The role of the *Candida albicans* histidine kinase [CHK1] gene in the regulation of cell wall mannann and glucan biosynthesis

Michael Kruppaa, Tresa Goinsb, Jim E. Cutlerb,c, Douglas Lowman d,e, David Williams d, Neeraj Chauhana, Veena Menona, Praveen Singhad, Dongmei Lia, Richard Calderonea,∗

a Department of Microbiology and Immunology, Georgetown University Medical Center, Washington, DC 20007, USA  
b Department of Microbiology, Montana State University, Bozeman, MT, USA  
c The Research Institute for Children, New Orleans, LA, USA  
d Department of Surgery, James H. Quillen College of Medicine, Johnson City, TN 37614, USA  
e Global Polymers and Research Analytical Services, Eastman Chemical Company, Kingsport, TN 37662-5150, USA

Received 24 July 2002; received in revised form 22 August 2002; accepted 23 August 2002

First published online 12 October 2002

Abstract

The human pathogen *Candida albicans* encodes at least three putative two-component histidine kinase signal transduction proteins, including Chk1p and a response regulator protein (Csk1p). Strains deleted in CHK1 are avirulent in a murine model of hematogenously disseminated disease. The specific function of Chk1p has not been established, but hyphae of the chk1 mutant exhibit extensive flocculation while yeast forms are less adherent to reconstituted human esophageal tissue, indicating that this protein may regulate cell surface properties. Herein, we analyze glucan, mannann and chitin profiles in strains deleted in chk1 (CHK21) compared to a gene-reconstituted strain (CHK23) and a parental strain CAF2. Total alkali-soluble hexose from the cell wall of the chk1 mutant (strain CHK21) was significantly reduced. Western blots of cell wall extracts from CHK21, CHK23 and CAF2 reacted with a Mab to the acid-stable mannann fraction revealed extensive staining of lower molecular mass species in strain CHK21 only. FACE (fluorophore assisted carbohydrate electrophoresis) was used to characterize the oligosaccharide side chains of β-eliminated (O-linked), acid-hydrolyzed (acid-labile phosphomannann) and acetolysis (acid-stable mannann) extracted fractions of total mannann. The profiles of O-linked as well as the acid-labile oligosaccharides were similar in both CAF2 and CHK21, but the acid-stable oligosaccharide side chains were significantly truncated. We also characterized the β-glucan from each strain using NMR, and found that both the degree of polymerization and the ratio of (1-3)/(1-6) linkages was lower in CHK21 relative to wild-type cells. The sensitivity of CHK21 to antifungal drugs and inhibitors was unaffected. In summary, our data have identified a new function for a histidine kinase two-component signal protein in a human pathogenic fungus.

© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

1. Introduction

*Candida albicans* is a commensal of humans that primarily causes invasive disease in the immunocompromised, neutropenic patient. In this setting, an estimated mortality of approximately 35% has been reported [1]. General surgery patients receiving prolonged anti-bacterial antibiotics during catheterization are likewise predisposed to invasive disease. In the AIDS patient, the disease occurs almost exclusively on mucosal surfaces. While generally not life-threatening in this patient, reports of drug resistance to standard therapies (such as fluconazole) are increasing in frequency. Many of the drug-resistant isolates are non-albicans species of *Candida*. Complicating the use of appropriate anti-*Candida* drugs is the obvious toxicity associated with amphotericin B therapy in the patient with invasive candidiasis. Thus, the search for new targets and drugs continues.

Two-component signal transduction has been identified in bacteria, fungi, higher plants and in the Archaea but is not found in *Caenorhabditis elegans*, *Drosophila melanogaster* or in mammals, indicating a specificity that might be exploited in drug discovery [2,3].
Signal events through two-component proteins are thought to provide a mechanism for adaptive responses to external stress conditions [2,4,5]. The two-component signal system is comprised of a membrane-associated sensor protein (histidine kinase, HK) and a cytoplasmic, response regulator (RR) protein. Mechanistically, an environmental signal causes an autophosphorylation of a key histidine residue within a domain of an HK that is referred to as the H-box. A kinase domain of the HK in cooperation with a proximal linker domain catalyzes phosphorylation of this histidine residue. Once the HK is phosphorylated, phosphotransfer to an aspartate residue within a domain of the RR is mediated by the HK. The phosphorylated RR protein can act directly as a transcription factor or can transfer the signal via a MAP kinase pathway. In *Saccharomyces cerevisiae*, for example, adaptive response to osmotic stress is regulated through the Sln1 (HK), Ypd1 (phosphohistidine intermediate), and Ssk1 (RR) HOG1 signal pathway (HOG = hyperosmotic glycerol) [4]. Under osmotic stress, unphosphorylated Ssk1p activates a downstream MAP kinase pathway that increases the intracellular content of glycerol, allowing cells to adapt to high osmolality. Two-component signal transduction protein homologs in *C. albicans* have been recently studied. It appears that the organism has at least three hybrid HK proteins and two RR proteins [6-18]. Recently, a HK gene has been identified in *Aspergillus fumigatus*, another human pathogen [19]. For a general review of signal transduction pathways in human pathogenic fungi, see Lengeler et al. [20].

While homologs of a Hog pathway (similar to the one described above) as well as other HKs have been identified in *C. albicans*, these proteins seem to have a minimal function in osmosensing; rather, strains deleted in these genes are defective in morphogenesis under some conditions and are delayed in a switch phenotype [6,8,13,17,18]. Importantly, deletion mutants are less virulent than parental strains in a hematogenously disseminated murine candidiasis model [11,13,15,18]. We have identified both a hybrid HK (*CHK1*) and an RR (*CSSK1*) gene. Deletion mutants in each gene have defective morphogenesis and are avirulent [11,13]. The structural, downstream proteins that are regulated by Chk1p have not been identified, but the chk1 mutant flocculates extensively in liquid media such as M-199, implying that cell surface changes may have occurred as a consequence of the gene deletion [8,13]. Also, recently we have shown that yeast cells of strains deleted in *CHK1* are less able to adhere to reconstituted human esophageal tissues in vitro [21,22].

The cell wall of *C. albicans* has been modeled structurally much like that of *S. cerevisiae* [23,24]. While differences in cell wall content exist between these two species, the current thinking is that cell wall β-1,3 glucan forms a three-dimensional network that surrounds the cell and serves as a scaffolding to which chitin and glucan (β-1,6) branch points are covalently linked. Cell wall proteins (CWPs) are either bound to the β-1,3 glucan (PIR, Proteins with internal repeats) or are linked to the β-1,3 glucan via β-1,6 glucan branch chains [23,24]. The latter group would include wall proteins that are transferred from GPI-anchored membrane sites to the β-glucan. For *C. albicans*, a number of genes have been identified that encode cell wall biosynthetic enzymes such as glucan and chitin synthases, mannosyl transferases, as well as proteins that cross-link and extend glucan chains [23-32]. Two-component signal transduction pathways that regulate wall biosynthesis in fungi have not been studied until recently. For *S. cerevisiae*, Skn7, a RR protein that serves as a branch point protein in the Hog1 pathway [33], has been suggested as a regulator of cell wall synthesis that may act in parallel with the PKC (protein kinase C) cell wall integrity cascade and mannosylation [34]. The *C. albicans* Skn7 homolog has been listed in the Candida genome database, but its function has not been determined. Likewise, a protein of the PKC cell wall integrity pathway (Mkc1p, = MAP kinase of *C. albicans*) has been identified and its function partially characterized [35,36]. Mkc1p is essential for cell growth at high temperatures and for survival in cells undergoing thermal shock [35]. A deletion mutant had several phenotypic changes compared to wild-type cells, including cell surface alterations (observed by scanning electron microscopy), an increase in O-glycosylated mannosyl epitopes, and an increased susceptibility to nikkomycin Z [35]. The Mck1p is an apparent homolog of the *S. cerevisiae* Mpk1p [35].

In this study, we have shown that the downstream events associated with a *CHK1* function includes mannan synthesis and β-1,6 glucan branching. The cell wall phenotype of strains deleted in *CHK1* is reported. These data represent the first description of a two-component, HK regulation of specific cell wall biosynthetic processes in a human pathogenic fungus.

2. Materials and methods

2.1. Strains of *C. albicans*

The strains of *C. albicans* used in this study have been described previously [8,13] and are listed in Table 1. The chk1 deletion mutant (strain CHK21) and gene-reconstituted strain (CHK23) were constructed following the ‘urablaster protocol’ [37].

2.2. Quantitation of cell wall hexose

Fig. 1 summarizes the general methods for extraction of cell wall polysaccharides that are described in detail in the subsequent sections. A matched set of wild-type (CAF2), a double allelle deletion mutant in *CHK1* (CHK21) and the gene-reconstituted strain (CHK23) containing a single copy of *CHK1* were grown in YPD medium to stationary
phase at 25°C, washed and then inoculated into fresh YPD broth at a density of 2.5 x 10^6 cells ml^-1. All cultures were incubated at 37°C for 24 h. For dry-weight determinations, cell samples in triplicate were collected on pre-weighed nitrocellulose filters, and dried in vacuo. For hexose analysis of cell wall fractions, we followed the procedures of Fonzi [28] and Boone et al. [32]. Cell samples were collected in triplicate from each culture by centrifugation, washed with ice-cold, sterile distilled water and stored at -70°C until needed. Purified cell wall was obtained following cell breakage with glass beads. Fractionation of the cell wall was accomplished as described below.

The cell wall from each strain was extracted three times in 0.5 ml of 0.75 M NaOH for 60 min at 75°C (Fig. 1). The alkali-soluble extracts were combined and neutralized. The alkali-insoluble cell wall pellet was washed once with 100 mM Tris (pH 7.5), then once with 10 mM Tris (pH 7.5), and suspended in 1 ml of 10 mM Tris (pH 7.5) containing 0.01% sodium azide and 1.0 mg of β-1,3 glucanase (Zymolyase 100T, ICN Pharmaceuticals). The mixture was incubated for 16 h at 37°C with gentle shaking. The alkalinsoluble, Zymolyase-soluble, and Zymolyase-insoluble (obtained by centrifugation and washed with water) fractions were analyzed for total hexose content by the phenol-sulfuric acid method of Dubois [38] using glucose as a standard. For the determination of chitin, yeast cells of all strains were grown in YPD broth at 30°C for 24 h. Cells were harvested by centrifugation and washed twice with phosphate buffer. The average cell wet weight yield was 1.63 g l^-1 (CAF2) and 1.76 g l^-1 (CHK21). The pellets were lyophilized and stored at -20°C. Extractions were done twice for each strain and similar results were observed by NMR.

### 2.3. Glucan extraction for NMR determinations

For nuclear magnetic resonance (NMR) determinations of β-glucan, strains were plated onto blood agar and incubated at 37°C for 48 h. Colonies selected from the plates were used for these experiments. Tubes (5 ml) containing brain–heart infusion broth were inoculated with one colony each from the blood agar plates and were incubated in a slanted position at 37°C with agitation for 18 h. Flasks containing 1 l of brain–heart infusion broth were inoculated with 1 tube of C. albicans. The flasks were incubated at 37°C with agitation (200 rpm) for 18–24 h. This provided a turbid suspension that was harvested by centrifugation and washed twice with phosphate buffer. The average cell wet weight yield was 1.63 g l^-1 (CAF2) and 1.76 g l^-1 (CHK21). The pellets were lyophilized and stored at 4°C for extraction of cell wall glucan. Water-insoluble microparticulate glucan was isolated from the cell wall of lyophilized cells by the method of Müller et al. [40]. Following extraction with 0.75 M NaOH, the cell pellet was then treated with 2.0 N phosphoric acid (Fig. 1). The glucan residue was then extracted with 1% (v/v) phosphoric acid in absolute ethanol to remove residual lipids [41], washed with 18 mOhm water (3 x), and the pH adjusted to 7.0 prior to lyophilization. The lyophilized product was stored at -20°C. Extractions were done twice for each strain and similar results were observed by NMR.

### 2.4. NMR spectroscopy

All NMR spectrometry was performed with the water-insoluble glucan fraction. Spectral data were collected using either a JEOL Model Eclipse+600 or a Model DEL-
TA-400 NMR spectrometer operating at 80°C in 5 mm OD NMR tubes. For each sample, 10–25 mg of each glucan was dissolved in 1 ml of DMSO-d$_6$ at 80°C. A few drops of trifluoroacetic acid (greater than 99.8% deuterated, Cambridge Isotope Labs) were added to the solution to shift the water resonance downfield [42]. Proton-chemical shifts were referenced to the residual DMSO-d$_6$ proton resonance at 2.50 ppm. Generally, NMR spectral collection and processing parameters were the following: 25 ppm spectral width centered at 7.5 ppm, 32768 data points, 1024 scans, 15 s relaxation delay, 2.18 s acquisition time, and exponential apodization. The number of scans was varied, based upon the sample size.

2.5. Preparation of cell wall mannoproteins extracts

CAF2 and the paired set of strains described above (Table 1) were grown for 18–24 h in 250 ml of YPD at 30°C with shaking (150 rpm). The cells were harvested by centrifugation for 10 min at 5000 × g. For samples grown in YPD, the pellet was processed further as described below. For growth in medium 199 (M-199, Sigma, St Louis, MO, USA), yeast cells were incubated overnight in YPD, washed and counted using a hemocytometer. 1 l of M-199 was inoculated to a final cell concentration of 1 × 10$^8$ cells ml$^{-1}$ and allowed to incubate for 3 h at 37°C, which was sufficient time to initiate germination of yeast cells. The cells were collected following centrifugation at 5000 × g for 10 min. The cell pellets from cultures grown in YPD or M-199 were suspended in 25 ml of digestion buffer (1 M sorbitol, 50 mM potassium phosphate, pH 7.8, 5 mM magnesium chloride and 1.8 mM β-mercaptoethanol) [43], and 2500 U of lyticase (Sigma) was added to each sample. The cells were incubated at 30°C for 90 min to allow for cell wall digestion, centrifuged at 7500 × g for 10 min, and the pellet was discarded, while the soluble fractions (containing the cell wall components) were subjected to dialysis (3500 MW cutoff) against 50 mM Tris (pH 8.0) in the presence of 1 mM PMSF for 24 h at 4°C. Protein concentration was determined by the Bradford dye method, in which bovine serum albumin was used as a control (Bio-Rad).

2.6. SDS–PAGE and Western analysis

Protein extracts were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [44]: 10 µg of protein from each extract was electrophoresed on a 7.5% SDS–PAGE gel. The gel was blotted to nitrocellulose (0.2-µm pore size), and the membranes were probed with the anti-mannan IgM monoclonal antibodies B6 and B6.1, which recognize the acid-stable (B6.0) and the acid-labile (B6.1) portions of the mannan polysaccharide [45,46]. We used the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Hercules, CA, USA) to detect reactive mannoproteins.

2.7. Preparation of mannann

Yeast cells of each strain were grown in a glucose–yeast extract–peptone broth (2% glucose, 0.3% yeast extract, 1% peptone, w/v) in shake culture for 24 h at 37°C, then transferred to the same medium for an additional 24 h at 37°C. The cells (stationary phase) were then centrifuged at 3000 × g for 10 min and washed extensively with cold, deionized water (dH$_2$O). Cells were autoclaved in water (20 ml per g wet weight of cells) for 2 h, the cooled supernatants were collected by centrifugation, and the total volumes were reduced to 20 ml by lyophilization. Short-term Fehling precipitation (fractionation completed within 2 h) was performed as described [47], and the mannan-protein (MP) was freeze-dried for shelf storage (Fig. 1). Aqueous stock solutions of the mannan-protein were prepared at 10 mg ml$^{-1}$ and stored at −20°C. Protein and carbohydrate contents were determined using the BCA reagent (Pierce, Rockford, IL, USA) and by the phenol-sulfuric acid assay, respectively [38]. MP was fractionated by acid-hydrolysis (50 mM HCl at 100°C for 10 min) to release β-1,2-oligo-mannosides (acid-labile moieties) and by β-elimination to release the O-linked α-1,2- and α-1,3-oligosaccharides [47] (Fig. 1). Following hydrolysis, the samples were neutralized with either 100 mM NaOH or 1 N HCl, freeze-dried, suspended in dH$_2$O at 10 mg ml$^{-1}$, and stored at −20°C. The acetylation procedure used was accomplished as follows. In brief, the MP was acetylated for 18 h at 40°C in formamide:pyridine:acetic anhydride, 1:5:5, v/v/v, then the MP was mixed with five volumes of ice water [48–50]. The acetates were collected by centrifugation. Mild acetylation was carried out for 36 h at 40°C with acetic anhydride:glacial acetic acid:sulfuric acid, 100:100:1 v/v/v, mixed with ice water as described above, and the acetates extracted into dry chloroform [51]. Following evaporation of the chloroform with mild heat (40°C), the acetates were suspended into dry methanol, deacetylated with sodium methoxide, extracted into water and freeze-dried [52].

2.8. FACE analysis of oligosaccharides

Labeling of oligosaccharides with 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) was done according to the methods of Goins et al. [53] and Jackson [54], using 100 nM aliquots of freeze-dried mannan fractions. Following tagging with ANTS, samples were dried under nitrogen, suspended in loading buffer, and stored at −70°C until analyzed by electrophoresis. Oligosaccharides were resolved in 32% acrylamide gels (PlusOne Ready Sol IEF40, Pharmacia Biotech, Piscataway, NJ, USA) according to the method of Goins et al. [53]. The preparation of carbohydrate standards from hydrolyzed dextran has been previously described [53]. Following electrophoresis, visualization of the ANTS-labeled oligosaccharides and data acquisition was accomplished by UV illumination using a
BioRad Multiflour Imager. The data were exported as tagged-image format files (TIFF)-based images and were analyzed using SigmaGel analysis software (SPSS Science, Chicago, IL, USA). Oligosaccharide concentrations of individual bands and relative migration indices (RMI) were determined as described [53].

2.9. Hydrophobicity and inhibitor assays

We used the assay described by Hazen and Hazen [55] to measure the cell surface hydrophobicity of C. albicans strains. In brief, yeast cells were grown in YPD broth medium for 18–24 h. Cells were collected by centrifugation, washed 3 times with cold, sterile water, and 4×10⁶ cells were transferred to a phosphate buffer (HMA buffer, hydrophobicity microsphere assay, 0.05 M Na–phosphate, pH 7.2). The cells were then centrifuged and suspended in 100 µl of HMA buffer from a stock suspension containing a total of 6 µl of low-sulfate, polystyrene microspheres (0.825 µm) in 2 ml of cold HMA buffer. The cell–bead mixture was vortexed and the % hydrophobicity determined in a hemocytometer by counting the number of yeast cells with three or more attached microspheres. A total of 100 yeast cells were counted. Data were analyzed using the Student’s t-test.

To measure the effects of inhibitors and antifungals on strains, we used the procedure of Navarro-Garcia et al. [35]. Cells of each strain were grown on YPD plates. Cells were collected in saline, washed and suspended to a cell concentration of 1×10⁶ ml⁻¹ in 10 ml of YPD broth. Cultures were grown at 30°C for 14 h. Cells were collected by centrifugation, washed twice with PBS–0.05% Tween 80 and suspended in PBS. The cell inoculum was adjusted to 2×10⁷ ml⁻¹ in 2×-RPMI broth medium. Stock solutions of micronazole, amphotericin B, cilofungin, nikkomycin Z, calcofluor white, hygromycin, neomycin, and caffeine were prepared and each was diluted to give a final stock concentration of 320 mg l⁻¹, except for nikkomycin Z which was diluted to a concentration of 625 mg l⁻¹. Serial two-fold dilutions were prepared with RPMI broth for each inhibitor in 96-well microtiter plates. A total volume of 100 µl of inhibitor in RPMI and 100 µl of yeast cells (2×10⁵ cells) in RPMI was incubated at 37°C for 24 h. The turbidity of each plate was read at 405 or 530 nm with a Winsleit 96-well reader and sensitivities were plotted to determine minimum inhibitory concentrations (MIC). The MICs for all drugs were determined as the concentration that resulted in 80% growth inhibition, except for amphotericin B where 100% growth inhibition was defined as the MIC.

3. Results

3.1. Cell wall analysis of total hexose

The hexose composition from the cell wall fractions of strains was determined (Table 2). For strain CHK21 (chk1 null) compared to wild-type cells (CAF2), we observed a significantly lower total hexose in the alkali-soluble fraction (CAF2 > CHK21, P = 0.02) and total cell wall hexose (CAF2 > CHK21, P = 0.004), while differences were not observed between strain CHK23 (gene-reconstituted strain) and CAF2. Also, no significant changes in the total hexose content in either the Zymolyase-soluble or Zymolyase-insoluble cell wall fractions of all strains occurred, and the chitin content appeared to be comparable among strains. Chitin content was measured either from the Zymolyase-insoluble fraction or from separately prepared chitin extracts using the procedure described by Johnson [39] for hexosamine determinations (Table 2).

3.2. NMR studies of cell wall β-glucan

Glucans from C. albicans CAF2 (wild-type) and CHK21 (chk1 null) were isolated by the alkali–phosphoric acid method according to published procedures [40,41]. Two extractions were done per strain and similar NMR profiles were observed for each extraction and for each strain.

(1-3)-β-D-Glucans are polymers of glucose connected through glycosidic oxygen between the 1-position (the anomeric position) and the 3-position of the neighboring anhydroglucose repeat unit (AGRU), forming a linear backbone of AGRUs (Fig. 2, structure 1). Kim and coworkers [56] have used NMR of the anomic protons to characterize several structural features of glucans which included the reducing terminus (RT), α- and β-anomer stereochemistry at RT (RT(α) and RT(β), respectively), the second repeat unit connected to the reducing terminus (SRT), backbone chain repeat units (BC), the non-reduc-

### Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alkali soluble (%)</th>
<th>Soluble Zymolyase (%)</th>
<th>Cell wall fraction insoluble Zymolyase (%)</th>
<th>Chitin (%)</th>
<th>Total hexose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF2</td>
<td>327.9 ± 1.5</td>
<td>220.5 ± 3.1</td>
<td>19.3 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>567.7 ± 3.5</td>
</tr>
<tr>
<td>CHK21</td>
<td>261.7 ± 6.8</td>
<td>233.1 ± 3.2</td>
<td>20.8 ± 2.7</td>
<td>2.0 ± 0.1</td>
<td>515.6 ± 8.0</td>
</tr>
<tr>
<td>CHK23</td>
<td>326.0 ± 6.0</td>
<td>231.4 ± 5.7</td>
<td>16.7 ± 0.5</td>
<td>3.0 ± 1.1</td>
<td>549.1 ± 8.3</td>
</tr>
</tbody>
</table>

*Values are the average micrograms hexose per milligram (dry weight) of cell wall ± standard deviations of the results for three experiments.

*Values are the average micrograms glucosamine per milligram (dry weight) of cell walls ± the standard deviation.

CAF2 > CHK21, P = 0.02.

CAF2 > CHK21, P = 0.04.
Side chain AGRUs are characterized as those attached to the RT (reducing-terminus side chain, RTSC) (Fig. 2, structure 2) and those attached along the backbone (SC) (Fig. 2, structure 3) at the 6-position of the AGRU in the linear glucan chain. We used these structural assignments to describe differences among strains in isolated glucans and to quantify several structural features, including the degree of polymerization (DP) and degree of branching (DB), as well as levels of (1-3)- and (1-6)-linkages by methodologies described elsewhere [56,57]. Two different RT end groups, each containing α and β anomers, are evident in the *C. albicans* spectra (Fig. 3). These structures are supportive of the glucan polymer chain growth model that adds AGRUs through linear (1-3)-linkages with an occasional (1-6)-linked branch point [58].

The % distribution of structures 1 and 2 in CHK21 was altered compared to CAF2 (Fig. 3 and Table 3). For strain CHK21, DP was reduced by 50%, the level of (1-6) linkages increased four-fold, and the RT level increased two-fold relative to (1-3) linkages compared to CAF2 (Fig. 3 and Table 3). Thus, the β-glucan from strain CHK21 has reduced β-1,3 linkages but there is more β-1,6 branching.

### 3.3. Western blot analysis of hyphal and yeast extracts

β-Mercaptoethanol and lyticase were used to obtain cell wall extracts from strains CAF2 (wild-type), CHK21 (*chk1* null mutant), and CHK21 (Fig. 3). Western blot analysis was performed to detect the expression levels of the β-glucan biosynthesis-related genes RT1, RT2, and SRT1 in CAF2 and CHK21 strains. The blots showed a decrease in the expression of RT1 and RT2 in CHK21 compared to CAF2, while SRT1 expression remained similar in both strains. This suggests a reduction in the expression of RT1 and RT2 in the β-glucan biosynthesis pathway in CHK21.
null) and CHK23 (gene-reconstituted). When examined by Western blot, we observed that the reactivity of the extracts from CHK21 with Mab B6 (acid-stable epitope) was different from that of similarly prepared extracts from CAF2 and CHK23. Those differences were characterized by the presence of reactive components from CHK21 of lower molecular mass (range of 50 to 150 kDa) in addition to the higher molecular mass proteins that were common to both CHK21 and CAF2 (Fig. 4). The lower molecular mass reactive manoprotein specific for CHK21 were apparent in both yeast (Y) and mycelium (M) preparations. On the other hand, the Western blot profiles of extracts from both yeast and hyphae of CAF2 and CHK21 were similar when reacted with Mab B6.1 (acid-labile epitope) (Fig. 4). It should also be mentioned that similar profiles between CAF2 and CHK21 were observed with Mab C3.1, an IgG Mab that reacts with the acid-labile oligosaccharides of L-1,2 phosphomannan derived from the acid-labile fractions of total mannan (data not shown).

In comparison, the Western blot profiles of CHK23 (revertant) were very similar to those of CAF2 with Mabs B6, B6.1 and C3.1 (data not shown). Thus, these observations suggest that the acid-stable mannan oligosaccharides of mannoproteins are altered in CHK21. An explanation for this result might include a partial loss of mannosyl transferase activity that is associated with elongation of the acid-stable oligosaccharide side chains. This partial loss may not necessarily be associated with just one mannosyl transferase since an analysis of strains with the polyclonal antiserum to the major mannan epitopes of C. albicans using the IATRON diagnostic kit (Iatron Diagnostics, Tokyo, Japan) suggests an overall reduction in the ability of CHK21 to agglutinate in the presence of antisera to the 1.4,5 and 6 epitopes (data not shown).

In addition to the differences described above, it is also important to point out that not all mannoproteins from wild-type cells (CAF2) contain both acid-labile and acid-stable side chains (Fig. 4, compare reactivity to Mab B6.0 with Mab B6.1). It would appear that the lower molecular mass mannoproteins contain only acid-labile side chains, since these mannoproteins only reacted with Mab B6.1 and not Mab B6.0.

3.4. FACE analysis of mannan side chains

FACE analysis provides a method to examine the relative abundance of oligosaccharide side chains present in the cell wall. The charged fluorophore, ANTS, binds to the free terminal aldehyde of individual oligosaccharides. Comparisons can be made between species, or as in our study, between wild-type and a gene-deleted strain. Our results indicate that the acid-stable oligosaccharides obtained by acetylation (which include the epitope recognized by Mab B6.0) were truncated in the chk1/chk1 null (CHK21) mutant but not in CAF2 (wild-type cells) or in the revertant strain, CHK23 (Fig. 5). Moreover, the oligosaccharide side chains of the acid-labile (obtained by acid hydrolysis, phosphate-linked) as well as the O-elimination fractions (O-linked via serine/threonine residues) appeared similar in all three strains (Fig. 6). Recall that the Western blot profiles of extracts from both yeast and germ tubes appeared altered when reacted only with Mab B6.0 (acid-stable) and not Mab B6.1 (acid-labile), the latter of which reacts with the acid-labile fractions of mannan. Thus, the altered mannan phenotype of CHK21 was detected by both Western blot and FACE.

3.5. Hydrophobicity and drug sensitivity of strains

We used the hydrophobic microsphere assay (HMA) to measure the cell surface hydrophobicity of the paired set of CHK1 mutant strains compared to CAF2 [55]. Our results indicate that the cell surface hydrophobicity of CHK21 was unchanged compared to CAF2 (% hydropho-
bicity = 81.1 ± 9.6 for CHK21 and 63.5 ± 9 for CAF2, no statistical difference). We compared the sensitivity of the chk1 deletion mutant to the antifungal drugs (amphotericin B, micronazole) that inhibit non-cell wall targets, as well as inhibitors of protein synthesis (neomycin, hygromycin), cell wall inhibitors (calcofluor white, cilofungin), or caffeine. We did not observe changes in the sensitivity of strain CHK21 to any of these compounds.

4. Discussion

Two-component signal transduction systems are believed to enable bacteria and fungi to adapt to stress conditions that exist in the host and in the environment. Among fungi, sensor HK-RR proteins activate pathways that participate in resistance to both oxidative and osmotic stress in *S. cerevisiae* and *Schizosaccharomyces pombe* [4,5]. In *Neurospora crassa*, the HK nik-1 is required for hyphal development [59]. The HKs of *C. albicans* participate in functions related to morphogenesis, as stated above. Likewise, a *hog1* mutant had defective morphogenesis, characterized by abnormal growth in hyphal-inducing media [60]. More recent data point to a role for the RR protein Skn7p of *S. cerevisiae* in cell wall biosynthesis, signifying that this adaptive pathway may regulate cell wall mannosylation and also protect cells from osmotic shock [34].

Our interest in cell wall biosynthesis and the role of CHK1 in these events was precipitated by previously published observations [8,13,21,22]. First, hyphal forms of the CHK1 mutant flocculate extensively in liquid media, implying that strains have an altered cell surface. Second, we have shown that yeast cells of the CHK1 mutant are less able to adhere to reconstituted human esophageal tissues [21,22], again implying that a cell surface change is associated with each deletion. More recently, we have observed by transmission electron microscopy that the cell surface of the *chk1* mutant (CHK21) is smooth in comparison to the fibrillar cell wall of wild-type cells, CAF2 (unpublished). Thus our efforts in this study were focused upon characterizing the changes in mannan, glucan and chitin content of mutant cell wall.

The current study in part explains the observations on the cell surface of CHK21 mentioned above. Thus, Chk1p probably regulates directly or indirectly the expression of cell wall components. Our results indicate that the hexose content is statistically lower in the alkali-soluble fraction of purified cell wall from CHK21 (see Table 2) compared to CAF2 and the gene-reconstituted strain (CHK23). The reduction in hexose content would appear to be due to the truncation in acid-stable mannan side chains but not in the side chains of the acid-labile or O-linked side chains (see Fig. 5). We speculate that the truncation of the acid-stable side chains reflects the lower molecular mass of mannoproteins seen in the *chk1* mutant by Western blot analysis.

Our observations extend beyond a comparison of mutant and parental cells, since the reactivity of parental

![Fig. 5. FACE analysis of the oligosaccharides from strains of *C. albicans*. O-linked (β-eliminated oligosaccharide side chains (left), acid-solubilized oligosaccharides (center), and acid-stable oligosaccharides obtained by acetolysis (right) are shown. Molecular markers of hydrolyzed dextran are indicated on the left. The relative migration index is assigned unit values based upon the number of hexose residues (G2 = two hexose units); G1–G12 represent increasingly larger oligosaccharides.](image)

![Fig. 6. A comparison of the β-glucans of CAF2 (wild-type cells, A) and CHK21 (*chk1* null, B) are represented. The darker line drawing represents the β-1,3 glucan and the lighter, shorter lines are meant to indicate the relative abundance of β-1,6 glucan side chains. The latter are illustrated to reflect the four-fold increase in the β-1,6 side chains in strain CHK21, while the reduced size of the β-1,3 glucan in CHK21 is also shown. Both CWPs and PIR proteins are shown as circles, and each type of protein is shown as covalently attached to (1-3) glucan (PIR) or (1-6) glucan (CWP). The reduction in acid-stable mannan side chains is indicated for the CHK21 mutant.](image)

A. CAF2

B. CHK21
and, in this study, strains of differences in cell wall composition among different species with the oligosaccharide truncation in the proteins observed by Western blot may be associated says for chitin content were compared. RTLinear ratio)). This may account for the similar hexose CHK21, the degree of CHS2 strain CHK21 was considerably reduced (see Table 3, (1-3/total chitin content of a strain of -1,3 glucan increased approximately four-fold in strain CAF2 versus -1,6 glucan. Thus, the NMR method used by Lowman and Zymolyase-insoluble cell wall fractions from the cells that display equal representation of acid-stable and -labile, phosphodiester-linked side chain oligosaccharides of mannoproteins. The total hexose content of both the Zymolyase-soluble and Zymolyase-insoluble cell wall fractions from the chk1 mutant was similar to parental cells. Likewise, the chitin content was statistically similar for all strains, and all strains were equally susceptible to chitin and glucan inhibitors. It should be stated that extractions of cell wall components often require different methodologies. In support of this hypothesis, Munro et al. [61] have found that the total chitin content of a strain of C. albicans deleted in CHS2 (chitin synthase 2) varied markedly when three assays for chitin content were compared.

While the extent of β-1,6 glucan branching to the linear β-1,3 glucan increased approximately four-fold in strain CHK21, the degree of β-1,3 linkages (polymerization) of strain CHK21 was considerably reduced (see Table 3, (1-3/RTlinear ratio)). This may account for the similar hexose content of the two strains as obtained by Zymolyase treatment following alkalai digestion (Table 2). Of importance, Lowman et al. [57] have shown that the NMR studies of β-glucan from C. albicans versus S. cerevisiae are supportive of the biochemical data presented by Klis et al. [62]. In the latter study, the major quantitative biochemical difference in the cell wall of these two yeasts was the level of β-1,6 glucan. Thus, the NMR method used by Lowman et al. [57] is a more than reasonable way of verifying differences in cell wall composition among different species and, in this study, strains of C. albicans.

As mentioned above, the difference in profile of mannoproteins observed by Western blot may be associated with the oligosaccharide truncation in the chk1 mutant. Alternatively, an explanation of the results we observed in the Western blots of extracts from CAF2 versus CHK21 is that the additional β-1,6 branching may account for a greater yield of extractable mannoproteins, as it is certain that GPI-CWPs bind to the β-1,3 glucan through β-1,6-linked glucan. Thus more branching results in more extractable cell wall proteins, but there seems to be some indication that in the mutant a specific class of lower molecular mass mannoproteins is now over-expressed.

In Fig. 5 we present a model of the cell wall β-glucans and mannan of strain CHK21 (chk1 null) compared to wild-type cells (CAF2). Interestingly, the changes observed in the cell wall of strain CHK21 were not correlated with any differences in hydrophobicity or sensitivity to antifungals and other inhibitors.

Of importance, we are currently measuring temporal events related to cell wall biosynthesis in CHK21, since compensatory changes occur as a consequence of deletions in genes encoding cell wall regulatory or structural genes. For example, Fonzi [28] has shown that deletions in PHR1, which processes β-1,3 glucans to facilitate attachment of β-1,6 glucans, resulted in an increased cell wall content of chitin that was preceded by an increase in cross-linking of chitin between β-1,6 glycosylated mannoproteins and chitin. Therefore, temporal studies with the chk1 mutant may provide observations on the cause–effect relationship between glucan and mannan that we described in the current study.

Strain CHK21, as indicated above, is avirulent in a murine model of hematogenously disseminated candidiasis while virulent in a rat model of vaginitis [11]. We have shown that CHK21 is more susceptible to the growth-inhibitory and killing effects of human polymorphonuclear leukocytes (PMNs) [63]. This observation fits well with the current dogma that PMNs are critical to immunity against invasive disease but not critical to protection against vaginitis [64–68]. Based upon the observations described herein, the glucan and mannan of the mutant may result in a heightened response of PMNs to the organism, since glucans have been suggested as biological response modifiers in candidiasis [69].

Acknowledgements

This work was supported by Public Health Service Grants from the National Institute of Allergy and Infectious Diseases, AI47047, AI07510, and AI43465 (R.C.), AI 24912 and AI 37194 (J.C.), CA88456 from the National Cancer Institute (R. Cihlar) and AI 45829 (D.W.).

References


