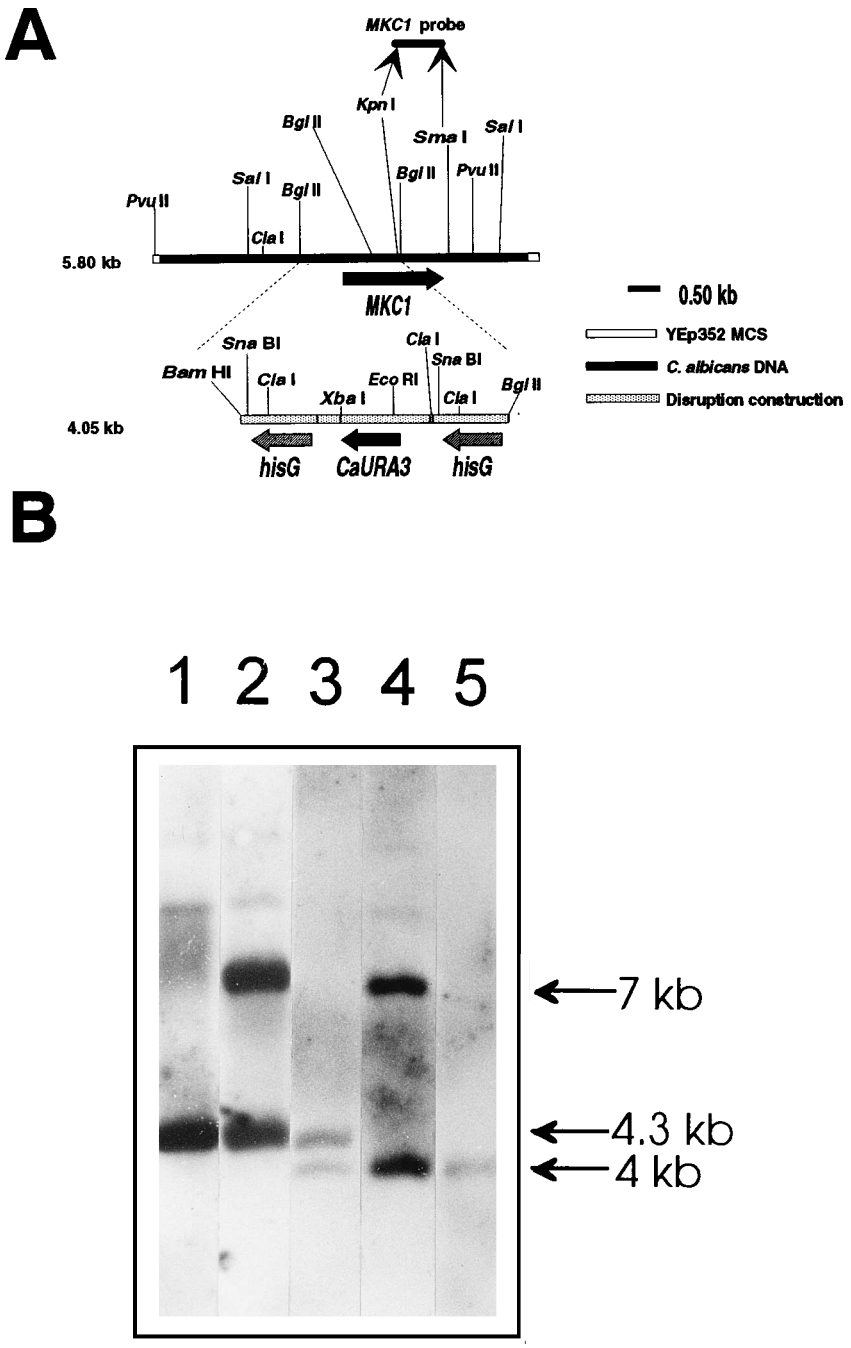

FIG. 3. Strategy used for deletion of the two alleles of the *MKC1* gene in *C. albicans* CAI4 and Southern hybridization analysis of the sequential process. (A) Deletion strategy. The disruption construction was used to substitute for the *Bgl*II-*Bgl*II fragment of the *MKC1* locus (see Materials and Methods). (B) Southern hybridization analysis of DNAs from strains obtained during the process of deletion. Genomic DNAs were *Sal*I digested, separated by electrophoresis, and probed with the 0.9-kb *Kpn*I-*Sma*I fragment. Lane 1, CAI4 (wild type Ura$^-$); lane 2, CAI-49 (*MKC1*/*mkc1*Δ Ura$^+$); lane 3, CM-16 (*MKC1*/*mkc1*Δ Ura$^-$); lane 4, CM-1613 (*mkc1*Δ/*mkc1*Δ Ura$^+$); lane 5, CM-1613C (*mkc1*Δ/*mkc1*Δ Ura$^-$). The 4.3-, 7-, and 4-kb bands correspond respectively to the wild-type *MKC1* locus, the *mkc1*Δ::*hisG-URA3 hisG*::*mkc1*Δ locus, and the *mkc1*Δ::*hisG*::*mkc1*Δ locus. The band of the *hisG*-disrupted allele is slightly smaller than the wild-type allele because of the elimination of a *Bgl*II-*Bgl*II fragment of 1.5 kb and the maintenance of 0.9 kb of the *hisG* gene after Ca*URA3* excision.

mation, more than 50 Ura$^+$ transformants were obtained, and 20 were checked for homologous replacement by Southern hybridization (Fig. 3). Of the 20 checked clones, eight were shown to carry the correct substitution that deleted the first allele. One of them, namely CAI-49, was chosen for isolation of Ura$^-$ revertants, which must arise by an excision event eliminating the *URA3* gene, presumably by homologous recombination involving the *hisG* repeats. After 20 days in 5-FOA selective medium, 34 clones were checked again for the desired (i.e., intrachromosomal instead of interchromosomal recombination) loss of the *URA3* gene by Southern hybridization. Of the 34, three had undergone the desired event, and we chose strain CM-16 (Fig. 3). This strain is an *MKC1*/*mkc1*Δ heterozygote, auxotrophic for uracil. Two independent trans-

formation experiments with this strain were carried out, with the *Pvu*II-digested pUCINT1 plasmid, in order to delete the second *MKC1* allele. The number of transformants in both cases was more than 100, and a total of 32 were checked to identify 7 homozygous disrupted transformants: strains CM-1610, CM-1613, CM-1615, CM-1620, CM-1622, CM-1624, and CM-1625. CM-1613 yielded strain CM-1613C, a Ura$^-$ auxotroph, by a procedure identical to the one described above. The results shown below were obtained mostly with SC5314, CAI-49, and CM-1613, as well as their Ura$^-$ derivatives. However, most of the experiments were also carried out with the other independently obtained homozygous deletion strains to show that the phenotype was the same. The gene replacements leading to deletion of both *MKC1* alleles as well as the elimination of the selective *URA3* gene in both transformants were checked by Southern hybridization analysis (Fig. 3B) as well as by PCR analysis (not shown). The development of heterozygous and homozygous deletion strains for the *MKC1* gene, both in Ura$^+$ and Ura$^-$ backgrounds, facilitated the analysis of the consequences of the deletion of the gene. Pulsed-field gel electrophoresis analysis of *mkc1*Δ/*MKC1* and *mkc1*Δ/*mkc1*Δ strains showed no apparent alterations in their karyotypes compared with those of the parental strain, SC5314 (data not shown).

Effects of *MKC1* deletion on sensitivities to high temperature and caffeine. *SLT2* function in *S. cerevisiae* is required for viability of growing cells at 37°C, but not at 24°C (38, 45, 66). Lysis at the nonpermissive temperature is prevented by osmotic stabilization with 1 M sorbitol. Another phenotype of these mutants is caffeine sensitivity owing to cell lysis, even at the permissive temperature (13). The degree of lysis in cell cultures (induced either by high temperature or by caffeine) is very much dependent on the genetic background (43, 44). We first addressed the effect of temperature on the *C. albicans mkc1*Δ strains, taking into account that normal growth temperatures for *C. albicans* are usually higher than those for *S. cerevisiae*. The cells were viable at 28°C, the usual laboratory temperature used for growing *C. albicans*. Heterozygous and homozygous deletion strains could also grow at 37 and 42°C in agar plates, indicating that viability at these higher temperatures was sufficient to support colony growth. To examine the effect of temperature in a more quantitative manner, we analyzed growth yield and viability of the cells in liquid cultures grown at 42°C. As shown in Fig. 4, the wild-type parental strain CAI4 grew well at this temperature, but viability of the cells was eventually reduced to 30% after 48 h. On the other hand, strain CM-1613C (*mkc1*Δ/*mkc1*Δ), grown at 42°C, gave a much lower growth yield, with a complete loss of viability earlier than that of the wild-type strain (24 h). The heterozygous deletion strain CM-16 (*mkc1*Δ/*MKC1*) gave intermediate results. Thermosensitivity of *C. albicans* cells deficient in *MKC1* function was therefore manifested as an intense loss of cell viability in cultures grown at 42°C. The strains discussed above had a Ura$^-$ phenotype. When the same experiments were repeated with Ura$^+$ strains, the effect of temperature on viability was not nearly as dramatic (data not shown). Therefore, we further explored the role of the *MKC1* gene in maintaining thermostability by studying the effect of heat shock treatments at a higher temperature, namely, 55°C. In order to use precise conditions, inocula of 10^5 exponentially growing cells were spotted onto YED and SD agar to be challenged at 55°C for periods of 0 to 90 min and further incubated at 28°C. The results (Fig. 5) showed that lack of *MKC1* function leads to thermal sensitivity. Heterozygotes were more sensitive to temperature shock than wild-type strains, and homozygotes were more sensitive still. Interestingly, parallel experiments with

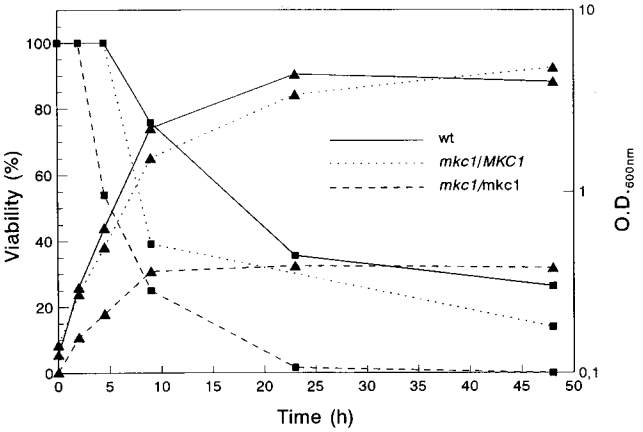

FIG. 4. Growth and viability at 42°C of *C. albicans* wild-type and *mkc1*Δ single- and double-deletion strains. Exponentially growing cells were taken and inoculated to prewarmed medium (YED). Cultures were grown at 42°C, and aliquots were taken to measure viability (■) and OD_{600} (▲) at different times. Solid lines, strain CAI4 (wild type); dotted lines, strain CM16 (*MKC1*/*mkc1*Δ); dashed lines, strain CM1613C (*mkc1*Δ/*mkc1*Δ). Viability was determined by plating dilutions from aliquots of the culture in YED agar in order to count the CFU.

stationary-phase cells revealed that their sensitivity to thermal shock was not significantly affected by deletion of the *MKC1* gene in either background (not shown).

We next investigated caffeine sensitivity, another phenotypic characteristic of *S. cerevisiae* deficient in *SLT2* function (13). Suspensions of cells were spotted onto SD agar supplemented with 10 mM caffeine. The *mkc1*Δ-homozygous deletion strain was clearly more sensitive than the heterozygote, which was more sensitive than the wild-type parental strain at 42°C (Fig. 6A). The enhanced sensitivity was again greater in the Ura$^-$ background and at higher-temperature incubation. These experiments were also carried out with several other homozygous deletion strains as well as several caffeine concentrations (data

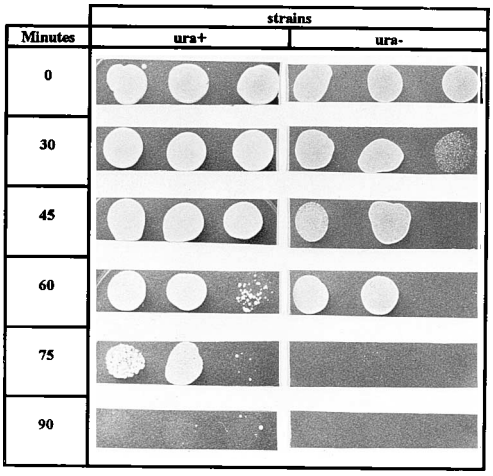

FIG. 5. Effects of 55°C thermal shocks on *C. albicans mkc1*Δ deletion strains. Suspensions of approximately 10^5 exponentially growing cells were spotted onto YED plates, the thermal shock was carried out for the periods indicated, and the plates were incubated at 28°C following the temperature challenge. Pictures show, from left to right, the growth, after 24 h, of *C. albicans* Ura$^+$ strains SC5314 (wild type), CAI-49 (*MKC1*/*mkc1*Δ), and CM-1613 (*mkc1*Δ/*mkc1*Δ) and Ura$^-$ strains CAI4 (wild type), CM16 (*MKC1*/*mkc1*Δ), and CM-1613C (*mkc1*Δ/*mkc1*Δ).

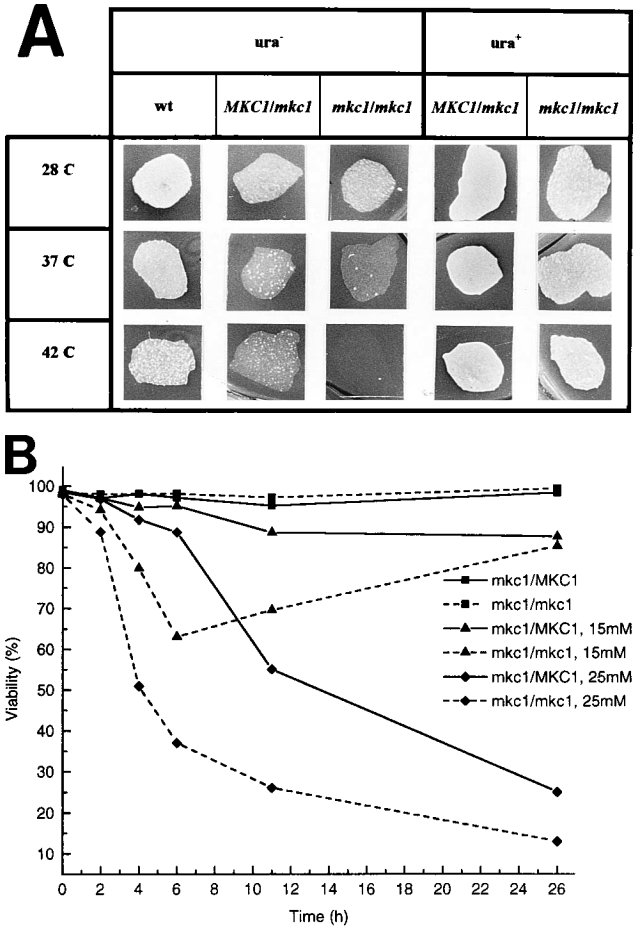

FIG. 6. Caffeine susceptibilities of *C. albicans mkc1*Δ strains. (A) Suspensions of approximately 10^5 exponentially growing cells of the indicated strains were spotted on SD–10 mM caffeine plates and photographed after 48 h of incubation at the indicated temperatures. (B) Exponentially growing cells were suspended in YED medium without caffeine (■) or with 15 mM (▲) or 25 mM (◆) caffeine, and the cultures were incubated at 28°C and 200 rpm. The Ura$^+$ strains CAI-49 (*MKC1*/*mkc1*Δ) (solid lines) and CM-1613 (*mkc1*Δ/*mkc1*Δ) (dashed lines) were used in these experiments.

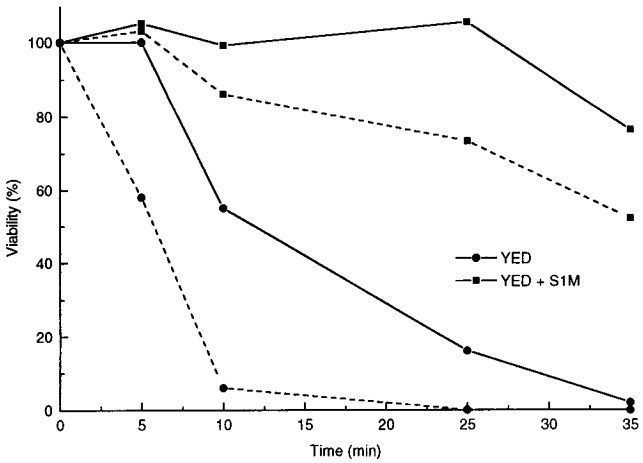

FIG. 7. Effects of cell wall-digesting enzyme preparations on viability of *C. albicans* wild-type and *mkc1*Δ double-deletion strains. The percentages of cells able to grow on YED plus 1 M sorbitol (■) and YED (●) plates following Glusulase treatment (see Materials and Methods) are represented for *C. albicans* strains CAI4 (wild type Ura$^-$) (solid lines) and CM1613C (*mkc1*Δ/*mkc1*Δ Ura$^-$) (dashed lines) as a function of time after addition of the lytic enzyme preparation.

not shown). Spotted cells of homozygous disrupted strains were unable to grow at 20 mM, and they grew poorly at 15 mM, whereas the heterozygous strain grew at 15 and 20 mM but not at 25 mM caffeine. Similar observations were made by checking the effect of 15 and 25 mM caffeine in liquid cultures and analyzing the proportion of nonviable lysed cells by the propidium iodide flow cytometry procedure (14). The effect of caffeine on Ura$^+$ versions of the heterozygous and homozygous *mkc1*Δ strains was also investigated by propidium iodide staining to measure the proportion of viable cells in liquid culture. The lack of *MKC1* function again conferred a significantly higher loss of viability, especially at 25 mM caffeine (Fig. 6B). In this case, we also observed osmotic protection of the lytic effect of caffeine with 1 M sorbitol. The observed recovery of viability of cultures after 6 h in 15 mM caffeine could be due to desensitization or to selection of resistant cells altered in transport of the drug. Thus, deletion of the *MKC1* gene in *C. albicans* results in thermosensitive growth and enhanced caffeine sensitivity, and osmotic stabilization of the medium leads to remediation of the phenotypic deficiency. Although alterations in cell morphologies were clearly apparent, attempts to correlate these defects with alterations in chitin deposition or in the pattern of actin filaments did not give clear results (data not shown).

***mkc1*Δ strains are highly sensitive to cell wall-lytic enzyme preparations.** As an approach to the analysis of the consequences of *MKC1* deficiency on the architecture of the cell wall, we next examined the effects of Glusulase treatment on cells. Glusulase consists of a complex mixture of enzymes that can degrade cell wall components. As shown in Fig. 7, wild-type cells remained viable after 5 min of Glusulase treatment whereas 40% of the population of the CM1613C strain (*mkc1*Δ/*mkc1*Δ) were killed during the same period of treatment. The loss of viability was also much more marked in this strain thereafter. Osmotic stabilization (with 1 M sorbitol) protected both strains from lysis by Glusulase (Fig. 7). We infer that the deletion of *MKC1* leads to cells with walls that are much less resistant to lytic enzyme preparations. Essentially identical results were obtained with Ura$^+$ strains. In all Glusulase sensitivity experiments, the heterozygous deletion strain behaved in the same way as the wild-type strain.

Effect of high osmolarity on *mkc1*Δ strains. The behavior of *MKC1*-deficient strains on high-osmolarity media was also analyzed. *mkc1*-homozygous deletion strains were able to grow at low water activity, a condition achieved by adding to the medium substances frequently used as osmotic stabilizers. For example, the *mkc1*Δ/*mkc1*Δ strains grew on plates containing up to 1.4 M sodium chloride, 1 M sorbitol, or 1.6 M potassium chloride. Indeed, the presence in the media of great amounts of NaCl (even 5 M) during short times (up to 1 h) did not diminish cell viability. However, we also observed two other interesting phenotypic characteristics. First, *mkc1*Δ/*mkc1*Δ Ura$^-$ strains were sensitive to high Ca^{2+} ion concentrations. As shown in Fig. 8A, concentrations of Ca^{2+} higher than 0.2 M killed the homozygous deletion strain CM-1613C, whereas the same concentrations had much less of an effect on CM-16 (*mkc1*Δ/*MKC1*) or wild-type cells. Second, on high-osmolarity media (sorbitol concentrations greater than 0.8 M or sodium chloride concentrations higher than 0.4 M), there was a significant change in colony morphology. Under these conditions, the otherwise normally smooth wild-type cells (strains CAI4 and SC5314) changed to rough colony morphology (Fig. 8B).

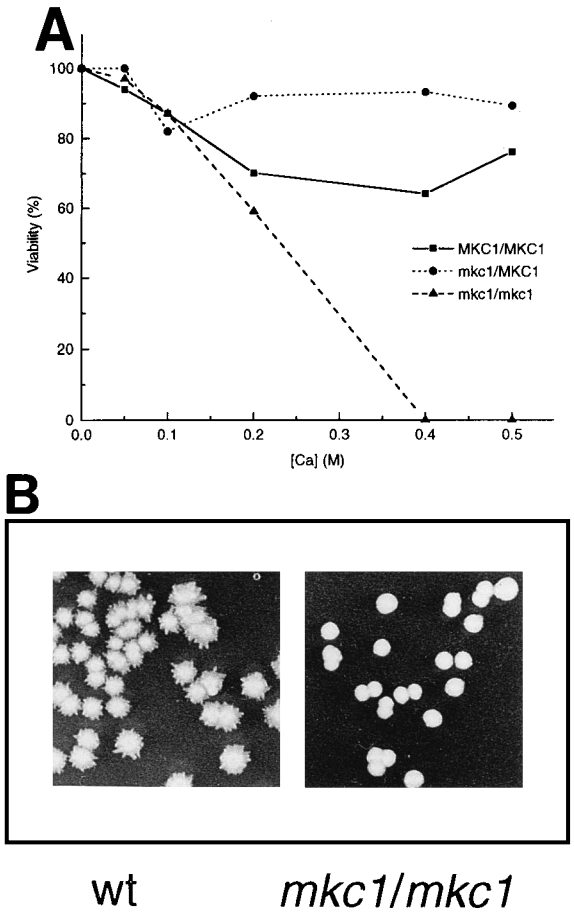

FIG. 8. Effects of high concentrations of calcium chloride on growth and of high-osmolarity media on colonial morphology of *C. albicans mkc1*Δ deletion strains. (A) Cell suspensions of exponentially growing cells on YED medium at 28°C were inoculated in YED-agar plates containing the indicated concentrations of $CaCl_2$. The plates were incubated at 28°C to determine the percentage of the cell population surviving at each concentration, with the number of colonies able to grow without $CaCl_2$ defined as 100%. The Ura$^-$ strains CAI4 (solid line), CM-16 (*MKC1*/*mkc1*Δ) (dotted line), and CM-1613C (*mkc1*Δ/*mkc1*Δ) (dashed line) were used in these experiments. (B) Effects of high salt concentration (0.4 M CINa) on the colony morphologies of strains CAI4 (left) and CM1613C (*mkc1*Δ/*mkc1*Δ) (right). Colonies are shown in YED agar after 3 days.

The microscopic appearance of the wild-type cells was also changed, most of them being observed as filaments. In contrast, colonies of the *mkc1*Δ/*mkc1*Δ strains were smooth (Fig. 8B).

DISCUSSION

The recent demonstration that a cascade of phosphorylation reactions governed by protein kinase Pkc1 plays an essential role in the generation of a stable cell wall in *S. cerevisiae* (20) represents an interesting new finding that leads to new questions and experimental strategies. The unraveling of details regarding the activating stimulus of this cascade, as well as the mechanisms of transmission of the signal, should increase the understanding of the connection between growth control and morphogenesis in yeasts. The characterization of homologous gene functions in other species should also help to advance the analysis of the biological role of the cascade. The clinical interest in *C. albicans* justifies the analysis of basic mechanisms that might control the generation of a stable cell wall because it could contribute to the identification of novel antifungal targets (48, 67).

Assuming that a similar signalling cascade might exist in *C. albicans* and that the corresponding homologous genes could be expressed in *S. cerevisiae* despite the deviation from universal translation rules for some *Candida* species (32, 60, 69), we were able to isolate a complementing *C. albicans* homolog of the *S. cerevisiae SLT2* gene, suggesting that a signalling cascade homologous to the Pkc1-controlled cascade of *S. cerevisiae* is functional in this opportunistic fungus. The isolated gene, named *MKC1*, replaced the function of *SLT2* in *S. cerevisiae* null mutants by restoring growth and preventing autolysis at 37°C, thus showing that the *Candida* gene is expressed in *Saccharomyces* cells. This idea that *C. albicans* contains a signalling pathway homologous to the one described in *S. cerevisiae* is also supported by the recent isolation of a *PKC1* homolog from this opportunistic fungus (52).

Analysis of the predicted amino acid sequence of Mkc1p confirmed that it is a MAP kinase homolog with a closer relationship to Slt2p than to any other of the known MAP kinases. Outstanding features of Mkc1p are the Thr-Glu-Tyr phosphorylation motif (22, 56), just downstream from kinase subdomain VIII (27), that is typical of MAP kinases and the C-terminal nonkinase domain that contains a highly charged glutamic acid-rich motif. This motif may be related to the glutamine region and the polyglutamine track of the C-terminal moiety of Slt2p (66). Studies to define the domains that are essential for function of both MAP kinases by development of hybrids of Slt2p and Mkc1p, among other strategies, are in progress in this laboratory.

MAP kinases are central elements in signal transduction cascades that elicit cellular responses to particular environmental stimuli. We undertook the disruption of the *MKC1* gene in a *C. albicans* strain in order to define the physiological consequences of the lack of gene function. Gene disruption in a diploid organism such as *C. albicans* is a complex process, and consequently only a limited number of disrupted strains have been reported in the literature (5, 21, 25). Elimination of the two alleles of a certain gene can be achieved only if the homozygous disrupted cells are viable at least under certain conditions. The method we used for disruption (21) leads to mutants that are deleted for the specific locus, and, in this case, we were able to obtain heterozygous and homozygous deletion strains in both Ura$^+$ and Ura$^-$ backgrounds. A thorough examination of gene function in *C. albicans* demands the comparison of heterozygous and homozygous deletion strains in both Ura$^+$ and Ura$^-$ backgrounds, despite the complications involved in the use of six strains in most experiments. This approach allows us to investigate the effects of both gene dosage and nutritional markers on the intensities of the phenotypes under study.

Our results show that although *C. albicans* cells deficient in *MKC1* function can grow under standard laboratory conditions they are limited in their capacity to withstand stress situations such as elevated temperatures or caffeine concentrations. This was observed in both Ura$^+$ and Ura$^-$ backgrounds, but more markedly in the latter. The fact that the heterozygous deletion strains displayed an intermediate sensitivity also represents a clear indication that the function of the *MKC1* gene is involved in the survival of the cells under the above-mentioned stresses. The Ras-adenylate cyclase pathway has been implicated in the capacity of *S. cerevisiae* stationary-phase cells to reenter growth phase (7, 8) after temperature shock treatments similar to the ones we use. However, it seems unlikely that the Ras pathway is affected in *mkc1*Δ strains, since the sensitivity to temperature stress was only observed in growing cells.

The caffeine sensitivity of *C. albicans mkc1*Δ strains represents another relevant phenotypic alteration. *S. cerevisiae* mutants defective for the Pkc1p cascade, such as *slk1* (*bck1*) (12) and *slt2* (13), as well as *ppz1* and *ppz2* (57), are also caffeine sensitive. Our results are consistent with these observations, but they do not shed light on what process or processes might be affected by the drug. In any case, caffeine has been implicated in several biological effects in *S. cerevisiae*, among them the inhibition of cAMP phosphodiesterases (3, 51).

Relevant to the analysis of the biological role of the *MKC1* gene in *C. albicans* is the observation that *mkc1*Δ cells were significantly more sensitive than wild-type cells to complex lytic preparations that degrade cell wall components. This sensitivity is reminiscent of the autolysis seen for *S. cerevisiae slt2* cells growing at the nonpermissive temperature (38, 43, 44, 66), but in this case we provide a direct demonstration of possible changes in cell wall architecture. It follows that the normal activity of Mkc1p is required for the generation of stable cell wall structure. The implicated mechanisms remain to be discovered, but the osmotic remediation of the deficiency implies that *mkc1*Δ cells have a stable plasma membrane but a defective cell wall. The complexity of pathways leading to the synthesis, assembly, and membrane transport of wall precursors and structural components makes it difficult to speculate about the link between the deficiency in the MAP kinase we describe and the generation of a weaker cell wall.

The simplest interpretation of the results presented in this paper is that the MAP kinase gene *MKC1* is required for growth of *C. albicans* under at least some stress conditions, such as high temperature, possibly because of the formation of weaker cell walls. *S. cerevisiae slt2* mutants appear to exhibit a more pronounced phenotype than *C. albicans mkc1*Δ/*mkc1*Δ strains. However, we have observed that *S. cerevisiae slt2* mutants of various types (deletion and point mutations) are very much dependent on their genetic background for the degree of autolysis that they express (44). For example, some *slt2* strains grow at 37°C in nutrient agar, although many of the cells in the population appear lysed, whereas other strains do not grow at all at this temperature. Relevant examples of the influence of the genetic background on the expression of a lytic phenotype are the observations showing that loss of *SSD1* function can increase the thermosensitivity shown by some *slt2* strains (38) and that the *C. albicans ADE2* gene can alleviate the thermosensitivity of *S. cerevisiae ade2 slt2*Δ strains; these provide further support for the idea that genetic background can influence the severity of the *slt2* phenotype. In view of these facts, it is perhaps not surprising that the severity of the phenotype of *C. albicans mkc1*Δ strains was higher in the Ura^- background. It must also be considered that *S. cerevisiae* laboratory strains currently used in genetic experiments usually have a higher number of genetic markers (auxotrophies and other deficiencies) than the so-called more wild *Candida* strains. This could also facilitate the expression of lethal phenotypes in *S. cerevisiae.*

Therefore, we can conclude that both *SLT2* (*MPK1*) of *S. cerevisiae* and *MKC1* of *C. albicans* are genes that are important for cell growth, especially under stress conditions. They may also play a role in dimorphic cell type transitions. The discovery of *C. albicans* homologs of the *S. cerevisiae* Pkc1 cascade genes opens a number of interesting questions. For us, the most interesting one is the connection of *MKC1* function to growth control and its influence on the generation of a stable wall structure. Some of the mechanisms that relate the function of the Pkc1p-controlled cascade with enzymes that can be relevant in the generation of the cell wall in *S. cerevisiae* are beginning to emerge (62). The discovery of conditions that kill the *mkc1*Δ/*mkc1*Δ strain, such as high concentrations of calcium or caffeine, should enable us to look for suppressors of this lethal phenotype with the use of a genetic transformation system developed in our laboratory (54). Such suppressors may allow us to identify genes acting downstream of the MAP kinase Mpk1p. On the other hand, we are also currently addressing the question of the influence of *MKC1* in the growth of the commensal yeast in the mammalian host with the use of experimental infection systems. The potential use of the cascade as an antifungal target should benefit from this approach.

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