Published in final edited form as:

Fungal Genet Biol. 2009 October ; 46(10): 731–741. doi:10.1016/j.fgb.2009.06.008.

The *Candida albicans* histidine kinase Chk1p: signaling and cell wall mannan

Dongmei Li¹, David Williams², Douglas Lowman^{2,3}, Mario A. Monteiro⁴, Xuan Tan⁴, Michael Kruppa¹, William Fonzi¹, Elvira Roman⁵, Jesus Pla⁵, and Richard Calderone^{1,*}

¹Georgetown University Medical Center, Department of Microbiology & Immunology, East Tennessee State University, Johnson City, TN, 37614

²Department of Surgery, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, 37614

³Global Analytical Services, Eastman Chemical Company, Kingsport, TN. 37662-5150

⁴Department of Chemistry, University of Guelph, Guelph, ON, N1G 2W1, Canada

⁵Universidad de Complutense Madrid

Abstract

Several published functions associated with the *CHK1* histidine kinase of *Candida albicans* resemble those of the MAPK Cek1p and its cognate receptor Sho1p (*SSU81*). To explore this further, we have compared mutants lacking the proteins mentioned above and have constructed a double *sho1/chk1Δ* null mutant to determine relationships among these proteins. We observed that the sensitivity to Congo red (CR), calcofluor white (CW), as well as clumping of cells, was slightly increased in the double mutant compared to the single *chk1Δ* or *sho1Δ* mutants. However, Cek1p phosphorylation via Sho1p, which occurs during log phase growth in the presence or absence of CR in Wt cells, does not require Chk1p. These data suggest that Chk1p and Sho1p are components of parallel but independent signal pathways. In addition, bulk mannan of strains was analyzed by GPC/MS and NMR. Compared to Wt and a *CHK1* gene-reconstituted strain (CHK23) that contained, high, intermediate and low Mw mannan species, we found that the mannan of strains CHK21 (*chk1Δ* null), the *cek1Δ* null, and the double mutant consisted only of low Mw mannan. The *sho1Δ* null mutant only demonstrated a reduced intermediate type of mannan. Alcian blue binding was lower in *cek1Δ*, *chk1Δ*, and the double *sho1/chk1Δ* null mutant lacking high and intermediate Mw mannan than in the *sho1Δ* null which had a partial loss of intermediate Mw mannan only. We conclude that the Chk1p HK is part of a functionally similar but parallel pathway to the Sho1p-Cek1p pathway that confers resistance to the cell wall inhibitors CR and CW. However, a functional relationship in mannan biosynthesis of Chk1p and Cek1p exists that only partially requires Sho1p.

Keywords

mannan; histidine kinase; signal transduction; *Candida albicans*

© 2009 Elsevier Inc. All rights reserved

*Corresponding author Richard Calderone Georgetown University Medical Center Department of Microbiology & Immunology 302 NW Med Dent Building 3900 Reservoir Rd, NW Washington, DC 20057 Tel # 202 687 1513 calderor@georgetown.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Signal transduction via MAPK pathways is critical to the adaptation of fungi and other microorganisms to their environment. For human pathogenic fungi, expression of virulence factors, morphogenesis, stress adaptation, and drug resistance are associated with signaling via MAPK pathways. In *Candida albicans*, at least 4 MAPK pathways have been identified and their functions partially described, including Cek1 (growth and cell wall construction), Cek2 (mating), Mkc1 (cell wall integrity and stress adaptation), and Hog1 (cell wall and stress adaptation) (Alonso-Monge et al., 2006). In yeast, the Hog1 MAPK (high osmolarity glycerol) consists of three upstream, phosphotransfer proteins, including a membrane-bound, histidine kinase (HK, Sln1p), a histidine intermediate protein (Hpt, Ypd1p) that in turn is critical to the phosphotransfer from Sln1p to the third protein, the Ssk1p response regulator protein. Ssk1p then activates the Hog1 MAPK pathway during stress (Hohmann, 2002). Compared to bacteria which accomplish phosphotransfer on 2 proteins (hence the name 2-component), the eukaryotic system is referred to as 3-component signal transduction (Beier and Gross, 2006). The role of the *C. albicans* Hog1 MAPK pathway in adaptation to high osmolarity, oxidative stress, morphogenesis, cell wall biosynthesis, and virulence has been documented (Alonso-Monge et al., 2006; Alonso-Monge et al., 2003; Arana et al., 2007; Arana et al., 2005; Calera et al., 2000a; Calera et al., 2000b; Chauhan et al., 2008; Chauhan et al., 2006; Eisman et al., 2006; San Jose et al., 1996).

C. albicans has two additional HK proteins that are found in other fungi but not in *Saccharomyces cerevisiae*, including Chk1p and Nik1p, both of which have been extensively studied (Alex et al., 1998; Calera et al., 1998; Calera and Calderone, 1999; Calera et al., 1999; Kruppa and Calderone, 2006; Kruppa et al., 2004a; Kruppa et al., 2004b; Kruppa et al., 2003; Li et al., 2004; Li et al., 2002; Nagahashi et al., 1998; Selitrennikoff et al., 2001; Srikantha et al., 1998; Torosantucci et al., 2002; Yamada-Okabe et al., 1999). Thus, in *C. albicans*, there are 3 HK proteins, although both the Chk1p and Nik1p have not been assigned to a MAPK pathway.

Three-component proteins of fungi may be important drug targets, an hypothesis based upon their conservation among human pathogens and important functions in virulence, such as the Sln1p homologue of *Blastomyces dermatitidis* that is required for dimorphism and transcription of virulence factors as well as in *Cryptococcus neoformans* of which functions have been assigned to stress response adaptation, drug sensitivity, sexual development, and virulence (Bahn et al., 2006; Nemecek et al. 2006). Interestingly, for *C. albicans*, a double histidine kinase mutant (*sln1/nik1Δ*) could not be isolated suggesting that these proteins play a major role in growth of the organism (Yamada-Okabe et al., 1999).

Functions have been assigned to the Chk1p of *C. albicans* based upon mutants lacking the gene (Calera and Calderone, 1999; Calera et al., 1999; Kruppa et al., 2004a; Kruppa et al., 2004b; Kruppa et al., 2003; Li et al., 2002; Nagahashi et al., 1998; Yamada-Okabe, 1999). For example, a deletion mutant lacking *CHK1* (strain CHK21) is avirulent in a murine model of hematogenously disseminated candidiasis and has decreased levels of adherence to human esophageal cells (Calera et al., 1999; Li et al., 2002). Further, the mutant heavily agglutinates *in vitro* (Calera and Calderone, 1999), and this phenotype, along with its inability to bind to host cells, suggests that the mutant has alterations in its cell surface. To that end, we have demonstrated that changes occur in the proportions of β -glucans and by Western blot that the acid-stable mannan side chain is truncated (Kruppa et al., 2003).

Common phenotypes have been reported for *CHK1* (herein), *SHO1*, and *CEK1* null mutants, including their sensitivity to Congo red that is not observed in other MAPK signal pathway mutants (Alonso-Monge et al., 2006; Eisman et al., 2006; Roman et al., 2005). Also, both the

chk1Δ and *sho1Δ* mutants clump extensively *in vitro*, but those data were reported using different media (Calera and Calderone, 1999; Roman et al., 2005). As stated above, Sho1p is an upstream protein of the Cek1p MAPK pathway. To determine functional relationships among these proteins, a double *chk1/sho1Δ* mutant was constructed and phenotypically compared to single mutants of *CEK1*, *CHK1*, and *SHO1*. We have demonstrated that Chk1p and the Cek1p MAPK (and partially Sho1p) have common functions in mannan biosynthesis, but Chk1p is part of a parallel but independent pathway of Sho1p or Cek1p in regard to CR and CW resistance.

2. Materials and Methods

2.1. Strains and growth conditions

All *Candida albicans* strains previously described or constructed in this study are listed in Table 1. Cells were grown at 30°C in YNB medium consisting of 2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with uridine (25 μg ml⁻¹) or in YPD broth. A *sho1/chk1Δ* double mutant (strain REP36-1) was constructed as described below. Null mutants in the MAPK genes *hog1Δ*, *mkc1Δ*, *cek1Δ*, and *cek2Δ* were used in agglutination experiments (Chen et al., 2002; Eisman et al., 2006; San Jose et al., 1996). The Sho1R (REP5) and Cek1R (CK43B-R1) are reintegrant strains of the *sho1Δ* and *cek1Δ* null mutants, respectively (Table 1).

2.2. Construction of the *sho1/chk1Δ* double mutant

Disruption of *SHO1* was completed in strain CHK22, a Uri⁻ mutant lacking the *CHK1* gene, described in Table 1. Standard molecular biology procedures were used for construction of a double *sho1/chk1Δ* null mutant (REP36-1) using the same disruption cassette described by Roman et al., 2005.

2.3. Drop plate assays with inhibitors

We followed standard procedures for these assays (Chauhan et al., 2003). To determine the sensitivity of strains to Congo red (CR) and calcofluor white (CW), we used drop plates containing 50 μg ml Congo Red (CR) (ICN Biomedicals Inc.) or 24 μg/ml of CW in YPD agar. Cells for assays were prepared as overnight cultures in YPD medium (30°C), centrifuged, washed in saline 3 times, adjusted to 50 - 5 × 10⁵ yeast cells in 5 μl, and spotted onto the YPD agar medium with/without inhibitors. Inoculated plates were incubated at 30°C for 48 h and photographed.

2.4. Cek1p phosphorylation

Stationary phase cells from overnight cultures in YPD broth medium (30°C) of the Wt strain (CAF-2), the *chk1Δ* or *sho1Δ* null mutants, or double null mutant (*sho1/chk1Δ*) were diluted in YPD medium or YPD medium supplemented with Congo Red (CR, 100 μg/mL). After 1 h, cells were collected and prepared for Western blot analysis as described by Roman et al. (2005). Samples were then blotted and p42-44 antibody was used to detect phosphorylation of the Cek1 MAP kinase. Other blots were reacted in a similar way using a rabbit polyclonal serum against the Hog1p protein as a loading control and then processed similarly.

2.5. P_{CHK1}-lacZ gene constructs and galactosidase assays

We used the plasmid (pChk1prlacZ) of Li et al (2004) to construct strains carrying the P_{CHK1}-lacZ reporter gene in CAI4, as well as the *sho1Δ*, *chk1Δ*, or *sho1/chk1Δ* null mutants, each of which lacked *URA3*. Transformation was done as follows: *Ava*I-linearized pChk1prlacZ (5 μg) was used to transform CAI4 and each of the mutants by using lithium acetate. The plasmid included a 1.4kb CHK1-pr sequence in-up stream of the *lacZ* reporter

gene. Three transformants of each mutant and strain CAI4 were chosen for determinations of β -galactosidase activity, which was performed by measuring the hydrolysis of the substrate ONPG from broth cultures using early-exponential (3h) cells obtained in the following manner. Fresh 2% glucose-YNB medium (5 ml) with uridine was inoculated with 100 μ l of an overnight culture of CAI4 or each mutant. Cultures were incubated at 30°C with vigorous shaking for 3 h to achieve an $OD_{600} = 0.3$. Cultures were then supplemented with 100 μ g/mL of CR. Control cultures consisted of cells without CR but treated similarly to cultures with CR. All cultures were incubated at 30°C for 20 min, cells of each strain were collected by centrifugation and suspended in 5 ml Z buffer (pH 7.0, 0.01 M sodium phosphate, supplemented with KCl, $MgSO_4$ and β -mercaptoethanol). Then, triplicate samples of cells (0.8 ml per strain and for each growth condition) were permeabilized with 25 μ l chloroform and 25 μ l 0.1 % SDS. Cells were equilibrated at 37°C for 5 min, 0.2 ml (4 mg ml^{-1}) of the ONPG substrate was added, and the cells were mixed and incubated at 37°C for 20 min, then processed for *lacZ* measurements as described previously (Li et al., 2004).

2.6. Cell clumping assays

All strains were grown overnight in YPD broth, cultures were centrifuged, cells were washed with saline, and transferred to M199 medium, pH 7.5, and SD medium (2% glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 0.07g amino acid mix), or YPD (total of 5-ml). All strains were adjusted to a final concentration of 2.5×10^5 cells. Cultures were also incubated in M199 medium at 37°C for 4 h and in YPD and SD medium at 30°C for 8 h.

2.7. Alcian blue binding

An Alcian blue binding assay was carried out using the method of Herrero et al., 2002, in which a standard curve was constructed (OD_{600}) using various Alcian blue (Sigma-Aldrich) concentrations, prepared in 0.02N HCl. To quantify Alcian blue binding to cells, exponential-phase yeast cells (OD_{600} 0.6) were centrifuged, the cells were washed twice with 2 ml of 0.02N HCl and suspended in 1 ml 0.02N HCl containing 50 μ g Alcian blue. The suspension was kept at RT for 10 min in shake culture, and the cells were collected following centrifugation. The OD_{600} of the supernatant was measured, and the amount of Alcian blue binding was determined from a standard curve mentioned above.

2.8. Mannan Extraction

All strains were grown overnight at 30°C in YPD medium. Yields of mannan varied depending upon the strain. For the *chk1* Δ , *sho1/chk1* Δ , and *cek1* Δ null mutants, cells were grown in 10 liters of YPD (due to lower yield of mannan precipitate, see below). Mannan from CAF2, *sho1* Δ , and strain CHK23 was obtained from cells grown in 2-liters of YPD medium. A typical yield of mannan from CAF2, *sho1* Δ , and CHK23 from 2 liters of YPD medium (11-12 g of dehydrated, acetone-washed cell mass), was ~1g, while the yield of mannan from the *chk1* Δ and the *cek1* Δ mutants from 10 liters of YPD medium (total dehydrated mass ~60g) was ~0.8g. Thus, the amount of precipitable mannan from CAF2, CHK23, and the *sho1* Δ mutant was about 5-fold more than the *chk1* Δ , *sho1/chk1* Δ , and *cek1* Δ mutants.

For extraction, we used a modification of the procedure of Kocourek and Ballou (1969), and Shibata et al. (2003, 1995). Cells were harvested, and the cell paste was washed and dehydrated with acetone. The dehydrated cell pellet was then extracted in 100-ml of water with bead agitation using a Bead Beater (Biospec Prod.). The 100-ml slurry was transferred to a second vessel and another 100-ml of water added to the mixture, which was then subjected to autoclaving for 3 h. After cooling, the solid portion of the extract was removed by centrifugation. The supernatant was then split in half. One-half of the sample was treated with 100mg of pronase (pronase was pre-heated to 70°C for 30 min before addition to eliminate any contaminating glycosidic activity) and both untreated and pronase treated samples were

incubated for 20 h at 37°C, then subjected to a Fehling precipitation (Kocourek and Ballou, 1969). An equal volume of Fehling's solution was added to the extracted mannan mixture with stirring. Within 1 h of stirring, a precipitate of copper-mannan formed and was allowed to settle. The remaining supernatant was removed, and the copper-mannan complex dissolved in 6 ml of 3N HCl. The resulting solution was then poured slowly, with stirring, into a 100 ml (8:1) mixture of methanol/acetic acid, and the resulting precipitate was allowed to settle overnight. The supernatant was decanted, and the precipitate then stirred with a fresh methanol/acetic acid mix to remove the copper complex. This was repeated until the supernatant appeared colorless. The precipitate was then collected and washed several times with methanol and allowed to dry.

2.9. GPC-MALLS analysis

GPC/MALLS analyses, performed essentially as described by Mueller *et al* (2000), was done with mannan from strains CAF2 (Wt), CHK23 (*chk1/CHK1*), *chk1Δ*, *sho1/chk1Δ*, *cek1Δ*, and *sho1Δ* null mutants. The only modification was the use of a Wyatt TriStar MALLS instead of the Wyatt Dawn MALLS (Wyatt Technology, Santa Barbara, CA). Prior to sample analysis, the GPC/MALLS system was validated by analysis of water soluble pullulan standards (Shodex P-82 standards, Showa Denko, Japan). The dn/dc for mannan samples was assigned as 0.19. Using the pullulan standards, the inter- and intra-experimental variability was 1 to 3%, depending upon the specific pullulan standard employed. Duplicate or triplicate *C. albicans* mannan samples (treated or not with pronase) were dissolved (3 mg/ml) in the mobile phase (50mM sodium nitrite, pH 7.0). The samples were incubated for 15 min at ambient temperature, followed by sterile filtration (0.45 μm) and injection into the GPC at a concentration of 600 μg in 200 μl of mobile phase.

2.10. Sugar Composition and Linkage Analysis

Mannan of Wt cells (CAF2) and CHK21 were prepared for monosaccharide composition analysis by the alditol acetate method (Dean, 1995; Ciucanu and Kerek, 1984; Kath and Kulicke, 1999; Sawardeker *et al.*, 1967). The glycosyl hydrolyses were carried out with 4M-trifluoroacetic acid at 105°C for 5 h followed by reduction in H₂O with NaBD₄ overnight at room temperature, and subsequent acetylation by acetic anhydride at 100°C for 2 h. The alditol acetate derivatives were analyzed by gas liquid chromatography (GLC) using a Varian 3400 gas chromatograph equipped with a 30-m DB-17 capillary column [210°C (30 min)→240°C at 2°C/min], and by GLC-mass spectrometry (GLC-MS) in the electron-impact and chemical-ionization modes in a ThermoFinnigan PolarisQ instrument. Sugar linkage analysis was performed by the methylation procedure (NaOH/Me₂SO/CH₃I) and with characterization of the permethylated alditol acetate derivatives by GLC-MS in the electron impact mode (DB-17 column, isothermally at 190 °C for 60 min) (Shibata *et al.*, 2003; Shibata *et al.*, 1995).

2.11. NMR spectroscopy

1D and 2D gradient-selective double-quantum-filtered homonuclear correlation spectroscopy (COSY) proton NMR spectra of the mannan isolates from each of the strains were collected on a JEOL Eclipse+ 600 NMR spectrometer in 5-mm OD NMR tubes at 80°C in D₂O. Internal chemical shift reference was provided by trimethylsilyl-2,2,3,3-d₄-propionic acid (TSP). Spectral collection and processing parameters for the 1D proton NMR spectra were the following: 25 ppm spectral width centered at 5.0 ppm, 32,768 data points, 256 scans, 15 sec relaxation delay, 2.18 sec acquisition time and exponential apodization. Spectral collection and processing parameters for the 2D COSY spectra were the following: 512 points in f2 and 128 points in f1 zero-filled 4 times for a 512 × 512 data array size, 5 ppm spectral width centered at 4.5 ppm in both dimensions, 4 prescans, 128 scans and 1.5 sec relaxation delay.

3. Results

3.1. Sensitivity of mutants to Congo red (CR) and calcofluor white (CW)

The sensitivity of strains to CR and CW was evaluated in YPD agar using drop plate assays. Cells from each strain were grown overnight at 30°C, washed and standardized to deliver 5×10^5 to 50 cells in 5 μ l of PBS in drop plates. Cultures then were incubated at 30°C for 48 h and growth inhibition determined in YPD + CR or YPD + CW or YPD agar lacking either inhibitor. All strains grew equally well in YPD agar (Figure 1, **left panel**), but when incubated with CR (Figure 1, **center panel**) compared to CAF2 cells (or the Rm100 parental strain of the *sho1* Δ null, not shown) all null mutants were equally sensitive to CR although the double mutant was slightly more sensitive to CR than the single mutants. The CW sensitivity of the *cek1* Δ null as well as the *sho1/chk1* Δ null was increased compared to CAF2 and the single null mutants (*chk1* Δ and *sho1* Δ) as well as the Rm100 Wt strain (not shown) (Figure 1, **right panel**). Since the *sho1/chk1* Δ null mutant was more sensitive to CW or CR (slightly) than either of the single mutants, our data indicate that Chk1p and Sho1p are part of parallel and independent signal pathways. For both CR and CW resistance, the gene-reconstituted strains regained near Wt levels (Figure 1).

3.2. Cek1p phosphorylation requires Sho1p but not Chk1p

Phosphorylation of Cek1p occurs when cells initiate exponential growth either in the absence or presence of the cell wall inhibitor CR (Roman et al., 2005). To determine the relationship of Chk1p to Cek1p phosphorylation, cells of Wt, *chk1* Δ , *sho1* Δ , and the *sho1/chk1* Δ null mutants were grown in the absence or presence of CR and then phosphorylation of Cek1p was determined by Western blotting (Figure 2). We show that with or without CR stress, phosphorylation of Cek1p occurred in Wt cells, the Sho1R reintegrand, and in the *chk1* Δ but not in the *sho1* Δ or double mutant, data which support our hypothesis that Chk1p is necessary for resistance to CR, but the Chk1p and Sho1p are part of different but parallel pathways, validating the phenotype data shown in Figure 1. Thus, the function of Chk1p in CR resistance does not include Cek1p as a downstream target.

3.3. P_{CHK1}-lacZ reporter activity in strains

In order to determine if Cek1p or the Sho1p regulate transcription of *CHK1*, we constructed a *P_{CHK1}-lacZ* reporter cassette, which was then used to transform strains CAI4, or the *sho1* Δ and *cek1* Δ mutants. Log phase cells were screened for *lacZ* activity in either untreated 2% glucose-YNB medium with/without CR. Expression of *lacZ* in all strains was similar in medium lacking or with CR (Figure 3). These data indicate that CR stress does not alter *CHK1* expression and that neither Sho1p nor Cek1p regulate transcription of *CHK1*.

3.4. Clumping of strains

Clumping of both the *chk1* Δ and *sho1* Δ null strains has been reported, but in those studies different media were used to evaluate this phenotype (Calera & Calderone, 1999; Roman et al., 2005). So, we compared clumping of these strains as well as knock-out mutants in the MAPKs *cek2* Δ , *hog1* Δ , *cek1* Δ , *mkc1* Δ , and the *sho1/chk1* Δ mutant to Wt strains as well as null strains that were reconstituted with their respective genes (Chk1R, Sho1R, Cek1R) (Figure 4A). The *chk1* Δ as well the *sho1* Δ null mutants clumped, but the *sho1/chk1* Δ and *cek1* Δ null mutants did so to a greater extent than either single mutant in M199 (37°C). In contrast, Wt, null mutants in each of the other MAPKs, as well as the gene reconstituted strains did not exhibit a clumping phenotype. Thus, of the MAPKs, only Cek1p appears to regulate a Wt cell surface manifested by its corresponding mutant. Clumping of these mutants occurred in SD medium (cells are both yeast and filaments) or M199 (mostly filaments) (Figure 4B). Interestingly, in SD medium, each mutant formed mixtures of yeast and hyphae, while the Wt

strain only formed yeast cells. Thus, the clumping phenotype is mostly associated with this morphology. These results again show that, like resistance to CR and CW, Sho1p and Chk1p operate in parallel but independent pathways in regard to the cell surface properties that prevent this phenotype.

3.5. Alcian blue binding is significantly reduced in the *chk1Δ*, *cek1Δ* and *sho1/chk1* double mutant but less so in the *sho1Δ* null mutant

The clumping phenotype of mutants suggested that cell surface changes had occurred. To explore this observation, we measured Alcian blue binding to all null strains with the clumping phenotype as well as Wt (CAF2 and RM100) and null strains with the *CHK1*, *CEK1*, or *SHO1* reintegrated (Chk1R, Cek1R, or Sho1R) (Figure 5). Our data indicate that compared to Wt and reintegrated strains, binding was significantly reduced in *chk1Δ*, *cek1Δ*, and the *sho1/chk1Δ* null mutants but much less in the *sho1Δ* null. As Alcian blue binds to phosphomannosyl residues, we surmised that the mannan component of these mutants was changed.

3.6. Chk1, Sho1, and Cek1p deletions alter the molecular weight and polymer distribution of cell wall mannan

We first demonstrated that the yield of extracted mannan from strains CAF2 and CHK23 (*chk1/CHK1*) (~0.5 g/L of cells) was significantly higher than that of the *cek1Δ*, *sho1/chk1Δ*, and *chk1Δ* mutants (~0.08 g/L) (data not shown). Correspondingly, the Mw of mannan (expressed as gram/mol) derived from CAF2 ($7.45 \pm 0.13 \times 10^5$ Mw) and strain CHK23 ($7.9 \pm 0.33 \times 10^5$ Mw) was higher than the mannan isolated from the *chk1Δ* null mutant ($1.6 \pm 0.27 \times 10^5$ Mw). The Mw of the mannan isolated from the *sho1/chk1Δ*, and *cek1Δ* null mutants was, $2.55 \pm 0.5 \times 10^5$ Mw and $1.6 \pm 0.04 \times 10^5$ Mw, respectively, reflecting a decrease compared to the Wt strain of 65.8 - 78.5% depending upon the mutant (Table 2). For the *sho1Δ* null mutant, a reduction in Mw of approximately 30% was also observed compared to CAF2 (Table 2). Further, the differences in Mw and RMS in the *chk1Δ*, *sho1/chk1Δ* *cek1Δ* strains correlated with their reduced polydispersity compared to CAF2 and CHK23 (Table 2).

These observations were pursued through an analysis by GPC-MALLS of the mannan profiles of CAF2, CHK23, and the *chk1Δ* null mutant (strain CHK21) which indicated a dramatically reduced profile in the mutant (Figure 6A). Therefore, we compared mannan profiles from both of these strains to the *sho1Δ*, *sho1/chk1Δ*, and the *cek1Δ* mutants. We show that there are also distinct differences in the polymer distribution of these mutants (Figure 6A-D). Mannans derived from the wild type, CAF2, and *CHK1* gene-reconstituted (CHK23) strains showed similar polymer distributions consisting of three partially separated peaks covering a broad Mw range (Fig. 6A). In striking contrast, mannan isolated from CHK21 showed primarily a single polymer peak in the lower Mw range that appeared higher in concentration compared to the low Mw Wt mannan (Fig. 6A). The data were analyzed using a single peak setting so that the entire polymer distribution could be evaluated and compared. In comparison, the *sho1Δ* mutant profile indicated a loss of the intermediate-sized polymer peak and a shift to a lower Mw of the low Mw polymer profile (Figure 6B), while the double (*sho1/chk1Δ*) (Figure 6C) and *cek1Δ* null mutants (Figure 6D) resembled that of the *chk1Δ* mutant (strain CHK21) except for a slight shift to a lower Mw in the *cek1Δ* and double null mutants.

3.7. Pronase treatment results in a loss of high molecular weight mannan polymer in strains of *C. albicans*

Kocourek and Ballou (1969) previously demonstrated that pronase treatment reduced the Mw of total mannan from *S. cerevisiae* indicating that a portion of the mannan is linked to protein. Similarly, in our study, pronase treatment, followed by GPC/MALLS analysis, revealed a relatively specific effect with respect to its ability to eliminate the high Mw peak in CAF2 cells (Figure 6E). A comparable effect was observed for strain CHK23 (data not shown). In fact,

pronase treatment reduced the Mw of the mannan from the *chk1Δ*, *cek1Δ*, *sho1Δ*, and the *sho1/chk1Δ* null mutants (Table 2), although, the magnitude of the effect (~40%) was much less than that observed for CAF2 (65.6%) or CHK23 (59.5%) (Table 2). This observation suggests that there is a lower amount of protein at the cell surface of strain CHK21. We also noted that following pronase treatment in CAF2 and CHK23 (data not shown), there appeared to be a redistribution of polymers to lower Mw regions (Figure 6E). Pronase-treated and untreated samples were also analyzed by proton NMR for differences in strains. Loss of protein in the pronase-treated mannoproteins was confirmed by a loss of aromatic resonances associated with the protein (data not shown).

3.8. Linkage and NMR analysis demonstrates differences in mannan structure in the CHK21 and other mutants

The Mw distributions observed between preparations from CAF2, CHK21 and CHK23 prompted us to study the monosaccharide composition and define the sugar linkage types in each sample. Monosaccharide composition analysis revealed that CAF2, CHK21 and CHK23 preparations were composed almost exclusively of mannose (Man). While not strictly quantitative, the linkage analysis combined with a generic mannan structural model from Shibata and coworkers (Shibata et al., 2003, 1995) provides guidance in relative terms to estimate side chain length (Table 3). While the polymer backbone ("BB") is composed primarily of 6-Man-1 and 2,6-Man-1 ("1Br") monomers, the percentages of branch repeat units in the backbone ("1Br in BB") of the three mannan isolates are comparable (Table 3 and see Supplementary Table S1 for linkages of all mutants). The mannan polymer side chains ("Total SC Units") are composed of Man-1 end groups, unbranched repeat units (2-Man-1 and 3-Man-1), singly branched repeat units (2,3-Man-1, 2,4-Man-1 and 3,6-Man-1), and doubly branched repeat units (2,3,6-Man-1). The level of singly branched repeat units in the side chains ("1Br in SC") is highest for CAF2 and about half that level for CHK21 and CHK23. The level of doubly branched repeat units in the side chains ("2Br in SC") is lowest in CHK21 and roughly twice as large in CAF2 and CHK23 (Table 3). In relative terms, there are considerably more 1Br repeat units ("Br Ratio: 1Br:2Br") in side chains of CHK21 than CAF2 and CHK23 suggesting that side chains on average in CHK21 are more linear with less branching. Finally the ratio of SC-to-BB repeat units ("SC:BB") is highest for CHK21 compared to CAF2 and CHK23 which provides an estimate of the average side chain length ("Average SC Length") for CHK21 being about 30-35% longer than for either CAF2 or CHK23. Thus, linkage analysis indicates that the mannan isolated from CHK21 is different from the mannan isolated from CAF2 and CHK23. The data suggest that the average mannan structure for CHK21 relative to CHK23 and CAF2 contains lower branching and longer uninterrupted side chains.

The presence or absence of N- and O-linked mannoproteins is difficult to determine from the COSY spectra since these mannoprotein isolates were pronase treated before running the COSY spectra. The cross peak for the anomeric proton resonating at 5.133 ppm, observed in *chk1Δ* and the *cek1Δ* and perhaps in the double mutant *sho1/chk1Δ*, may be assigned to Man in either M α 1-3 or -2M α 1-O-Ser. No other cross peak was observed that was assignable to an N-linked mannoprotein. The linkage analysis indicated the presence of 4-GlcNAc, consistent with this monomer being the connection point back to the associated protein.

Following the example of Kobayashi and co-workers (Kobayashi et al, 1997) and Gimenez-Abian and co-workers (Gimenez-Abian et al, 2007), we examined the 2D COSY NMR spectra of each isolate to determine mannan structural motifs present since a large number of backbone and side chain structural features have already been assigned after polymer degradation and isolation of the oligomeric structures resulting from the side chains. These mannans contain two parts (acid-stable and acid-labile) that are coupled through a phosphate diester linkage. The mannan side chain oligomers in the labile portion are connected to the phosphate group

through linkage to an α -conformer mannosyl repeat unit through the 1-position of the reducing terminus on the side chain oligomer. One or more additional mannosyl repeat units are connected sequentially to this first mannosyl repeat unit by (β -1,2)-linkages. The level of the labile portion of the polymer is lower in the mannans isolated from the *chk1* Δ and the double *sho1/chk1* Δ double mutant, as shown by the relative size of the anomeric proton resonances (5.578 and 5.550 ppm) connected to the phosphate group for these two mutants. These data are supported by our observation of reduced alcian blue binding by the same mutants. There are sufficient branching repeat units along the backbone to not allow multiple, sequential, unbranched (1-6)-Man repeat units in any of the isolated mannans. The only backbone branch repeat units observed in these isolates allow branching through (1-2)-linkages, not (1-3)-linkages.

Side chains are primarily composed of (α 1-2)-linked mannosyl groups with a small amount of (α 1-3)-linked mannosyl groups within the chain and one or more (β 1-2)-linked mannosyl end groups. Side chains are terminated with either β 1- or α 1-linked mannosyl groups. Evidence for (1-2)- and (1-3)-linked mannosyl repeat units in side chains is present. Secondary (1-6)-linked branches from primary side chains are also noted.

Comparing the proton NMR spectra for CAF2, CHK23, *chk1* Δ , *cek1* Δ , *sho1/chk1* Δ , and the *sho1* Δ null mutants (Figure 7), it is clear that CAF2 and CHK23 are very similar while the null strains are decidedly different. The *sho1* Δ null strain is more like Wt cells. In Figure 7, all resonances are normalized to the highest resonance at 5.065 ppm which is assigned to anomeric protons from the backbone. In this presentation, several noticeably larger resonances from CHK21 are assigned to side chain structures: 5.370 ppm (α 1-2M α 1-3M α 1-2), 5.267 ppm (M α 1-2(M α 1-2)_nM α 1-2), 5.242 ppm (probably related to M α 1-2,3(M α 1-6)M α 1-2), 5.154 ppm (unknown but may be related to M β 1-2M α 1-2 also at 5.170 ppm), 4.920 and 4.933 ppm (M α 1-6) and 4.843 ppm (M β 1-2M β 1-2 or M β 1-2(M β 1-2)_nM β 1-2M α 1-2). Several smaller resonances from CHK21 are assigned as follows: 5.551 ppm (M β 1-2(M β 1-2)_nM α 1-Phosphate), 5.578 ppm (M β 1-2M α 1-Phosphate), 5.103 ppm (brancher M α 1-6 with a single M α 1-2 side chain mannosyl repeat unit), 4.823 ppm (M β 1-2M α 1-Phosphate or M β 1-2M β 1-2M α 1-2). 4.782 - 4.764 ppm (M β 1-2M α 1-2 and M β 1-2M α 1-3).

These assignments are consistent with the null strains described above (except *sho1* Δ) having a lower level of labile side chains attached to phosphate and a higher concentration of long side chains attached to the backbone. Loss of part of the labile portion of the mannan and the presence of longer side chains may encourage clumping of cells.

In summary, mutant profiling of CR and CW sensitivity and clumping indicate that Chk1p and Sho1p as determined by their respective mutants are functionally similar but occupy parallel and independent pathways in regard to resistance to CR and CW. This interpretation (CR sensitivity) correlates well with Western blot analyses. However, Cek1p and Chk1p are required for mannan biosynthesis. It would also appear that Sho1p, the cognate receptor protein for the CEK1 MAPK pathway, is partially required for mannan synthesis. A major conclusion of our data is that the extent of Alcian blue binding correlates with the loss or presence of high and intermediate Mw mannan.

4. Discussion

We phenotypically compared strain CHK21, lacking the Chk1p HK, to MAPK mutants and found that mutants in the Cek1p MAPK and its cognate sensor, Sho1p, were each sensitive to CR and CW as is CHK21. As for the other MAPK mutants, the *hog1* Δ mutant is not sensitive to CR, and the cell wall changes associated with the *mkk1* Δ mutant are not consistent with that of the *chk1* Δ mutant (Alonso-Monge et al., 2006). The *sho1* Δ null mutant also clumped as did

the *chk1Δ* null mutant, but those data were not evaluated in the same medium (Calera and Calderone, 1999; Roman et al., 2005) nor have comparisons been collectively made with all other mutants depicted in Figure 4A. To establish a relationship of Chk1p with Sho1p and Cek1 MAPK, a double *sho1/chk1Δ* null mutant was constructed. We now show that the double mutant is slightly more sensitive to CR and more sensitive to CW than single mutants indicating that both proteins are in parallel but independent pathways. This was further proven by Western analysis in which Chk1p was shown not to be required for phosphorylation of Cek1p during log phase growth with or without CR while Sho1p was required.

We also show that strain CHK21 was compromised in the biosynthesis of cell wall mannan. That phenotype was also evaluated in the *sho1Δ*, *sho1/chk1Δ* and *cek1Δ* mutants. The mannan profile of the *sho1Δ* null mutant indicated that Sho1p is at least partially required for mannan synthesis, while the *sho1/chk1Δ* double null mutant profile resembled that of the *chk1Δ* null mutant (CHK21). Of importance, the mannan profile of the *cek1Δ* null mutant was similar to strain CHK21, indicating that the two proteins have related cellular functions. Of importance, the Alcian blue binding data correlate well with the mannan changes in *cek1Δ*, *chk1Δ*, and the double (*sho1/chk1Δ*) null mutants (less binding) and the *sho1Δ* null (more binding).

We also investigated the changes in mannan that occurred in strain CHK21 in order to determine the impact of these genes on the structure and composition of cell-wall mannans using 1D and 2D NMR for mannan compositional profiling, GPC/MALLS for molecular weight and polydispersity differences, and GC/MS for monomer identification and monomer linkage type analysis (see Table S1, supplementary data). The structure of cell-wall mannans generally consists of an acid stable portion and an acid labile portion connected by a phosphate ester linkage (Masuoka et al., 2004; Shibata et al., 2003; Kobayashi et al., 1997; Shibata et al., 1995). The acid stable portion contains a backbone of [1-6]- α -linked mannosyl repeat units with side chains of [1-2]- α -linked mannosyl repeat units connected to the backbone by a [1-2]- α -linkage. The acid labile side chains are attached to the forgonig through a phosphate ester linkage. The α -anomer glycosidic linkage configuration is the major conformation found with a low level of β -anomer glycosidic linkage configuration (Shibata et al., 2003; Shibata et al., 1995).

A major finding of this study was that the mannan isolated from the null strains described above is structurally different relative to the control strains as determined by NMR spectra, linkage, GPC analysis, and even Alcian blue binding. Based upon the relative area of the resonance at 5.55 ppm, the acid labile portion of the mannan in strain CHK21 appears to be reduced in content by about 50% relative to CAF2 and CHK23. These data suggest that the Chk1p histidine kinase (and probably Cek1p) play an important role in maintaining the structural integrity of cell wall mannan.

In this study, we did not assess the presence of phospholipomannan (PLM) in our mannan extractions. PLM has been shown to have important functions in virulence and the expression of cell wall protein β -mannosylation (Mille et al., 2004; Trinel, et al., 2002). The association of the *chk1Δ*, *cek1Δ* or *sho1Δ* null mutants with changes in the synthesis of PLM is a possibility. However, a deletion strain lacking a key gene in the synthetic pathway of PLM (*mit1Δ* null mutant, GDP-mannose:inositol-phospho-ceramide mannosyl transferase), was equally resistant to calcofluor white as Wt cells (Mille et al., 2004) and thus is phenotypically different from the null strains in this report which are calcofluor white sensitive. We surmise that the changes in mannan in these mutants are probably not associated with changes in PLM.

Our data indicate that deletion of *CHK1* or *CEK1* results in a loss of the high and intermediate Mw portions of the cell wall mannan in *C. albicans*. Specifically, the deletion causes a shift in the mannan polymer distribution resulting in a single peak that is substantially of lower Mw

when compared to the high Mw trimodal distribution seen in the wild type and gene reconstituted *C. albicans* strains. The decrease in Mw of bulk mannan from CHK21, polydispersity and RMS data support this conclusion by denoting a narrow polymer distribution and smaller mean radius. Based on these data it is reasonable to conclude that the Chk1p histidine kinase and Cek1p, and to a lesser extent Sho1p, play an important role in the biosynthesis of cell wall mannans and maintaining the structural integrity of cell wall mannan.

Pronase is a non-specific protease that breaks down diverse proteins into their constituent amino acids. The glycosidase activity in this protease was eliminated prior to its use; thus it is reasonable to conclude that the effect of the pronase in these experiments is directed against proteins as previously shown in *S. cerevisiae* by Kocourek and Ballou (1969). If so, then bulk mannan contains a substantial amount that is linked to protein, *i.e.* mannoprotein, rather than pure mannans. Furthermore, the data indicate that cleavage of the protein component of this mannoprotein complex primarily results in a loss of the highest Mw component of the complex. The effect of pronase was observed in all of the cell wall mannan fractions, but it was most pronounced for the wild type (CAF2) and gene reconstituted (CHK23) mannans. This is reasonable given that the gene deleted CHK21 fraction contains much less of the high Mw component. Nevertheless, our results indicate that even the CHK21 mutant strain contains protein-complexed mannan, albeit at lower amounts. In previous published studies in which mannoproteins were extracted differently from bulk mannan preparations herein reported, coomassie-stained protein profiles of CHK21 and Wt cells by SDS-PAGE were similar. However, Western blot using antibody to the acid-stable and acid-labile fractions were altered in the former to reveal a lack of reactivity with high Mw glycoproteins (Kruppa et al., 2003). Based upon this observation, our hypothesis can be modified to include that the defect in *chk1Δ* and *cek1Δ* results from a failure to glycosylate proteins. In that same study, we postulated that the acid-stable mannan was affected in the mutant but not the acid-labile. The decreased binding of *chk1Δ*, *chk1/sho1Δ* and *cek1Δ* null mutants to alcian blue in this study would argue against a change in acid-stable mannan and rather point to change in acid-labile mannan since alcian blue binds to mannosyl phosphate. However, a decrease in alcian blue binding could occur as an indirect effect since the loss of acid-stable mannan could affect the structure of the acid-labile mannan also.

It appears, therefore, that the polymer distribution of mannan isolated from mutants' *chk1Δ* and *cek1Δ* null mutants and to a much lesser extent, *sho1Δ* of *C. albicans* is less complex than Wt strains and is biased toward lower Mw polymers. Of equal importance to the interpretation of our data is that these mutants may lack the ability to glycosylate protein(s) with high and intermediate Mw mannan. In summary, we postulate that the loss of virulence and recognition of some types of host cells in strain CHK21 is associated with an altered cell wall that includes a loss in high and intermediate Mw mannan. That phenotype is also observed in the *cek1* mutant. Thus, we suggest that both proteins are essential to the Wt mannan synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by a Public Health Service grants NIH-NIAID 47047 and AI43465 to RC and NIH-NIGMS 53522 to DLW. MAM was supported by NSERC. We wish to thank Doreen Harcus & Malcolm Whiteway for providing the *CEK1* reintegrant strain.

References

- Alex L, Korch C, Selitrenikoff C, Simon M. *COS1*, a two-component histidine kinase that is involved in hyphal development in the opportunistic pathogen, *Candida albicans*. *Proc. Natl. Acad. Sci* 1998;95:7069–7073. [PubMed: 9618540]
- Alonso Monge R, Roman E, Nombela C, Pla J. The MAP kinase signal transduction network in *Candida albicans*. *Microbiology* 2006;152:905–912. [PubMed: 16549655]
- Alonso-Monge R, Navarro-Garcia E, Roman E, Negredo B, Eisman C, Nombela C, Pla J. The HOG1 MAP Kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. *Eukaryot. Cell* 2003;2:351–361. [PubMed: 12684384]
- Arana DM, Alonso-Monge R, Du C, Calderone R, Pla J. Differential susceptibility of mitogen-activated protein kinase pathways to oxidative-mediated killing by phagocytes in the fungal pathogen *Candida albicans*. *Cell Microbiol* 2007;9:1647–1659. [PubMed: 17346314]
- Arana DM, Nombela C, Alonso R, Pla J. The Pbs2 MAP kinase kinase is essential for the oxidative stress response in the fungal pathogen *Candida albicans*. *Microbiology* 2005;151:1033–1049. [PubMed: 15817773]
- Bahn Y, Kojima K, Cox G, Heitman J. A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development and virulence of *Cryptococcus neoformans*. *Mol. Biol. Cell* 2006;7:3122–3135. [PubMed: 16672377]
- Beier D, Gross R. Regulation of bacterial virulence by two-component systems. *Curr. Opin. Microbiol* 2006;9:143–152. [PubMed: 16481212]
- Bernhardt J, Herman D, Sheridan M, Calderone R. Adherence and invasion studies of *Candida albicans* strains, using *in vitro* models of esophageal candidiasis. *J. Infect. Dis* 2001;184:1170–1175. [PubMed: 11598840]
- Calera JA, Zhao X-J, Calderone R. Defective hyphal formation and avirulence caused by a deletion of the *CSSK1* response regulator gene in *Candida albicans*. *Infect. Immun* 2000a;68:518–525. [PubMed: 10639412]
- Calera JA, Herman D, Calderone R. Identification of *YPD1*, a gene of *Candida albicans* which encodes a two-component phospho-histidine intermediate protein. *Yeast* 2000b;16:1053–1059. [PubMed: 10923027]
- Calera JA, Choi G, Calderone R. Identification of a putative histidine kinase two-component phosphorelay gene (*CaHK1*) in *Candida albicans*. *Yeast* 1998;14:665–674. [PubMed: 9639313]
- Calera JA, Calderone R. Flocculation of hyphae is associated with a deletion in the putative *CaHK1* two-component histidine kinase gene of *Candida albicans*. *Microbiology* 1999;145:1431–1442. [PubMed: 10411270]
- Calera JA, Zhao X-J, Sheridan M, Calderone R. Avirulence of *Candida albicans* *CaHK1* mutants in a murine model of hematogenously disseminated candidiasis. *Infect. Immun* 1999;67:4280–4284. [PubMed: 10417206]
- Chauhan N, Calderone R. Two-component signal transduction proteins as potential drug targets in medically important fungi. *Infect. Immun* 2008;76:4795–4803. [PubMed: 18765727]
- Chauhan N, Inglis D, Pla J, Li D, Calera J, Calderone R. The *SSK1* of *Candida albicans* is associated with oxidative stress adaptation and cell wall biosynthesis. *Eukaryot. Cell* 2003;2:1018–1024. [PubMed: 14555484]
- Chauhan N, Latge J-P, Calderone R. Signaling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. *Nature Microbiol. Rev* 2006;4:435–444.
- Chen J, Chen J, Lane S, Liu H. A conserved mitogen-activated protein kinase pathway is required for mating in *Candida albicans*. *Mol. Microbiol* 2002;46:1335–1344. [PubMed: 12453219]
- Ciucanu K, Kerek F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Res* 1984;131:209–217.
- Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, Thomas D, Whiteway M. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, *Cek1p*, in hyphal development and systemic candidiasis. *Infect. Immun* 1998;66:2713–2721. [PubMed: 9596738]
- Dean N. Yeast glycosylation mutants are sensitive to aminoglycosides. *Proc. Natl. Acad. Sci* 1995;92:1287–1291. [PubMed: 7877969]

- Eisman B, Alonso-Monge R, Roman E, Arana D, Nombela C, Pla J. The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot. Cell* 2006;5:347358.
- Fonzi WA, Irwin M. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 1993;134:717–728. [PubMed: 8349105]
- Gimenez-Abian M, Bernabe M, Leal J, Jimenez-Barbero J, Prieto A. Structure of a galactomannan isolated from the cell wall of the fungus *Lineolata rhizophorae*. *Carbohydrate Res* 2007;342:2599–2603.
- Herrero A, Uccelletti D, Hirschberg C, Dominguez A, Abeijon C. The golgi GDPase of the fungal pathogen *Candida albicans* affects morphogenesis, glycosylation, and cell wall properties. *Eukaryot. Cell* 2002;1:420–431. [PubMed: 12455990]
- Hohmann S. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev* 2002;66:300–372. [PubMed: 12040128]
- Kath F, Kulicke WM. Polymer analytical characterization of glucan and mannan from yeast *Saccharomyces cerevisiae*. *Die Angewandte Makromolekulare Chemie* 1999;268:69–80.
- Kobayashi H, Suzuki J, Tanaka S, Kiuchi Y, Oyamada H, Iwadata N, Suzuki H, Shibata N, Suzuki S, Okawa Y. Structure of a Cell wall mannan from the pathogenic yeast, *Candida catenulate*: assignments of ¹H Nuclear magnetic resonance chemical shifts of the inner α -1,6-linked mannose residues substituted by a side chain". *Arch. Biochem. Biophys* 1997;341:70–74. [PubMed: 9143354]
- Kocourek J, Ballou C. Method for fingerprinting yeast cell wall mannan. *J. Bacteriol* 1969;100:1175–1181. [PubMed: 5361210]
- Kruppa M, Calderone R. Two-component signal transduction in human fungal pathogens. *FEMS Yeast Res* 2006;6:149–159. [PubMed: 16487338]
- Kruppa M, Jabra-Rizk M, Meiller T, Calderone R. The histidine kinases of *Candida albicans*: regulation of cell wall mannan biosynthesis. *FEMS Yeast Res* 2004a;4:409–416. [PubMed: 14734021]
- Kruppa M, Krom B, Cihlar R, Calderone R. The histidine kinase Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot. Cell* 2004b;3:1062–1065. [PubMed: 15302838]
- Kruppa M, Goins T, Cutler J, Lowman D, Williams D, Chauhan N, Menon V, Singh P, Li D, Calderone R. The role of the *Candida albicans* histidine kinase (*CHK1*) in the regulation of cell wall mannan and glucan synthesis. *FEMS Yeast Res* 2003;3:289–299. [PubMed: 12689636]
- Li D, Gurskova V, Sheridan M, Calderone R. Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous *lacZ* reporter gene. *Microbiology* 2004;150:3305–3313. [PubMed: 15470110]
- Li D, Bernhardt J, Calderone R. Temporal expression of the *Candida albicans* genes *CHK1* and *CSSK1*, adherence and morphogenesis in a model of reconstituted human esophageal candidiasis. *Infect. Immun* 2002;70:1558–1565. [PubMed: 11854244]
- Masuoka J. Surface glycans of *Candida albicans* and other pathogenic fungi: physiological roles, clinical uses, and experimental challenges. *Clin. Microbiol. Rev* 2004;17:281–310. [PubMed: 15084502]
- Millet C, Janbon G, Delplace F, Ibata-Ombetta S, Gaillardin C, Strecker G, Jouault P, Poulain D. Inactivation of *CaMIT1* inhibits *Candida albicans* phospholipomannan β -mannosylation, reduces virulence, and alters cell wall protein β -mannosylation. *J. Biol. Chem* 2004;279:47952–47960. [PubMed: 15347680]
- Mueller A, Raptis J, Rice P, Kalbfleisch H, Stout D, Ensley H, Browder W, Williams DL. The influence of glucan polymer structure and solution conformation on binding to (1 $\rightarrow$ 3)- β -D-glucan receptors in a human monocyte-like cell line. *Glycobiology* 2000;10:339–355. [PubMed: 10764821]
- Nagahashi S, Mio T, Ono M, Yamada-Okabe T, Arisawa M, Bussey H, Yamada-Okabe H. Isolation of *CaSLN1* and *CaNIK1*, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus, *Candida albicans*. *Microbiology* 1998;144:425–432. [PubMed: 9493379]
- Nemecek J, Wuthrich M, Klein BS. Global control of dimorphism and virulence in fungi. *Science* 2006;312:583–588. [PubMed: 16645097]
- Novarro-Garcia F, Eisman B, Fluza S, Nombela C, Pla J. The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. *Microbiology* 2005;151:27373–2749.

- Roman E, Nombela C, Pla J. The Sho1p adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*. *Mol. Cell Biol* 2005;25:10611–10627. [PubMed: 16287872]
- San Jose C, Alonso-Monge R, Perez-Diaz R, Pla J, Nombela C. The mitogen-activated protein kinase *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J. Bacteriol* 1996;178:5850–5852. [PubMed: 8824643]
- Sawardeker JS, Sloneker J, Jeanes A. Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. *Anal Chem* 1967;37:1602–1604.
- Selitrennikoff CP, Alex L, Miller T, Clemons K, Simon M, Stevens D. *COS1*, a putative two-component histidine kinase of *Candida albicans*, is an *in vivo* virulence factor. *Med. Mycol* 2001;39:69–75. [PubMed: 11270409]
- Shibata N, Kobayashi H, Okawa Y, Suzuki S. Existence of novel β -1,2 linkage-containing side chain in the mannan of *Candida lusitanae*, antigenically related to *Candida albicans* serotype A. *Eur. J. Biochem* 2003;270:2565–2575. [PubMed: 12787022]
- Shibata N, Ikuta K, Imai T, Satoh Y, Satoh R, Suzuki A, Koima C, Kobayashi H, Hisamichi K, Suzuki S. Existence of branched side chains in the cell wall mannan of the pathogenic yeast, *Candida albicans*. *J. Biol. Chem* 1995;270:1113–1122. [PubMed: 7836369]
- Srikantha T, Tsai L, Daniels K, Enger L, Kingley K, Soll D. The two-component hybrid kinase regulator *CaNIK1* of *Candida albicans*. *Microbiology* 1998;144:2715–2729. [PubMed: 9802013]
- Trinel P-A, Maes E, Zanetta J-P, Delplace F, Coddeville B, Jouault T, Strecker G, Poulain D. *Candida albicans* phospholipomannan, a new member of the fungal mannose inositol phosphoceramide family. *J. Biol. Chem* 2002;277:37260–37271. [PubMed: 12138092]
- Torosantucci A, Chiani P, DeBernardis F, Cassone A, Calera J, Calderone R. Deletion of the two-component histidine kinase gene (*CHK1*) of *Candida albicans* contributes to enhanced growth inhibition and killing by human neutrophils *in vitro*. *Infect. Immun* 2002;70:985–987. [PubMed: 11796636]
- Yamada-Okabe T, Mio T, Ono N, Kashima Y, Matsui M, Arisawa M, Yamada-Okabe H. Roles of three histidine kinase genes in hyphal development and virulence of the pathogenic fungus, *Candida albicans*. *J. Bacteriol* 1999;181:7243–7247. [PubMed: 10572127]

Figure 1.

Drop plates of the Wt, reintegrants, and mutant strains on YPD agar (**Top set of panels**), YPD agar supplemented with 50µg/ml CR (center), or 24µg/ml of CW (right). All plates were inoculated with 50 to 5×10^5 cells of each strain (upper designations), then incubated at 30°C for 48 h, and photographed. Genotypes are indicated on the left side of the figure. All mutants are more sensitive than Wt cells to CR and CW. However, the *sho1/chk1*Δ double mutant is slightly more sensitive to CR and more sensitive to CW than either of the single mutants, *chk1*Δ or *sho1*Δ. (**Bottom set of panels**). Similar to that described above, except that the strains are reintegrants (*Chk1R*, *Sho1R*, and *Cek1R*); their sensitivity of each to CR or CW is restored to near levels of Wt cells.

Figure 2.

Phosphorylation of Cek1p does not require Chk1p. Western blot analysis of CAF2 vs. the *chk1Δ*, *sho1Δ*, and *chk1/sho1Δ* null mutants. Phosphorylation of Cek1p was determined in the absence (upper left panel) or presence of 100 µg of CR/ml (upper right panel). In the absence of *CHK1*, Cek1p phosphorylation still occurs, but the protein is not phosphorylated in the *sho1* mutant or in the double mutant *sho1/chk1Δ* but is in the *Sho1R* (reintegrant, far right lane of panel). Non-phosphorylated Hog1p serves as a loading control (lower panel).

Phosphorylation of Cek1p occurs only in log phase cells or in log phase cells stressed with CR (Roman et al., 2005).

Figure 3.

The expression of a lacZ reporter (P_{CHK1} -lacZ) is shown as a function of parental (CAI4), and the *sho1Δ* and *cek1Δ* mutant backgrounds, each grown in medium alone (No CR) or in the presence of 100μg/ml of CR. In the absence or presence of CR, transcription of *CHK1* (as measured by lacZ reporter activity) in either the *cek1Δ* or *sho1Δ* mutant is similar to CAI4.

Figure 4.

A) Microscopic observations of clumping in Wt, gene-reconstituted, and mutant strains of *C. albicans* grown in various media. Compared to CAF2 (Wt), the *chk1*Δ, *sho1*Δ, *chk1/sho1*Δ, and *cek1*Δ mutants clumped in each medium, while the MAPK mutants (*cek2*Δ, *mkc1*Δ and *hog1*Δ) and gene-reconstituted strains (Chk1R, Cek1R, Sho1R) grew like the Wt cultures.

B). All strains which clumped, as shown in Fig. 4A, were also examined microscopically in

YPD, SD, and M-199 medium. Clumping of mutant strains occurred in SD and M199. In YPD, some evidence of clumping was observed in the *cek1*Δ null mutant.

Figure 5.

Alcian blue binding in strains of *C. albicans*. The assay was done as described in the Methods and Methods. The amount of Alcian blue binding to the cells of each strain is shown as a percentage of that binding to the wild type cells (CAF2 or RM100). The strains used were *chk1Δ*, *sho1Δ*, *chk1Δ sho1Δ*, *chk1Δ*, CHK23 (*chk1/CHK1*), RM100 (*C.albicans* wild-type), Sho1R (*sho1/SHO1* revertant strain) and Cek1R (*cek1/CEK1* revertant strain). Data reflect 3 experiments.

Figure 6.

GPC/MALLS analysis of bulk mannan from *C. albicans* strains. **A.** Strains CAF2 (solid line), CHK23 (dashed line), and CHK21 (*chk1*Δ, dotted line) are shown. Both the CAF2 and CHK23 display a trimodal profile of high (left), intermediate (middle), and low (right) Mw mannans. CHK21 lacks both the high and intermediate profile peaks. **B.** Mannan profiles in CAF2 (dashed line) and the *sho1*Δ mutant (solid line) are shown, the latter of which displays a profile in which the intermediate sized mannan has been reduced in amount and the low Mw mannan has shifted to a slightly smaller sized mannan. **C.** Strain CHK21 (*chk1*Δ) and the *sho1*/*chk1*Δ double mutant are shown. The phenotype of the double mutant (solid line) is deficient in both high and intermediate sized mannan, like that of strain CHK21 (dashed line). A slight decrease in the low Mw mannan is also seen in the double mutant. **D.** Both the *cek1* (solid line) and *chk1* mutant (dashed line) mannan profiles are similar, except for the slight change to a lower Mw in the *cek1* mutant. **E.** Pronase treatment of mannan from strain CAF2, untreated (solid line) and treated (dashed line). Treated mannan no longer has the high Mw mannan (indicated by a ↓), which is reflected by an increase in intermediate and low Mw mannans.

Figure 7. Proton NMR spectra expanded to detail the anomeric proton spectral regions for mannan isolates. All strains are indicated by each respective profile from top to bottom: CAF2, CHK23, CHK21 (*chk1*Δ), *sho1*Δ, *cek1*Δ, and *sho1/cek1*Δ.

Table 1*Candida albicans* strains used in this study.

Strains	Relevant genotype	Reference
CAF2	<i>ura3Δ::imm434/URA3</i>	Fonzi and Irwin, 1993
CAI4	<i>ura3Δ::imm434/Δura3Δ::imm434</i>	Fonzi and Irwin, 1993
CHK21	<i>ura3Δ::imm434/ura3Δ::imm43</i> <i>chk1Δ::hisG/chk1Δ::hisG-URA3-hisG</i>	Calera and Calderone, 1999
CHK22	<i>ura3Δ::imm434/ura3Δ::imm43</i> <i>chk1Δ::hisG/chk1Δ::hisG</i>	Calera and Calderone, 1999
CHK23	<i>ura3Δ::imm434/ura3Δ::imm434</i> <i>chk1Δ::hisG/CHK1::URA3-hisG</i>	Calera and Calderone, 1999
Rm100	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG-URA3-hisG</i>	Roman et al., 2005
REP3	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>sho1Δ::SHO1-GFP</i>	Roman et al., 2005
REP5	<i>ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/his1Δ::hisG</i> <i>sho1Δ::hisG/sho1Δ::hisG-URA3-hisG::SHO1-GFP</i>	Roman et al., 2005
REP36-1	<i>ura3Δ::imm434/ura3Δ::imm434 chk1Δ::hisG/chk1Δ::hisG</i> <i>sho1Δ::hisG/sho1Δ::hisG-URA3-hisG</i>	This study
REP1	<i>ura3Δ::imm434/ura3Δ::imm434 hisG/hisΔ::hisG</i> <i>SHO1/sho1::hisG-URA3-hisG</i>	
CK43B-16	<i>cek1Δ::hisG/cek1Δ::hisG-URA3-hisG</i>	Csank et al., 1998
CK43B-RI	<i>ura3/ura3 cek1Δ::hisG/cek1Δ::CEK1-URA3</i>	Csank et al., 1998
CM1613	<i>mkc1Δ::hisG/mkc1Δ::hisG-URA3-hisG</i> <i>ura3Δ::imm434/ura3Δ::imm434</i>	Navarro-Garcia et al., 2005
CNC13	<i>ura3Δ::imm434/ura3Δ::imm434his1Δ::hisG/his1Δ::</i> <i>hisGhog1::hisG-URA3-his/hog1::hisG</i> <i>P_{CHK1}-lacZ-URA3-CHK1/CHK1</i>	Eisman et al., 2006
CHK-lacZ	<i>ura3Δ::imm434/ura3Δ::imm434</i> <i>P_{CHK1}-lacZ-URA3-CHK1/CHK1</i>	Li et al., 2004
REP3-lacZ	<i>sho1Δ::hisG/sho1Δ::hisGura3Δ::imm434/ura3Δ::imm434</i> <i>P_{CHK1}-lacZ-URA3-CHK1/CHK1</i>	This work
CK43B-lacZ	<i>cek1Δ::hisG/cek1Δ::hisG ura3Δ::imm434/ura3Δ::imm434</i>	This work

Table 2

Characteristics of cell wall mannan in strains of *C. albicans* and the effect of pronase treatment on the mannan.

Sample ID	Mw (g/mol) ¹	Polydispersity ² Mw/Mn	RMS radius ³ (nm)
CAF2	7.45×10^5	3.55	40.6
CHK21(<i>chk1</i> Δ)	1.60×10^5 (↓78.5%) ⁴	1.93	23.7
CHK23	7.90×10^4	3.13	41.6
SCHK1 (<i>sho1/chk1</i> Δ)	2.55×10^5 (↓65.8%) ⁴	2.65	27.6
CK43B-16 (<i>cek1</i> Δ)	1.62×10^5 (↓76.8%) ⁴	1.87	25.7
REP3 (<i>sho1</i> Δ)	5.13×10^5 (↓30%) ⁴	4.82	31.7
CAF2 + pronase	2.56×10^5 (↓65.6%) ⁴	2.27	30.6
CHK21 + pronase	0.96×10^5 (↓40%) ⁴	1.34	26.8
CHK23 + pronase	3.20×10^5 (↓59.5%) ⁴	2.12	32.2
SCHK1 + pronase	1.25×10^5 (↓51.0%) ⁴	1.59	28.3
CK43B-16 + pronase	0.91×10^5 (↓43.8%) ⁴	1.27	25.7

¹ Average molecular weight of the mannan samples expressed as gram/Mole (Mueller et al., 2000).

² Polydispersity is a measure of the distribution of molecular mass in a given polymer sample. Polydispersity is calculated as the weight average molecular weight (Mw) divided by the number average molecular weight (Mn) (Mueller et al., 2000).

³ RMS radius is a measure of polymer size weighted by the mass distribution about its center of mass (Mueller et al., 2000).

⁴ The values in the parentheses indicate the percent decrease in Mw for the mannan from CHK21 compared to parental (CAF2) mannan, or the percent decrease in Mw following pronase treatment when compared to the corresponding untreated mannan sample.

Table 3

Measurements of relative amounts of the various structural repeat units in the mannan isolates.

Structural Unit	CAF2	CHK21	CHK23
BB ^a	42.5	26.5	36.0
1Br ^b	32.5	20.0	30.0
1Br in BB	76.5%	75.5%	83.3%
Total SC Units ^c	170	159	172
1Br in SC ^d	7.4%	4.4%	4.1%
2Br in SC ^e	1.5%	0.6%	1.2%
Br Ratio: 1Br:2Br	500.0%	700.0%	350.0%
SC:BB	4.0	6.0	4.8
Average SC Length	5.2	8.0	5.7

^aBB = 6-Man-1 + 2,6-Man-1 monomer units, without including end groups.^b1Br = 2,6-Man-1 monomer units.^cTotal SC Units = Man-1, 2-Man-1, 3-Man-1, 2,3-Man-1, 2,4-Man-1, 3,6-man-1 and 2,3,6-Man-1 monomer units.^d1Br on SC = 2,3-Man-1, 2,4-Man-1 and 3,6-man-1 monomer units.^e2Br on SC = 2,3,6-Man-1 monomer units.