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KRE5 Gene Null Mutant Strains of *Candida albicans* Are Avirulent and Have Altered Cell Wall Composition and Hypha Formation Properties

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The UDP-glucose:glycoprotein glucosyltransferase (UGGT) is an endoplasmic reticulum sensor for quality control of glycoprotein folding. *Saccharomyces cerevisiae* is the only eukaryotic organism so far described lacking UGGT-mediated transient reglucosylation of N-linked oligosaccharides. The only gene in *S. cerevisiae* with similarity to those encoding UGGTs is *KRE5*. *S. cerevisiae KRE5* deletion strains show severely reduced levels of cell wall β-1,6-glucan polymer, aberrant morphology, and extremely compromised growth or lethality, depending on the strain background. Deletion of both alleles of the *Candida albicans KRE5* gene gives rise to viable cells that are larger than those of the wild type (WT), tend to aggregate, have enlarged vacuoles, and show major cell wall defects. *C. albicans kre5*/*kre5* mutants have significantly reduced levels of β-1,6-glucan and more chitin and β-1,3-glucan and less mannoprotein than the WT. The remaining β-1,6-glucan, about 20% of WT levels, exhibits a β-1,6-endoglucanase digestion pattern, including a branch point-to-linear stretch ratio identical to that of WT strains, suggesting that Kre5p is not a β-1,6-glucan synthase. *C. albicans KRE5* is a functional homologue of *S. cerevisiae KRE5*; it partially complements both the growth defect and reduced cell wall β-1,6-glucan content of *S. cerevisiae kre5* viable mutants. *C. albicans kre5*/*kre5* homozygous mutant strains are unable to form hyphae in several solid and liquid media, even in the presence of serum, a potent inducer of the dimorphic transition. Surprisingly the mutants do form hyphae in the presence of *N*-acetylglucosamine. Finally, *C. albicans KRE5* homozygous mutant strains exhibit a 50% reduction in adhesion to human epithelial cells and are completely avirulent in a mouse model of systemic infection.

Candida albicans is a common human commensal. However, when the host-commensal balance is disturbed, infections of oral, vaginal, and gastrointestinal tracts may occur. In immunocompromised and other high-risk patients, *C. albicans* can enter the bloodstream and invade internal organs, leading quite often to death. The incidence of fatal *C. albicans* infections has increased dramatically in recent years (28), prompting great interest in the discovery of new antifungal drug targets. Two such potential new targets are genes involved in the synthesis of the cell wall, an essential organelle in fungal species that is not present in mammalian cells, and genes necessary for the yeast-to-hypha dimorphic transition. The ability of *C. albicans* to switch its mode of growth has been shown to be critical for its virulence (6, 18).

The cell walls of fungi are essential for maintaining the osmotic balance of the cell and for normal cell growth, cell division, and morphogenesis. Chitin, β-1,3-glucan, β-1,6-glucan, and highly mannosylated glycoproteins are the main cell wall polymers in yeast (25, 29). β-1,6-Glucan is a critical cell wall component. It is highly branched, covalently associated with all of the other cell wall polymers, and essential for the retention of many cell wall proteins (13). In *C. albicans* cell walls, β-1,6-glucan is particularly abundant, being present at almost double the amounts found in *Saccharomyces cerevisiae* (23). β-1,6-Glucan synthesis is not yet understood at the biochemical level, but based on genetic analyses of null strains of *S. cerevisiae*, including the *kre* mutants that are resistant to K1 killer toxin, many genes appear to be involved in the process (reviewed in reference 25). The proteins encoded by some *KRE* genes are located along the secretory pathway, suggesting that synthesis of β-1,6-glucan starts inside of the cell or requires the action of proteins located in the endoplasmic reticulum (ER) and Golgi apparatus. On the other hand, one study done with anti-β-1,6-glucan antibodies could not detect polysaccharide inside the cell (24). Recently, a screen for altered sensitivity to K1 killer toxin of *S. cerevisiae* mutants with individual deletions of 5,718 genes demonstrated that mutation in 268 genes led to a phenotype of resistance or hypersensitivity to the toxin compared with the wild type (WT) (26). Many of these genes affect specific areas of cellular activity, including secretory pathway trafficking, protein N glycosylation, lipid and sterol biosynthesis, and cell surface signal transduction, suggesting that biosynthesis of β-1,6-glucan depends, at least in part, on reactions occurring inside the cell (26).

One significant gene involved in β-1,6-glucan biosynthesis is *KRE5*, which is epistatic to all other *KRE* genes that have so far been isolated. *KRE5* is essential for *S. cerevisiae* viability in certain genetic backgrounds. Viable mutants have extremely reduced levels of β-1,6-glucan polymer, show aberrant morphology, are unable to retain cell wall mannoproteins, and

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have compromised growth. Kre5p is a luminal ER protein of 150 kDa that contains an HDEL ER retention signal in its COOH terminus that is required for its function (16, 21). It has been proposed that Kre5p may be a glucosyltransferase that is involved in the initiation of β-1,6-glucan synthesis (29). Kre5p has limited but significant similarity to UDP-glucose:glycoprotein glucosyltransferases (UGGTs). The homology is greater towards the COOH terminus, where the UDP-glucose binding site of the enzyme resides (8). The UGGT enzyme resides, as Kre5p, in the lumen of the ER, where it is central to the process of facilitation of glycoprotein folding and quality control. Using UDP-glucose as substrate, UGGT transiently reglucosylates N-linked oligosaccharides that are present in unfolded or misfolded proteins. This reglucosylation allows glycoprotein recognition by lectin chaperones, attainment of mature conformation, and exit from the ER to the final cellular destinations. This reaction exists in every eukaryotic cell studied so far, from trypanosomatids to humans and including the fission yeast *Schizosaccharomyces pombe*, with the sole exception of the budding yeast *S. cerevisiae* (27). UGGTs are 150-kDa proteins, as is Kre5p, and are organized in two domains: a highly conserved catalytic COOH terminus, comprising one-fourth of the protein, and a large amino terminus that maintains its size but shows less sequence conservation.

Here we describe the characterization of *C. albicans KRE5*, a functional homologue of *S. cerevisiae KRE5*. Deletion of both alleles of *C. albicans KRE5* gives rise to viable cells that have aberrant morphology and cell wall β-1,6-glucan reduced to one-fifth of WT levels. The remaining polymer still contains all characteristic structural features, including branch points and linear stretches, strongly suggesting that Kre5p is not a β-1,6-glucan synthase but rather that its role in polymer biosynthesis is indirect. Both β-1,3-glucans and β-1,6-glucans from *C. albicans* are more linear that the *S. cerevisiae* polymers. *C. albicans* Kre5p is involved in morphogenesis, cell wall construction, dimorphic transition, and adhesion to epithelial cells, and it is essential for the virulence of *C. albicans*. Thus, Kre5p is potentially a good target for the development of new antifungal drugs.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *C. albicans* strains used were SC5314 (prototrophic), its Ura$^-$ derivative, CAI4 (Δ*ura3*::*imm434*/Δ*ura3*::*imm434*) (10), and the strains generated by this work, all derived from strain CAI4: strains KAH1 (*ura3*/*ura3 KRE5*/*kre5*::*hisG-URA3-hisG*), KAH2 (*ura3*/*ura3 KRE5*/Δ*kre5*::*hisG*), KAH3 (*ura3*/*ura3 kre5*::*hisG-URA3-hisG*/Δ*kre5*::*hisG*), and KAH4 (*ura3*/*ura3* Δ*kre5*::*hisG*/Δ*kre5*::*hisG*). The *S. cerevisiae* strains used were WT HH2 (*MAT***a** ura3 lys2 ade2 his3 trp1 leu2[r]) and OCY6 (Δ*kre5*::*HIS3*), both on the YPH274 background (3). Strains were grown in yeast extract-peptone-dextrose (YEPD) or synthetic minimal dextrose (SD) medium (30), which for Ura$^-$ strains was supplemented with 50 μg of uridine/ml. Solid medium was obtained by adding agar (2%). Solid medium for inducing the yeast-hypha transition in *C. albicans* was Lee medium in which glucose was replaced by mannitol (1.25%), Spider medium (1% nutrient broth, 1% mannitol, 0.2% K_2HPO_4), or agar plus 10% bovine calf serum (BCS; GIBCO) medium. Cells were grown at 30°C in SD medium, and approximately 50 cells were spread on different agar medium plates. The dimorphic transition in liquid medium was induced by growing cells in YEPD at 30°C and changing them at a density of 2×10^7 cells/ml to 37°C in YEPD plus 10% BCS (GIBCO). Alternatively, cells were grown in SD medium at 30°C and then transferred to Lee medium, to Lee medium with 10% BCS (GIBCO), or to Lee medium with 1.25% *N*-acetylglucosamine (GlcNAc) instead of glucose and incubated at 37°C.

Disruption of *KRE5* alleles. Gene disruption was performed by the Ura-blaster protocol (10). *C. albicans* CAI4 genomic DNA was used as template for PCR using the oligonucleotides KSC (5′-TACCTAAGGTTAAGAGCTCATCACA ATG) and KBGL (5′-CATTTCAAAGATCTGTGTCGTAGTGTGA). Oligonucleotide KSC corresponds to nucleotides −853 to −825 relative to the ATG in the *KRE5* sequence and adds a SacI site to the PCR product, while oligonucleotide KBGL corresponds to nucleotides −25 to + 3 and adds a BglII site. The 856-bp PCR fragment was cut with SacI and BglII and ligated to the plasmid pMB7 (10), which had been previously digested with SacI and BglII. The resulting plasmid was designated pMB7-5′. *C. albicans* genomic DNA was used again as a template for PCR using the oligonucleotides KSAL (5′-GCCAAAGAAG TTGGTCGACAGATAGAAA) and KHIN (5′-TGTTAAGCTTTGTGAAAC TG). Oligonucleotide KSAL corresponds to nucleotides +4243 to +4251 relative to the start codon in the *KRE5* sequence and also adds a SalI site to the PCR product. Oligonucleotide KHIN corresponds to nucleotides +448 to +467 relative to the stop codon and contains the HindIII site present in the *KRE5* sequence. The 539-bp PCR fragment was cut with SalI and HindIII and ligated to the plasmid pMB7-5′ previously digested with SalI and HindIII. From the resulting plasmid, pMB7-5′+3′, a 5.3-kb SacI-HindIII fragment was isolated and used to transform strain CAI4. Correct integration of the cassette into the *KRE5* locus of the Ura$^+$ transformants was verified by PCR and Southern blot analysis. Spontaneous Ura$^-$ derivatives of one of the heterozygous disruptants were selected on medium containing 5-fluoroorotic acid (US Biological). These clones were screened by PCR and Southern blot hybridization to identify those which had lost the *URA3* gene via intrachromosomal recombination mediated by the *hisG* repeats. The procedure was then repeated to delete the remaining functional allele of *KRE5*. The *KRE5* gene was then reintroduced into KAH4 by transforming this strain with plasmid pLC14KRE5. This plasmid was constructed by inserting the 6.2-kb SacI-PstI fragment obtained from plasmid pCanKRE5 into the SacI-PstI site of *C. albicans* plasmid pLC14 (37).

Vacuolar staining with FM4-64. Yeast cells were grown in YEPD at 30°C to late exponential phase. Staining of yeast vacuoles with FM4-64 was carried out using the protocol described by Vida and Emr (35).

Spot assay for analyzing sensitivity to different substances on plates. Methods for testing the *C. albicans* strains were similar for all effectors. Cultures were grown in 100 ml of YEPD medium until the exponential phase and then diluted to an optical density at 600 nm (OD_{600}) of 0.1. Four microliters of undiluted cell culture and 1/5 serial dilutions of each cell culture were spotted onto YEPD plates containing the following: NaCl (0.5 to 1.5 M), Calcofluor White (10 to 40 μg/ml), caffeine (5 to 15 mM), sodium dodecyl sulfate (SDS; 0.005 to 0.07%), sodium orthovanadate (5 to 15 mM), hygromycin B (100 to 300 μg/ml), EGTA (2 to 5 mM), dithiothreitol (20 to 40 mM), and tunicamycin (5 to 15 μg/ml). Differences in growth were recorded after incubation of the plates at 30°C for 72 h.

Cell wall analysis. To determine cell wall composition and structure, *C. albicans* yeast cells (50-ml cultures in YEPD or Lee medium) were labeled with 50 μCi of [U-^{14}C]glucose (310 mCi/mmol; NEN) until they reached the exponential growing phase. Cells (100 to 300 mg) were washed with water and resuspended in 400 μl of 0.1 M phosphate-buffered saline (PBS) containing protease inhibitors (Complete; Roche). Glass beads were added, and cells were then broken in a bead beater three times for 2 min at 4°C. Glass-bead-free homogenate was recovered, diluted to 10 ml with PBS plus protease inhibitors, and centrifuged at 1,000 × *g* for 20 min. The cell wall pellet was washed twice with 10 ml of chilled PBS plus protease inhibitors and immediately boiled for 5 min in order to inactivate endogenous lytic enzymes. Cell wall polysaccharides were fractionated and quantified as previously described (20). Briefly, radiolabeled cell walls were resuspended in 1 ml of 0.1 M potassium phosphate buffer, pH 6.5, containing 8 μl of β-mercaptoethanol, 10 mM sodium azide, Quantazyme (500 U; Interspex Products, Inc.), and *Serratia* chitinase (0.14 U; Sigma C 1650) and were incubated for 48 h at 37°C (step 1). Separation of high-molecular-weight (high-MW) material from the low-MW digestion products was performed by dialysis in 3-ml dialysis cassettes (MW cutoff, 3,500). The high-MW fraction was recovered from the bags and digested with endo-β-1,6-glucanase (step 2). Separation of high-MW material from low-MW β-1,6-glucan digestion products was again performed by dialysis. The high-MW fraction recovered from the dialysis cassette was subjected to the third and last enzymatic step with the enzymes β-glucosidase and laminarinase (step 3). The high-MW material remaining after the third step represents the mannan fraction.

Adherence to epithelial cells. Human cervical epithelial (HeLa) cells were grown to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 100 μg of ciprofloxacin/ml at 37°C (5% CO_2). Monolayers were established in six-well culture dishes and used for the adhesion studies. Adhesion was determined according to Timpel et al. (34). Briefly, mono-

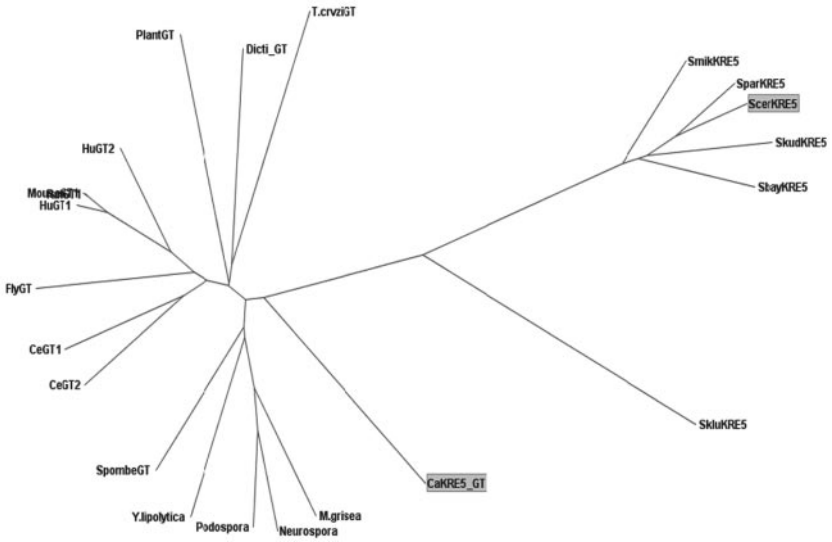

FIG. 1. Phylogenetic relationships of Kre5p, UGGT, and UGGT-similar proteins. The proteins shown are from species with the following short names, species names, and GenBank accession numbers (if applicable): PlantGT, *Arabidopsis thaliana*, AC016162; CeGT2, *Caenorhabditis elegans*, Z81516; CeGT1, *Caenorhabditis elegans*, U28735; CaKRE5_GT, *C. albicans*, AX046917; Dicti_GT, *Dictyostelium discoideum* AX4, AC116548; FlyGT, *Drosophila melanogaster*, U20554; HuGT1, *Homo sapiens*, AF227905; HuGT2, *H. sapiens*, AF227906; M.grisea, *Magnaporthe grisea* 70-15, XM_360967; MouseGT1, *Mus musculus*, BC062936; Neurospora, *Neurospora crassa* OR74A, XM_331124; Podospora, *Podospora anserine*, BX088700; RatGT1, *Rattus norvegicus*, AF200359; ScerKRE5, *S. cerevisiae* S288C, M33556; SpombeGT, *S. pombe* 972 h, U38417; T.crvziGT, *Trypanosoma cruzi* CL Brener, AJ555866; Y.lipolytica, *Yarrowia lipolytica*, AJ547797; SmikKRE5, *Saccharomyces mikatae*; SparKRE5, *Saccharomyces paradoxus*; SbayKRE5, *Saccharomyces bayanus*; SkluKRE5, *Saccharomyces kluyveri*; SkudKRE5, *Saccharomyces kudriavzevii*; and Scast KRE5, *Saccharomyces castellii*. Sequences of *KRE5* are from http://db.yeastgenome.org/cgi-bin/FUNGI/nph-showAlign?locus = YOR336W.

layers were sequentially washed twice with 2 ml of Dulbecco's phosphate-buffered saline (DPBS), overlaid with either 100 or 200 individual *C. albicans* cells in 1 ml of DPBS, and incubated at 37°C for 45 min in an atmosphere of air containing 5% CO_2. Following the incubation, monolayers were washed twice with 2 ml of warm DPBS to remove nonadhering cells, and the monolayer in each well was then covered with 2 ml of warm YEPD-agarose (1% agarose). Yeast colonies appearing after 48 h of growth at 28°C were counted (each colony was assumed to be derived from a single cell). The total inoculum of fungal cells (100%) applied to the monolayers was determined by covering the yeast cells, placed in empty culture dishes, with the warmed YEPD-agarose. Adherence was determined as the percentage of fungal cells attached to monolayers of HeLa cells.

Virulence studies. Pathogen-free 6-week-old BALB/c mice were purchased from Jackson Labs (Bar Harbor, Maine). Mice were cared for and housed under specific-pathogen-free conditions at Boston University Medical Center's Laboratory Animal Science Center. For inoculation, fresh cultures of *C. albicans* were washed, resuspended in ice-cold PBS, briefly sonicated, and counted with a hemocytometer. Cell concentration was adjusted with PBS to 5×10^6 yeast cells per ml. Microscopic examination showed the cell suspension to be predominantly composed of single cells, with minimal clumping. Yeast suspensions were placed on ice until ready for injection. Assessment of the number of CFU pre- and postexperiment indicated that the yeast remained viable and did not significantly divide for the duration of the experiment (2 to 3 h; data not shown). Immediately prior to injection, the yeast suspensions were vortexed for 10 to 15 seconds and loaded into 1-ml syringes fitted with a 30G needle. Mice were warmed under an infrared heating lamp and placed into a Plexiglas restrainer. One hundred microliters of suspension was introduced into the tail vein, delivering a total of 5×10^5 yeast cells. Mice were returned to their cages and monitored daily for signs of disease.

Statistical analysis. Kaplan-Meier survival curves were compared using the log rank test (NCSS Statistical Software, Kaysville, Utah). A P value of <0.05 with the Mann-Whitney test was used as a measure of statistical significance.

RESULTS

***C. albicans* contains a single gene similar to both *S. cerevisiae KRE5* and genes encoding UGGT.** Close homologues of *KRE5* exist in all hemiascomycetes recently sequenced by the Genolevures project (http://cbi.labri.fr/Genolevures/). The carboxy-terminal one-fourth of Kre5p shows homology to family 8 glycosyltransferases of Henrissat's classification (http://afmb.cnrs-mrs.fr/CAZY/GT_8.html) and to UGGT family 24 glycosyltransferases.

The *C. albicans* predicted protein shows greater sequence similarity to UGGTs than to Kre5p, as shown in Fig. 1. Nevertheless, as we demonstrate below, *C. albicans* Kre5p is a functional homologue of *S. cerevisiae* Kre5p. To gain insight into the physiological role of this unusual Kre5p, we constructed *C. albicans KRE5* gene null mutants.

Disruption of *KRE5* alleles. The Ura-blaster technique was used to sequentially disrupt both copies of the *C. albicans KRE5* gene in the *C. albicans* strain CAI4 (10). Chromosomal DNA isolated from wild-type and transformant strains was double digested with BclI and HindIII (Fig. 2A) and analyzed by Southern hybridization (Fig. 2B). An 856-bp SacI-BglII DNA fragment derived from plasmid pMB7-5′ was used as a specific probe (see Materials and Methods). The wild-type *KRE5* allele showed a 1.3-kb band (Fig. 2B, lane 1). A new 2.4-kb band which represented the Ura blaster integrated into one *KRE5* allele appeared in the transformants (Fig. 2B, lane 2). After selection on 5-fluoroorotic acid-containing medium, the loss of the *URA3* gene and one copy of the *hisG* element resulted in a 2.7-kb band (Fig. 2B, lane 3). The remaining intact *KRE5* allele was disrupted similarly, leading to homozygous *kre5*::*hisG*/Δ*kre5*::*hisG-URA3-hisG* strains (Fig. 2B, lane 4) and corresponding Ura$^-$ derivatives (Fig. 2B, lane 5). Phenotypes reported in this paper were observed in at least two independently isolated disrupted or reconstituted strains.

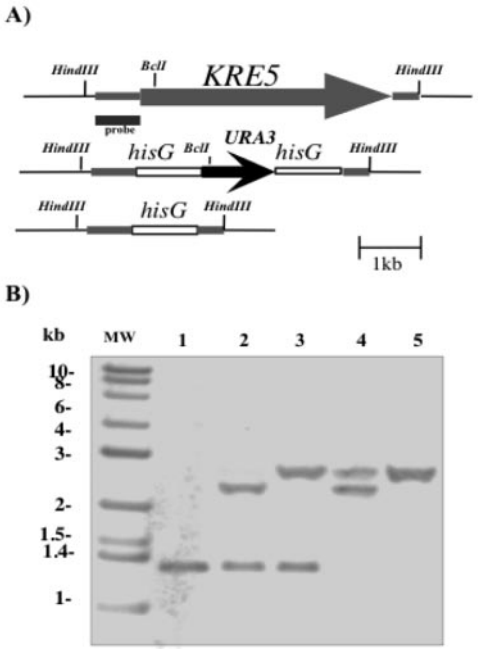

FIG. 2. Deletion of *C. albicans KRE5* alleles. (A) Structures of different alleles. The WT *KRE5* gene and the alleles disrupted by the *hisG-URA3-hisG* cassette or by *hisG* alone are shown. (B) Southern blot analysis of genomic DNA double digested with BclI and HindIII was performed with the following strains: CAI4 (*ura3*/*ura3*), lane 1; KAH1 (*ura3*/*ura3 KRE5*/*kre5*::*hisG-URA3-hisG*), lane 2; KAH2 (*ura3*/*ura3 KRE5*/*kre5*::*hisG*), lane 3; KAH3 (*ura3*/*ura3 kre5*::*hisG-URA3-hisG*/*kre5*::*hisG*), lane 4; KAH4 (*ura3*/*ura3 kre5*::*hisG*/*kre5*::*hisG*), lane 5.

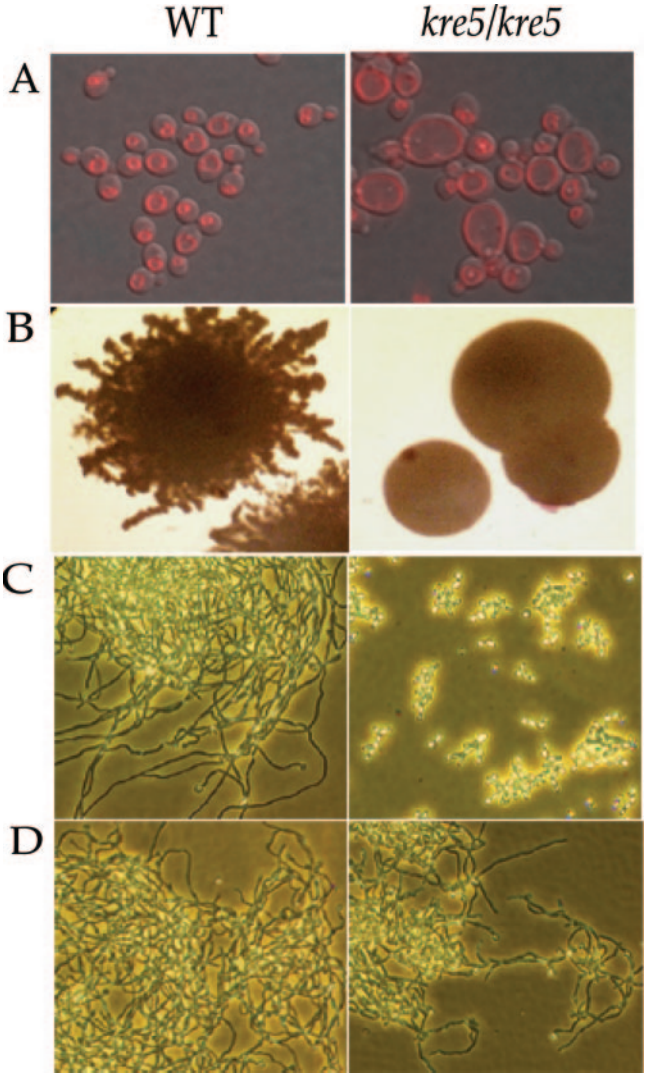

FIG. 3. Morphology of *C. albicans* strains. (A) Vacuolar staining with FM4-64. Pictures correspond to an overlaid image of cells viewed under fluorescent microscope and differential interference contrast optics. (B) Colonies grown for 7 days at 37°C in solid Lee-mannitol medium. (C) Hypha induction in liquid medium. Cells were grown for 18 h at 37°C in YEPD–10% FBS. (D) Hypha induction in liquid medium. Cells were grown for 18 h at 37°C in Lee medium plus *N*-actylglucosamine. The magnification was the same for all panels.

***C. albicans* Kre5p is required for normal cell growth and morphology.** Our ability to generate viable *kre5*/*kre5* double mutant strains indicates that *KRE5* is not an essential gene in *C. albicans*. *S. cerevisiae KRE5* deletion strains showed either extremely compromised growth or lethality, depending on the strain background (21). Growth curves of the WT, hemizygous, and reconstituted *Candida* strains showed no difference in doubling time in either YEPD or SD medium. *C. albicans kre5* homozygous strains grew at about half the rate of the WT during the logarithmic phase but continued to grow so as to reach at saturation 80% of the wild-type OD (data not shown). This growth defect could not be suppressed by providing osmotic support to the medium. The growth phenotype observed in the *C. albicans kre5*/*kre5* strains is not as severe as the defect displayed by *S. cerevisiae kre5* mutants that grow at a rate about one-fourth that of the WT (3). Since homozygous disruption of *KRE9*, a gene involved in β-1,6-glucan synthesis, in *C. albicans* was lethal when the organism was grown on glucose (19), we decided to analyze the effect of the carbon source on the growth of *C. albicans kre5* strains. We found that glucose is the preferred carbon source for both wild-type and *kre5* null mutant strains, followed by mannose, maltose, galactose, and *N*-acetylglucosamine. *C. albicans kre5* homozygous mutants grew at half the wild-type rate in all media tested, with the surprising exception of those media in which *N*-acetylglucosamine was used as a carbon source. Unexpectedly, when *N*-acetylglucosamine was offered, *C. albicans kre5*/*kre5* mutant strains grew at nearly the same rate as the WT strains (data not shown). The exogenous *N*-acetylglucosamine might suppress the growth defect by strengthening the cell wall, elevating the UDP-*N*-acetylglucosamine pool, and favoring deposition of chitin stress fibers on the lateral wall.

The disruption of the *C. albicans KRE5* gene produced cells with aberrant morphology; when grown vegetatively in liquid media, null mutant cells were larger than cells from the WT strain, tended to aggregate, and possessed enlarged vacuoles (Fig. 3A). Growth with *N*-acetylglucosamine as carbon source partially suppressed these morphological aberrations. The aggregates were easily disrupted by mild sonication, indicating that cells were not physically attached. These morphological phenotypes resembled those previously described for *S. cerevisiae* Δ*kre5* cells but not those described for *S. pombe* strains

with mutations of the homologous gene, *GPT1*. *S. pombe* cells lacking UGGT encoded by *GPT1* are viable and have no differences in growth rates or cell morphology compared with WT strains (9).

***C. albicans* Kre5p is required for hyphal morphogenesis.** *C. albicans* cells undergo morphological conversion from yeast to hyphae in both solid and liquid media when stimulated with 37°C temperature, serum, and *N*-acetylglucosamine. In order to determine whether the absence of *C. albicans* Kre5p affected hyphal morphogenesis in *C. albicans*, we examined the ability of WT, hemizygous, and homozygous *kre5* strains to undergo hyphal transition under several conditions on solid and liquid media. Cells were grown at 30°C in SD medium, and approximately 50 cells were spread on different agar medium plates containing Spider medium, Lee plus mannitol, and agar plus 10% FBS medium. We found that the homozygous mutant lacked the ability to form lateral hyphae at 37°C on all three solid media, even in the presence of serum, a strong inducer of the dimorphic transition. The appearance of colonies after 6 days of growth at 37°C in Lee plus mannitol medium can be seen in Fig. 3B. The hemizygous and reconstituted strains, KAH2 and KAH4-KRE5, behaved similarly to the WT strain (data not shown).

Hypha development in liquid media was induced at 37°C in YEPD plus 10% FBS or in Lee medium with glucose or *N*-acetylglucosamine as carbon sources (see Materials and Methods). We found that whereas WT, hemizygous, and reconstituted strains formed long hyphae, the *C. albicans kre5*/*kre5* mutants had a complete block of hypha formation in both YEPD serum (Fig. 3C) and Lee medium (data not shown). In both media, only some aberrant morphologies with short, thick, stubby protrusions resembling pseudohyphae were seen. Surprisingly, the mutant still formed normal hyphae in the presence of *N*-acetylglucosamine (Fig. 3D), indicating that at least one pathway of the several involved in the yeast-to-hyphae transition is still functional in the *kre5* null mutant.

***C. albicans* Kre5p is involved in cell wall morphogenesis.** Possible defects in the cell wall of *C. albicans kre5* mutants were first investigated by experiments including sensitivity to different effectors. Among them, the *C. albicans kre5*/*kre5* mutant appeared to be hypersensitive to caffeine, Calcofluor White, and SDS (Fig. 4), moderately sensitive to EGTA and tunicamycin (data not shown), and slightly more resistant to hygromycin B than the WT strain. No differences in sensitivity were found towards dithiothreitol (Fig. 4) or NaCl (data not shown). The hemizygous *KRE5*/*kre5* strain showed an intermediate phenotype for Calcofluor White and SDS, suggesting that proper levels of Kre5p are required to maintain normal cell physiology. Surprisingly, the hemizygous strain was found to be more resistant to hygromycin B than the homozygous strain or the WT strain. Hygromycyn B is an aminoglycoside antibiotic to which glycosylation and cell wall mutants tend to be hypersensitive. *S. cerevisiae kre5* mutants have also been reported to be more sensitive to Calcofluor White and SDS, but, in contrast to *C. albicans*, they are more sensitive to hygromycin B (2).

Cell wall composition analyses indicated that *C. albicans kre5*/*kre5* mutants maintained about 20% of WT levels of β-1,6 polymer (Fig. 5). When we analyzed the endoglucanase digestion pattern of this remaining β-1,6-glucan for the *kre5*/*kre5*

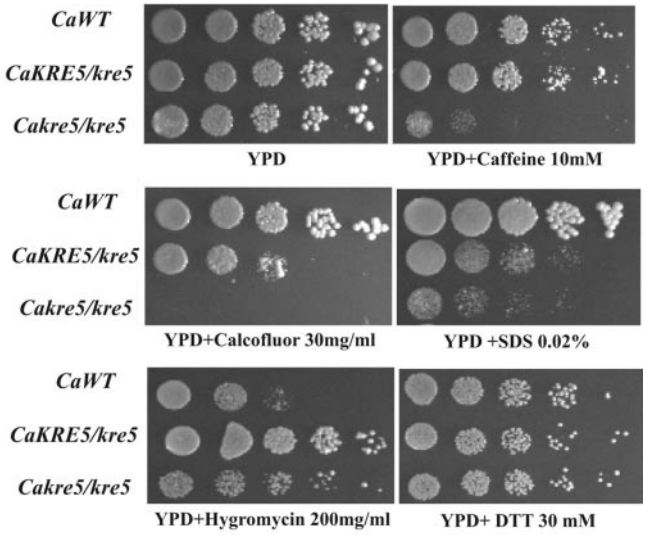

FIG. 4. Sensitivity of *C. albicans kre* mutants to different effectors. Four-microliter suspensions at an OD of 0.1 and 1/5 serial dilutions of *C. albicans* strains were spotted on YEPD plates containing the indicated drug. Growth differences were monitored after 3 days at 30°C.

mutant strain, we found it to be identical to that observed for the *C. albicans* WT strain (data not shown). *S. cerevisiae* Δ*kre5* mutants contain very little β-1,6-glucan detectable by the serial enzymatic digestion method that we employ (Table 1), which was not enough for structural analysis. Differing from *S. cerevisiae* WT strains, where β-1,6-glucan constitutes about 12% of the wall polysaccharides, *C. albicans* WT strains contain more than 20% β-1,6-glucan in their walls (Table 2). Similarities with *S. cerevisiae* Δ*kre5* mutants were found regarding all other wall polymers (Table 1), as *C. albicans kre5*/*kre5* mutants showed an increased level of chitin and β-1,3-glucan and a reduced amount of mannoproteins compared to WT strains (Fig. 5). The cell wall composition of the hemizygous *KRE5*/*kre5* strain was very similar to that of the WT strain; the only difference was the level of β-1,6-glucan, which was slightly but significantly reduced (Fig. 5). The gene dosage effect of *KRE5* on the levels of cell wall β-1,6-glucan suggests a primary role of this gene in β-1,6-glucan synthesis, assembly, or delivery.

In order to compare the cell wall compositions of WT yeast

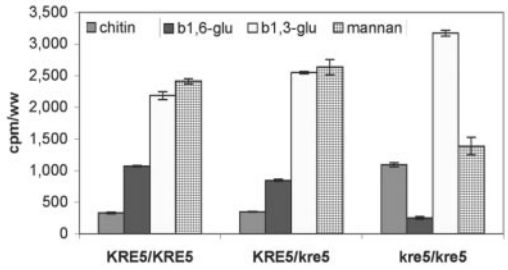

FIG. 5. Cell wall composition of *C. albicans* strains. Cell walls were prepared from cells grown in YEPD medium containing [U-^{14}C]glucose and fractionated by serial enzymatic digestions as described in Materials and Methods. cpm, counts per minute; ww, wet weight. Results are the averages ± standard deviations from four independent determinations.

TABLE 1. Cell wall composition of *S. cerevisiae kre5* mutant complemented with *C. albicans KRE5*[a]

Strain	Chitin	β-1,3-Glucan	β-1,6-Glucan	Mannan
WT	5.0 ± 0.6	37.0 ± 3.6	12.0 ± 1.6	46.0 ± 3.1
Δ*kre5*	18.6 ± 1.4	54.5 ± 2.4	1.4 ± 0.6	25.4 ± 2.1
Δ*kre5* + *C. albicans* Δ*KRE5*	8.5 ± 0.1	43.5 ± 0.3	7.0 ± 0.9	41.0 ± 0.5

[a] Wild-type (HH2) cells, Δ*kre5* (OCY6) cells, and Δ*kre5* cells complemented with *C. albicans KRE5* were labeled on SD medium containing [U-^{14}C] glucose and then collected after 20 h by centrifugation. Cell walls were obtained and fractionated by serial enzymatic digestions as described in Materials and Methods. The results are presented as the average percent of the total cell wall ± the standard deviation from triplicate experiments.

and hyphal cells and to distinguish the effects of the dimorphic stage from the change in temperature needed to induce it, we used Lee medium at different pHs and temperatures. Yeast cells were obtained at 25°C, pH 6.7, as well as at 37°C, pH 4.5. Hyphae were obtained at 37°C, pH 6.7. We found that hyphal cells contained twice as much chitin as the yeast cells (Table 2), in agreement with previous reports (4, 7, 32). Levels of β-1,6-glucan were also found to be increased in hyphal cells compared to yeast cells grown at 25°C but not compared to yeast cells grown at 37°C (Table 2). Mannoproteins appeared less abundant in both hyphae and yeast grown at 37°C than in yeast grown at 25°C (Table 2). These results suggest that the differences in the levels of β-1,6-glucan and mannoproteins are not due to the morphogenetic change but rather to the change in the growth temperature. The structure of the glucans in the hyphae, as revealed by the endoglucanase digestion patterns, was indistinguishable from that in yeast cells (data not shown). The *C. albicans* yeast cell wall composition obtained with the enzymatic method we used showed good correlation with published data using traditional methods (32). To our knowledge, this is the first report of a complete wall composition of *C. albicans* hyphae.

***C. albicans* glucans are more linear than *S. cerevisiae* polymers.** As minor changes in the architecture of the yeast cell wall can be associated with dramatic differences in the host immune response (36), we decided to look at the fine structure of *C. albicans* glucans by analysis of the chromatographic profiles of products released by endoglucanase treatment of cell walls (20).

The β-1,3-glucans from *C. albicans* WT and *kre5*/*kre5* strains appear to have significantly more linear stretches than those of *S. cerevisiae*, as indicated by the abundance of 5-glucose laminaripentose oligosaccharides (L5) (Fig. 6A). This conclusion is based on the cleavage specificity of the recombinant β-1,3-endoglucanase Quantazyme that produces L5 from a high degree of polymerization-linear β-1,3-linked glucan. The four-glucose oligosaccharides, L4, indicate branching or substitutions in the chains. Contrary to *C. albicans*, where 95% of the glucose label was in L5 and just 3% of the label was in L4, *S. cerevisiae* had 66% as L5 and 26% as L4, indicating a more branched and/or substituted polymer in this yeast (Fig. 6A). The only difference between the patterns of WT and *C. albicans kre5*/*kre5* strains is that the latter shows an increased amount of GlcNAc (Fig. 6A) originating from the degradation by chitinase–*N*-acetylglucosaminidase of the elevated chitin content in its wall (Fig. 5).

The β-1,6-glucan from *C. albicans* appears less branched than that from *S. cerevisiae*, as it has less than half (6.7 versus 15.5%) the relative amount of the branch point tetrasaccharide G4 in its chromatographic pattern (Fig. 6B). The major product that originated from the endo-β-1,6-glucanase action against the β-1,6 linear backbone, G2, is elevated in *C. albicans*, at 63.3%, versus 56.2% in *S. cerevisiae* (Fig. 6B). The G3 peak contains two species clearly distinguishable by thin-layer chromatography: G3a is Glc-β-1,3-Glc-β-1,6-Glc originating from single intrachain β-1,3 linkages present sporadically in the β-1,6 linear backbone, and G3b is a β-1,6 linear trisaccharide from the ends of the chains. In *C. albicans*, G3a represents 31.5% and G3b represents 68.5% of the peak, whereas in *S. cerevisiae* the values are 80 and 20%, respectively (Fig. 6C). This result was extremely reproducible among different experiments. The data taken together revealed that the β-1,6-glucan polymer is more linear and contains fewer intrachain β-1,3 linkages in *C. albicans* than in *S. cerevisiae*.

***C. albicans KRE5* encodes a functional homologue of *S. cerevisiae* Kre5p.** In order to determine whether *C. albicans* Kre5p was a functional homologue of *S. cerevisiae* Kre5p, we transformed *S. cerevisiae* Δ*kre5* cells with plasmid pCanKRE5 containing the *C. albicans KRE5* gene under its own promoter. We found that *C. albicans KRE5* complements the growth of *S. cerevisiae kre5* mutants in the YPH274 background to about 80% of the WT rate (data not shown). Then we analyzed the cell wall composition and found that *C. albicans KRE5* increased the β-1,6-glucan content of the *S. cerevisiae* Δ*kre5* cells to more than 50% of the WT levels (Table 1). Moreover, the P4 chromatography profiles of the β-1,6-glucan fraction of *S. cerevisiae* WT and Δ*kre5* cells complemented with the *Candida* gene were identical to each other (data not shown) and identical to the β-1,6-glucan pattern previously shown for *S. cerevisiae* (20). These results indicated that the *C. albicans KRE5* gene, when expressed in *S. cerevisiae*, created a bona fide β-1,6-glucan. Thus, *C. albicans* Kre5p is a functional homologue of *S. cerevisiae* Kre5p. Previously, it was shown that the *C. albicans KRE5* gene rescued the lethality of *S. cerevisiae kre5* mutants in the SEY6210 background (16).

TABLE 2. Cell wall composition of *C. albicans* yeast and hyphae forms[a]

Medium	Temp (°C)	Morphology	Chitin	β-1,6-Glucan	β-1,3-Glucan	Mannan
Lee, pH 6.7	25	Yeast	5.0 ± 0.4	20.9 ± 2.0	31.5 ± 2.5	42.5 ± 0.8
Lee, pH 4.5	37	Yeast	6.0 ± 0.6	33.2 ± 0.4	28.5 ± 3.0	30.2 ± 0.7
Lee, pH 6.7	37	Hyphae	11.2 ± 0.9	26.6 ± 0.6	35.0 ± 2.0	27.2 ± 0.4

[a] Wild-type CAI4 cells were labeled on the indicated medium containing [U-^{14}C] glucose and were collected after 18 h by centrifugation. Cell walls were obtained and fractionated as described in Materials and Methods. Results are presented as the average percentage of total cell wall ± the SD from triplicate experiments.

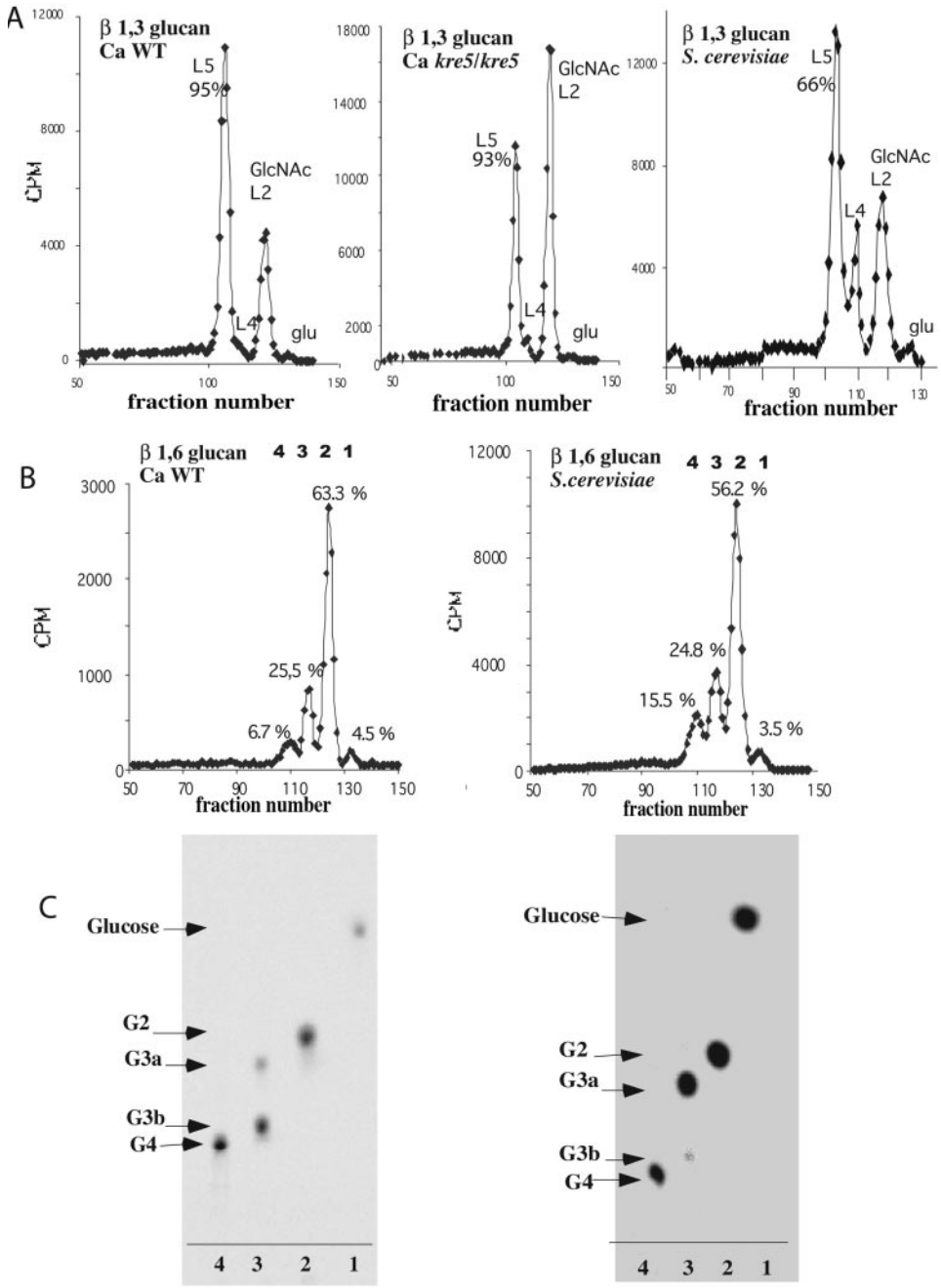

FIG. 6. Fine structure of *C. albicans* glucans. (A) Bio Gel P4 chromatography (column, 1.5 by 120 cm) of β-1,3-endoglucanase digestion products from step 1 of the serial enzymatic wall fractionation. Fractions of 1.3 ml were collected. Standards were laminaribiose (L2), laminariterose (L4), laminaripentose (L5), and GlcNAc. (B) Bio Gel P4 chromatography (column, 1.5 by 120 cm) of β-1,6-endoglucanase digestion products from step 2 of the serial enzymatic wall fractionation. Fractions of 1.3 ml were collected. (C) Thin-layer chromatography in silica gel plates, *n*-butanol–ethanol–H_2O (5/3/2) solvent system of peaks 1 to 4 from panel B, followed by autoradiography. Standards were gentiobiose (G2), glucose-β-1,3-glucose-β-1,6-glucose (G3a), gentiotriose (G3b), and branch point tetrasaccharide (G4).

Absence of *C. albicans* Kre5p reduces adherence to human epithelial cells. Several studies have shown that mannoproteins are necessary for adhesion of *C. albicans* to the surfaces of host cells (33). Because *KRE5* mutants showed reduced levels of mannoproteins, we decided to measure their adherence to monolayers of human HeLa cells. Yeast cells were placed on an epithelial monolayer for 45 min, followed by removal of nonadhering cells by washing. The number of adhering cells was determined by growth in a YEPD agar overlay after washing. Adherence was determined as the percentage of fungal cells attached to monolayers of HeLa cells. The adherence of WT *Candida* cells was 49% ± 4%, while for *kre5*/*kre5* mutant

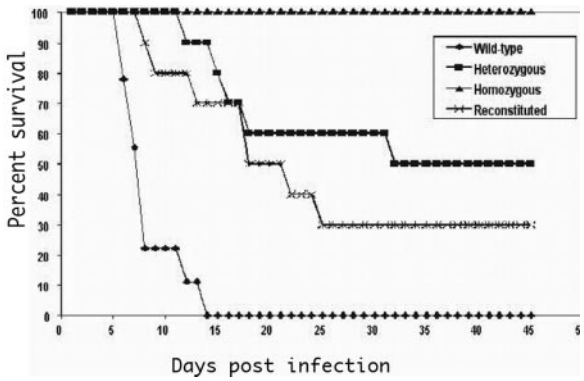

FIG. 7. Virulence of *C. albicans* strains. The survival of mice (n = 10/group) infected with 5×10^5 cells was determined. The following strains were used: SC5314 (WT), KAH1 (*ura3*/*ura3 KRE5*/*kre5*::*hisG-URA3-hisG*; heterozygous), KAH3 (*ura3*/*ura3 kre5*::*hisG-URA3-hisG*/*kre5*::*hisG*; homozygous), and the reconstituted strain KAH4+ pLC14KRE5.

cells adherence decreased to 20% ± 5%, with a P value of <0.001. Experiments were done in triplicate, with two starting amounts of fungal cells.

***C. albicans KRE5* homozygous mutant strains are avirulent in a mouse model of systemic infection.** To test whether *C. albicans* Kre5p influences virulence in systemic animal models, 5×10^5 cells of the prototrophic WT, heterozygous, and homozygous disruptant strains, SC5314, KAH1, and KAH3, respectively, were injected intravenously into the lateral tail veins of BALB/c mice. Seven and 14 days following injection of the WT strain, 50 and 100%, respectively, of the mice had died. In contrast, following infection with the heterozygous KAH1 strain, the survival rate was 50% at day 45, when the animals were euthanized (Fig. 7). The homozygous *kre5*/*kre5* mutant was completely avirulent under these conditions, but reintroduction of *KRE5* into this mutant restored virulence to a level in between the WT and the heterozygous mutant (Fig. 7). A second experiment that did not include the reconstituted strain yielded similar results (data not shown).

DISCUSSION

Our study has shown that in spite of strong similarities to genes encoding UGGTs from different organisms, *C. albicans KRE5* is a functional homologue of *S. cerevisiae KRE5* and that it is indirectly involved in the synthesis of cell wall β-1,6-glucan. Kre5p is not a glucan synthase, because the reduced amount of β-1,6-glucan made by *C. albicans kre5*/*kre5* mutants contains every structural feature present in the WT polymer: branch points, intrachain single β-1,3-linked glucose units, and linear stretches. Studying *C. albicans* was instrumental in reaching the above conclusion, because this species has more abundant β-1,6-glucan than *S. cerevisiae*. Structural studies of the β-1,6-glucan remaining in *S. cerevisiae kre5* mutants were not feasible due to the limited amount of material obtainable. On the other hand, Kre5p has a critical cellular role because its deletion either in *C. albicans* or in *S. cerevisiae* has more severe consequences than UGGT (*gpt1*) elimination in *S. pombe*. Loss of *C. albicans KRE5* function leads to growth, morphology, and wall-associated defects resembling but less severe than those described for *S. cerevisiae kre5* mutants (21). *S. pombe gpt1* cells grow normally even though they have induced the unfolded protein response pathway (9). It is only when additional stress, such as underglycosylation, is added by an *alg6* mutation that UGGT becomes essential for growth at high temperature, as the *gpt1 alg6* double mutant is not viable at 37°C (8).

The involvement of the initial steps of N glycosylation in the biosynthesis of cell wall β-1,6-glucan is well documented (29). Recently, mutations in the oligosaccharyltransferase subunit *stt3* were shown to be synthetically lethal with *KRE5*. Several *stt3* mutants exhibited a 60 to 70% reduction in the content of cell wall β-1,6-glucan compared to WT cells (5). It is tempting to speculate that Kre5p might have some kind of substrate-specific, UGGT-like chaperone function in the maturation of a limited set of glycoprotein substrates in the ER lumen. These substrates may be required for β-1,6-glucan synthesis, assembly, or delivery. The enlarged vacuoles that we observed on *C. albicans kre5* mutants might be a consequence of the accumulation of polypeptides that failed to acquire a mature conformation and are degraded in this organelle. The rate-limiting step for the delivery of the majority of glycoproteins to organelles or the cell surface is their export from the ER. Addition of N-glycans in the ER plays a pivotal role in protein folding and oligomerization (27). Certain glycoproteins require chaperone assistance for folding only under stress conditions. Other glycoproteins somehow implicated in β-1,6-glucan production might require folding assistance constitutively. *S. cerevisiae* Kre5p is likely to be a diverged relative of UGGT (1) and thus probably a glycoprotein-specific glycosyltransferase that creates a different product rather than a β-1,6-glucan synthase. We are currently investigating whether *C. albicans* Kre5p has UGGT activity in vivo or in vitro.

In agreement with previous reports for *C. albicans* WT cells, we have observed that β-1,6-glucan is more abundant in *C. albicans* than in *S. cerevisiae* (23). Because it is generally accepted that the innate immune system identifies pathogens based on molecular patterns formed by carbohydrates, lipids, and proteins expressed on their surfaces (22), we have also investigated the architecture of the β-glucans in the cell wall of *C. albicans*. Both β-1,3- and β-1,6-glucans were found to be significantly more linear in *C. albicans* than in *S. cerevisiae*. On the other hand, these architectural features of the β-glucans were not altered in *C. albicans kre5* mutants. *C. albicans* hyphae and yeast cells had similar amounts of mannoproteins and β-glucans.

There is rapid reshaping and expansion of the cell wall during hypha formation. For this reason, we examined whether *C. albicans kre5* mutants could undergo yeast-hypha transitions. The homozygous mutant, which has a complete loss of *C. albicans KRE5* function, failed to form hyphae in solid and liquid media, even in the presence of the strong inducer serum. Surprisingly, the *C. albicans kre5*/*kre5* mutants could form hyphae in liquid media when GlcNAc was the carbon source. Unlike *S. cerevisiae*, *C. albicans* can utilize GlcNAc as a carbon source for growth (14). The cluster of catabolic genes induced by GlcNAc encodes a GlcNAc permease, a GlcNAc kinase, a GlcNAc-6-phosphate deacetylase, and GlcNAc-6-phosphate deaminase, which act sequentially on GlcNAc to generate fructose-6-phosphate which can then enter the glycolytic pathway. Besides inducing the enzymes of its catabolic pathway, GlcNAc

induces changes in cellular morphology and formation of germ tubes from the yeast-phase cells (14). Probably cytosolic, nonglycosylated proteins are the effectors of these changes and do not require Kre5p for proper function. The hemizygous and reconstituted strains behaved as the wild type, indicating that the reduced amount of Kre5p present in those strains is enough to sustain the dimorphic transition.

The yeast-to-hypha transition in *C. albicans* occurs in response to a variety of stimuli and growth conditions, such as temperature, presence of serum, and presence of GlcNAc. Several signal transduction pathways that promote the morphogenetic switch have been identified (17). The pathways include a *CPH1*-mediated mitogen-activated protein kinase pathway, an *EFG1*-mediated cyclic AMP/PKA pathway, and a *CPH2* pathway. Genes turned on during filamentous growth do not respond to a central regulator. Rather, they respond individually to various pathways, suggesting a network of signaling pathways extending down to target genes. This means that different hyphal signaling pathways can respond to each specific medium or growth condition and then converge to regulate a common set of differentially expressed genes (15). Serum still stimulates hypha formation in mutants defective in elements of a conserved mitogen-activated protein kinase signaling pathway as well as in *C. albicans* Pmt1 O glycosylation mutants, all of which manifest a partial block in filamentation (34). The atypical hyphal induction pattern manifested by *C. albicans kre5*/*kre5* mutants should aid in deciphering the cues that trigger morphogenesis in *C. albicans*.

Deletion of *C. albicans KRE5* leads to the avirulence of the organism in a mouse model of systemic infection. The mutant has defects in two major virulence factors, in that it has an inability to form hyphae in the presence of serum and reduced adhesion to human epithelial cells. The ability of *C. albicans* to switch from yeast to hyphal forms has been shown to be required for the pathogenicity of the fungus (18). The agglutinin-like sequence (ALS) gene family encodes cell surface glycoproteins that are implicated in the adhesion of *C. albicans* to host tissues that are linked to cell wall β-1,6-glucan (12). In *S. cerevisiae*, the glycosylphosphatidylinositol-cell wall proteins are attached via β-1,6-glucan to the β-1,3-glucan chains. It has been described that *S. cerevisiae kre* mutants, which have reduced levels of β-1,6-glucan, secrete large amounts of cell wall proteins into the medium (11). The hemizygous *C. albicans* Δ*kre6* mutant has reduced levels of β-1,6-glucan and secretes into the medium large amounts of Als1p, a glycosylphosphatidylinositol-linked cell wall protein involved in cell adhesion (12). Hwp1p, another β-1,6-glucan-anchored cell wall protein of candidal germ tubes and hyphae, was demonstrated to mediate covalent attachment between *C. albicans* and host epithelial tissue by serving as a substrate for human transglutaminase activity (31).

Interestingly the *C. albicans KRE5*/*kre5* hemizygous strain manifested attenuated virulence, with 50% of the infected mice alive at the end of the experiment, in spite of having a WT capability to execute the dimorphic transition from yeast to hyphae in vitro. The hemizygous strain also has WT levels of bulk mannan and a small but significant reduction in β-1,6-glucan in its cell wall. Study of the subtle changes on the yeast surface could help in the identification of specific adhesins and/or other structural elements which mediate yeast-immune interactions.

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