Glucan and glycogen exist as a covalently linked macromolecular complex in the cell wall of Candida albicans and other Candida species

Douglas W. Lowman\textsuperscript{a,b}, M. Sameer Al-Abdul-Wahid\textsuperscript{c}, Zuchao Ma\textsuperscript{a,b}, Michael D. Kruppa\textsuperscript{b,d}, Elena Rustchenko\textsuperscript{e}, David L. Williams\textsuperscript{a,b,*}

\textsuperscript{a} Department of Surgery, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA
\textsuperscript{b} Center of Excellence in Inflammation, Infectious Disease and Immunity, PO Box 70442, East Tennessee State University, Johnson City, TN, USA
\textsuperscript{c} Department of Surgery, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA
\textsuperscript{d} Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, NY, USA
\textsuperscript{e} Department of Biomedical Sciences, Quillen College of Medicine, East Tennessee State University, Johnson City, TN, USA

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\textbf{ABSTRACT}

The fungal cell wall serves as the interface between the organism and its environment. Complex carbohydrates are a major component of the Candida albicans cell wall, i.e., glucan, mannan and chitin. β-Glucan is a pathogen associated molecular pattern (PAMP) composed of β-(1 → 3,1 → 6)-linked glucopyranosyl repeat units. This PAMP plays a key role in fungal structural integrity and immune recognition. Glycogen is an α-(1 → 4,1 → 6)-linked glucan that is an intracellular energy storage carbohydrate. We observed that glycogen was co-extracted during the isolation of β-glucan from C. albicans SC5314. We hypothesized that glucan and glycogen may form a macromolecular species that links intracellular glycogen with cell wall β-(1 → 3,1 → 6)-glucan. To test this hypothesis, we examined glucan-glycogen extracts by multi-dimensional NMR to ascertain if glycogen and β-glucan were interconnected. \textsuperscript{1}H NMR analyses confirmed the presence of glycogen and β-glucan in the macromolecule. Diffusion Ordered Spectroscopy (DOSY) confirmed that the β-glucan and glycogen co-diffuse, which indicates a linkage between the two polymers. We determined that the linkage is not via peptides and/or small proteins. Our data indicate that glycogen is covalently linked to β-(1 → 3,1 → 6) glucan via the β-(1 → 6)-linked side chain. We also found that the glucan-glycogen complex was present in C. dublinensis, C. haemulonii and C. auris, but was not present in C. glabrata or C. albicans hyphal glucan. These data demonstrate that glucan and glycogen form a novel macromolecular complex in the cell wall of C. albicans and other Candida species. This new and unique structure expands our understanding of the cell wall in Candida species.

\textbf{Introduction}

The fungal cell wall is the primary interface between the fungus and its environment (Cassone et al., 1987). The cell wall of Candida albicans is composed primarily of a layer of β-(1 → 3)-linked glucosyl repeat units in a linear backbone chain with side chains containing β-(1 → 6)-linked glucosyl repeat units, i.e., a β-(1 → 3,1 → 6)-glucan. In addition to glucan, approximately 1% of the cell wall composition contains chitin, which may be attached to the glucan through a (1 → 6)-linkage between the reducing terminus of the glucan and chitin (Kapteyn et al., 2000; Surarit et al., 1988). The reducing terminus of chitin has also been postulated to be attached to (1 → 3)-linked glucan chains through (1 → 2)- or (1 → 4)-linkages (Kollár et al., 1995). Exterior to the glucan and chitin, on the outer surface of the fungal cell wall, is a layer of mannanprotein and mannans which plays a role in innate immune recognition of the fungus and may also aid in attachment to environmental surfaces (Kruppa et al., 2011).

Glycogen is an α-(1 → 4,1 → 6)-linked glucan that is an intracellular energy storage carbohydrate in yeast (Arvindekar and Patil, 2002). In 2002, Arvindekar and Patil (2002) reported that glycogen was present in both the cell wall and cytoplasm of Saccharomyces cerevisiae. They proposed that glycogen was part of a cell wall complex that linked to glucan.
through the β-(1→6) linked side chain. However, Arvindekar and Patil did not provide direct evidence in support of their proposed structure.

We have studied cell wall carbohydrates derived from a variety of fungi (Kruppa et al., 2009; Lowman et al., 2011b; Mueller et al., 1997). Most recently, we have employed \textit{C. albicans} as our model system for examining the composition and structure of the fungal cell wall (Kruppa et al., 2011; Lowman et al., 2011a). \textit{C. albicans} is an important opportunistic fungal pathogen, thus making it a logical choice for detailed study (Brown et al., 2012; Delaloye and Calandra, 2014; Gow and Hube, 2012). We and others have reported that the \textit{C. albicans} cell wall is a highly dynamic organelle that modulates cell wall carbohydrate structure and composition in response to environmental cues (Kruppa et al., 2011; Lenardot et al., 2020; Lowman et al., 2011a).

During the course of our studies on \textit{C. albicans}, we found that glycogen was routinely co-extracted with cell wall β-(1→3,1→6) glucan. Our extraction methodology is optimized for glucan and should result in the degradation or solubilization of glycogen (Lowman et al., 2003, 2014). Furthermore, glycogen is reported to be intracellular in \textit{C. albicans} and is not thought to be located in the cell wall (Rejasingham and Cawson, 1980). Thus, it was not clear why glycogen would co-extract with glucan. Herein, we report that glycogen is covalently linked to β-(1→3,1→6) glucan in the cell wall of \textit{C. albicans} SC5314, via the β-(1→6)-linked side chain, to form a macromolecular complex. We also confirmed the presence of the glucan-glycogen complex in \textit{C. dubliniensis}, \textit{C. haemulonii} and \textit{C. auris}. To the best of our knowledge, this is a new and novel finding, which expands our understanding of the cell wall of \textit{Candida} species.

Methods

Glycogen

Bovine liver glycogen Type IX was purchased from Sigma-Aldrich (St. Louis, MO) and was used as received.

Fungal strains and their growth

\textit{C. albicans} SC5314, \textit{C. dubliniensis}, \textit{C. glabrata} and \textit{C. haemulonii} were passaged for 6 days at 37 °C on sheep blood agar (Remel). The conditioned cells were grown in 2L of YPD (1% yeast extract, 2% peptone, 2% dextrose) at 37 °C for 20 h and harvested by centrifugation, washed 1x with dH2O, lyophilized and stored (-20 °C) until used for extraction. \textit{C. albicans} deletion strains \textit{fks1}+/−, \textit{fks2}−/− and \textit{fks3}−/− that were generated independently in parallel experiments. See Supplemental Table 1 for the description of these strains and their controls. The strains were cultivated as described above.

To determine whether the glucan-glycogen complex was found in \textit{C. auris}, we cultivated a reference strain (KCTC17810) and seven clinical isolates from different geographic regions and sources. The conditions were grown in 2L of YPD (1% yeast extract, 2% peptone, 2% dextrose) at 37 °C for 20 h and harvested by centrifugation, washed (5x) in Type I ultrapure H2O, and pelleted by centrifugation. The supernatant was discarded. Pronase (D.W. Lowman et al., 2011b) was used as received.

| C. auris strain identification | Drug resistance | Side chain length | Branching frequency | Glycogen (%) |
|-------------------------------|-----------------|------------------|---------------------|-------------|
| KCTCI7810                     | Single          | 4.5              | 20.7                | 6.1%        |
| 10-03-11-60                   | Single          | 6.3              | 39.5                | 3.7%        |
| 2012                          | Single          | 4.1              | 30.2                | 2.0%        |
| 10-05-12-56                   | Double          | 4.9              | 23.8                | 3.3%        |
| 2013                          | Single          | 4.6              | 17.5                | 6.5%        |
| 2014                          | Single          | 3.5              | 20.3                | 1.2%        |
| 2015                          | Single          | 2.5              | 24.7                | 2.5%        |
| 2015-22                       | Single          | 3.6              | 21.0                | 5.2%        |

1 Single - resistant to a single anti-fungal drug. Double – resistant to two anti-fungal drugs.

Table 1 Description of the \textit{C. auris} strains employed in this study: drug resistance, glucan structure and glycogen content in the glucan-glycogen macromolecular complex.

particulate. The product was ≥ 95% carbohydrate. The water-insoluble particulate, i.e., the glucan-glycogen complex, was washed (3x) with Type I H2O, lyophilized to dryness and stored (−20 °C) until analyzed. To confirm the reproducibility of our results, at least seven separate experiments were performed on \textit{C. albicans} SC5314 and other fungal strains.

Glucon was isolated from \textit{C. albicans} SC5314 hyphae as previously described by our group (Lowman et al., 2013). Briefly, lyophilized hyphal cell walls were extracted with 0.1 N NaOH (15 min at 100 °C) followed by neutralization to pH 7.0. The neutral residue was extracted with 1.0 N H3PO4 (15 min at 100 °C), then neutralized to pH 7.0. The lipids were removed from the hyphal cell wall with boiling absolute ethanol (15 min).

Amylase treatment of the glucan-glycogen complex

To determine whether the glycogen could be removed from the glucan-glycogen complex, we employed α-amylase (Sigma Aldrich Cat. # A4551, Lot # SLBX3672). Amylase (1 mg = −500–1500 U) was dissolved in Dulbecco’s PBS at 1 mg/mL. The α-amylase (0.5 mL = −250–750 U) was added to the complex and incubated at 37 °C for 60 min on a rotary shaker (50 rpm). The resulting product was harvested by centrifugation, washed (5x) in Type I ultrapure H2O, followed by lyophilization and stored (−20 °C) until analyzed.

Pronase Treatment of the glucan-glycogen complex

Pronase (Roche Mannheim, Germany) from \textit{Streptomyces griseus} (specific activity = 7 U/mg) was dissolved in ultrapure water at 10 mg/mL. The pronase solution was incubated for 20 min at 65 °C to reduce non-specific glycosidase activity. The glucan-glycogen complex (~20 mg) was rehydrated (24 hrs) with Type I ultrapure water (~3 hr) and pelleted by centrifugation. The supernatant was discarded. Pronase (1...
mg (~10 mg of carbohydrate) was added (2 mL total volume) to the pelleted carbohydrate and incubated for 4 hrs at 37 °C. The glucan-glycogen complex was washed (5×) with Type I water, harvested by centrifugation and lyophilized.

**NMR sample preparation, data collection and analysis**

Glycogen and the glucan-glycogen complex samples were dissolved in DMSO-d6 (Cambridge Isotope Laboratories) and 1D 1H NMR spectral data acquired with a Bruker Avance III 400 NMR spectrometer at 80 °C, using a 5-mm NMR tube, 65,536 data points, 100 scans, sweep width of 20.69 ppm centered at 6.175 ppm, a 1-s relaxation delay and processed with exponential apodization using a 0.3 Hz line broadening. Either trifluoroacetic acid-d2 (TFA) (Cambridge Isotope Laboratories) or formic acid-d2 (Cambridge Isotope Laboratories) was added to the NMR solution to shift the exchangeable proton resonance downfield. 1H 2D Diffusion Ordered SpectroscopicY (DOSY) spectra were collected on a Bruker Avance III 600 NMR spectrometer at the University of Guelph, Guelph, Ontario, Canada. The NMR sample solution was prepared by dissolving 5 mg of the sample in 600 μL DMSO-d6, stirred at 60 °C for 2 hr. TFA (50 μL) was added to the solution prior to transfer to the NMR tube, to again shift the exchangeable proton resonance away from the carbohydrate spectral region and to improve solubility in the DMSO-d6.

The DOSY experiment used a bipolar pulse pair gradient sequence (Bruker pulse sequence ledpp2ga) (Wu et al., 1995). Each encoding/decoding gradient pair was 2 msec in duration with 5% smoothing at the start and end of the gradient pulse (Bruker shape file SMSQ10.100), the gradient strength was arrayed in 48 linear steps from 5% to 95% of the maximum gradient strength (nominal maximum strength is 53.5 G/cm), and the diffusion time between gradient pairs was 200 msec. The NMR sample volume was 560 μL to minimize the effects of convection on the diffusion measurement. For each diffusion step, 4 individual datasets using 28 scans each were collected, then summed for a total of 112 scans, using a relaxation delay of 5 sec, an acquisition time of 3.04 sec, and a sweep width of 17.96 ppm centered at 6.176 ppm. DOSY spectra were processed with the Topspin dosy2d processing macro, with baseline correction and line broadening of 0.2 Hz applied in f1. Spectra were processed using Bruker TopSpin software version 4.0.9 on a MacBook Pro running the Catalina operating system (version 10.15.7) and with TopSpin software resident on the NMR spectrometer. DOSY spectra were also processed with GNAT (https://www.nmr.chemistry.manchester.ac.uk/?q=node/430) at the University of Guelph.

**Treatment of the glucan-glycogen complex with TFA to enrich for (1 → 6)-α glucan**

TFA was added (50 μL) to the glucan-glycogen complex, followed by incubation for 9.5 days at 6 °C. TFA hydrolysis of the glucan-glycogen complex reduced the number of β-(1 → 3) linkages in the glucan, thus enriching for β-(1 → 6) glucan. Following incubation with TFA the complex was analyzed by DOSY as described above.

**Quantification of the glycogen content in the glucan-glycogen complex**

The percent glycogen was calculated by measuring the integral area of the resonance for the α-(1 → 4)-linked anomic protons in glucogen relative to the total integral areas of the resonances for the α-(1 → 4)-linked anomic protons of glycogen and β-(1 → 3) and β-(1 → 6)-linked glucan anomic protons. The integral area of the resonance for the α-(1 → 6)-linked anomic protons of glycogen was not used in this calculation due to their very low abundance in these glycogen polymers.

**Results**

**Identification of glucan and glycogen in the macromolecular complex**

The 1H NMR spectrum of the glucan portion of the glucan-glycogen complex (Fig. 1A) is characteristic of β-(1 → 3,1 → 6)-glucan. Resonances between 4.0 and 4.6 ppm arise from the anomeric proton and one of the methylene protons of the β-(1 → 6)-linkage in the side chain and the anomeric proton in the β-(1 → 3)-linkage of the glucan backbone (Lowman et al., 2011b). In addition to glucan, the complex contains glycogen by comparison with an authentic glycogen sample (Fig. 1B). Glycogen is a branched helical polymer of glucosyl repeat units (RU’s) connected by α-(1 → 4)- and α-(1 → 6)-linked glycosidic bonds. Randomly placed along the linear α-(1 → 4)-linked branches are branch points where additional chains of linear α-(1 → 4)-linked glucosyl RU’s are connected by α-(1 → 6)-linkages at the branch point. The anomeric proton chemical shifts in the α-(1 → 4)- and α-(1 → 6)-glycosidic linkages of glycogen are consistent with previously reported literature (Zang et al., 1991). Treatment of the glucan-glycogen complex with α-amylase, which reacts with α-linked carbohydrates, resulted in complete loss of glycogen (Fig. 1C), leaving the β-linked glucan intact. These observations strongly support the glycogen identification. We also examined whether the glucan-glycogen complex might be connected by a peptide or small protein. To eliminate any protein components, we treated the glucan-glycogen complex with pronase. Pronase treatment did not change the NMR spectrum of the complex, thus demonstrating that the linkage is not via peptides and/or small proteins (Fig. 1D).

**Diffusion Ordered SpectroscopicY (DOSY) demonstrates that glucan and glycogen exist as a complex**

To determine if the glycogen and glucan are one macromolecule or separate molecules, a DOSY (Groves et al., 2004; Politi et al., 2006) spectrum was collected (Fig. 2A). In this experiment, compounds in solution diffuse based upon their hydrodynamic volumes. Since dimethylsulfoxide (DMSO) and ethanol are the smallest molecules, they diffuse the most while the larger glucan and glycogen polymers diffuse slower.
the least. Glucan and glycogen both diffuse at the same rate as indicated by their resonances falling on the same row in the DOSY spectrum (red line, Fig. 2A). This confirms that glucan and glycogen exist as an interconnected macromolecule.

To gain greater insight into the linkage between the glucan and glycogen, the glucan-glycogen complex was treated with TFA following DOSY analysis (Fig. 2B). The gentle TFA hydrolysis of the glucan-glycogen complex at low temperature reduced the number of \( \beta-(1 \to 6) \) linkages in the glucan, thus enriching for \( \beta-(1 \to 6) \) glucan. As can be seen, the \( \beta-(1 \to 6) \) glucan enriched material diffuses at the same rate as the glycogen, indicating that the enriched \( \beta-(1 \to 6) \) glucan and glycogen exist as a complex (Fig. 2B). This data suggests that the glucan and glycogen are linked via the \( \beta-(1 \to 6) \)-linked side chain.

**C. albicans glucan and glycogen are covalently linked via the \( \beta-(1 \to 6) \)-linked glucan side chain**

Of the possible structural motifs that might explain the interconnection between glucan and glycogen, it is most likely that glycogen and the glucan are linked between the \( \alpha-(1\text{-}4) \)-linked reducing terminus of glycogen and the non-reducing terminus of the \( \beta-(1 \to 6) \)-linked glucan side chain through an \( \alpha-(1 \to 6) \)-linked glycosidic bond, \( -4\text{Glc} \text{o}_1-6\text{Glc} \beta 1- \). This structural fragment resembles the structure of \( \beta \)-isomaltose (\( \text{Glc} \text{o}_1-6\text{Glc} \beta \)). The chemical shift of the anomeric proton of the non-reducing terminus of the \( \beta \)-isomaltose is 4.91 ppm in \( \text{D}_2\text{O} \) (Arnold and Repeta, 1995). There is a small, broad proton resonance at 4.93 ppm in the \( ^1\text{H} \) NMR spectrum of the glucan-glycogen complex (Fig. 2A and B). This resonance declines in size in the spectrum of C. dublinensis (Fig. 3B) and is virtually non-existent in the spectrum of C. haemulonii (Fig. 3D). Therefore, due to the deshielded nature of this resonance, we assign the resonance at 4.93 ppm to the glucan reducing terminus anomeric proton in the \( \alpha \)-configuration (Glc\( o \)) that is attached to the non-reducing terminus of the \( (1 \to 6) \)-linked glucosyl side chain of the glucan through an \( \alpha-(1 \to 6) \)-linked glycosidic bond (-4\text{Glc} \text{o}_1-6\text{Glc} \beta 1-) forming a glucan-glycogen macromolecule. These results demonstrate that glycogen and the glucan form a novel covalently linked structure in the C. albicans cell wall.

The glucan-glycogen complex is not present in all Candida species

An important question was whether the glucan-glycogen macromolecular complex was present in Candida species other than C. albicans. To address this question, we examined C. dublinensis, C. glabrata, and C. haemulonii along with C. albicans SC5314 (Fig. 3) and C. auris (Fig. 4 and Table 1) for the presence of the glucan-glycogen complex. We found that C. dublinensis (Fig. 3B) possesses the glucan-glycogen complex at a level comparable to C. albicans (Fig. 3A). In contrast, C. glabrata did not contain the glucan-glycogen complex (Fig. 3C). C. haemulonii showed trace amounts of the glucan-glycogen complex (Fig. 3D).

We also examined one reference strain (KCTC17810) and seven clinical strains of C. auris for the presence of the glucan-glycogen complex (Fig. 4). All eight C. auris strains show evidence of the glucan-glycogen complex, albeit at much lower levels than that observed in C. albicans and C. dublinensis (Figs. 1 and 3). Interestingly, the levels of glucan-glycogen in C. auris were comparable to or slightly higher than C. haemulonii (Fig. 3D). \(^1\text{H} \) NMR spectra from three representative C. auris strains (Fig. 4) demonstrate the range of glycogen found in the eight C. auris strains. The amount of the glycogen in the C. auris glucan-glycogen complexes ranged from 1.2 to 6.5% across all eight strains (Table 1), which is approximately 97 to 82% less than that found in the control strain (CAF 2–1) of C. albicans (Table 2).

**Glycogen is complexed with glucan in C. albicans yeast, but not hyphal forms**

We have previously shown that there are structural differences
between glucan derived from the yeast and hyphal forms of *C. albicans* SC5314 (Lowman et al., 2014). This prompted us to ask the question - is glycogen complexed with hyphal glucan in a fashion similar to yeast glucan? We found that in contrast to yeast glucan, hyphal glucan does not show the presence of glycogen (Fig. 5). This indicates that glucan in *C. albicans* hyphae is not complexed with glycogen.

The glucan-glycogen complex is present in *C. albicans* grown under different conditions

To determine if growth conditions impact the presence of the glucan-glycogen complex, we grew *C. albicans* SC5314 in YPD, blood and serum (Fig. 6). The $^1$H NMR spectra of the isolated glucans grown under the three growth conditions demonstrate the presence of the glucan-glycogen complex.

Table 2

| Strain         | Glucan (%) | Glycogen (%) | Glucan:Glycogen Ratio |
|----------------|------------|--------------|-----------------------|
| CAF2-1 ura +   | 63.3       | 36.7         | 1.7:1                 |
| CAF4-2 ura -   | 63.4       | 36.6         | 1.7:1                 |
| NF1-4 fks1 +   | 74.8       | 25.2         | 3.0:1                 |
| NF133 fks1 +   | 83.7       | 16.3         | 5.1:1                 |
| NFK2-2-12 fks2- | 53.7     | 46.3         | 1.2:1                 |
| NFK2-5-9 fks3- | 50.3       | 49.7         | 1.0:1                 |
| NFK3-7-19 fks3- | 58.6     | 41.4         | 1.4:1                 |
| NFK3-8-1 fks3- | 53.9       | 46.1         | 1.2:1                 |

1 A description of the *C. albicans* strains employed in this study is given in Supplemental Table 1. See also Suwunnakorn et al. (2018)

*C. albicans* hyphae is not complexed with glycogen.

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examined for the presence of the glucan-glycogen complex. C. albicans glucan synthase fks deletion strains
The ratio of glucan to glycogen in the macromolecular complex is altered in
Fig. 5.

D.W. Lowman et al.

C. albicans glucan synthase fks deletion strains show glucan to glycogen ratios that are
compared to either the control or parental strains (Table 2). In contrast, due to the lack of one copy of the essential FKS1 (Suwunnakorn et al., 2018), showed a higher glucan content versus glycogen content, when
compared to the parental, fks2−/− or fks3−/− strains (also see Table 2).

Glycogen content is based upon the integral area of the α-(1→4) resonance at
5.1 ppm. The x-axis is the chemical shift dimension.

Discussion

Herein, we present evidence that β-(1→3, 1→6)-glucan and glycogen exist as a novel macromolecular complex in the cell wall of C. albicans SC5314 and other Candida species. The 1D 1H NMR data of the glucan-glycogen complex clearly shows the presence of β-(1→3, 1→6)-glucan and α-(1→4, 1→6) glycogen. In Fig. 1, resonances between 4.0 and 4.6 ppm arise from the anomeric proton in the β-(1→3)-linkage and the anomeric proton and one of the methylene protons of the β-(1→6)-linkage in the glucan (Lowman et al., 2014). The chemical shifts of the resonances for the anomeric protons of the α-(1→4) and
α-(1→6)-glycosidic linkages in glycogen, H1-4 and H1-6, respectively, are consistent with a previous report from the literature (Zang et al., 1991). These data confirm the presence of glycogen and glucan in the complex. Our data also confirm that the macromolecular structure of the complex is not dependent upon proteins or peptides. It is important to note that our analyses did not reveal any compounds within the complex other than glucan and glycogen.

DOSY is a well-established NMR method that provides insights into diffusion of macromolecular species in solution (Wu et al., 1995). In the context of this study, DOSY was used to analyze the glucan-glycogen complex. Our DOSY spectra show that the glucan and glycogen diffuse at the same rate (Fig. 2), indicating that they exist as a single macromolecular complex. We incubated the glucan-glycogen complex with TFA, which preferentially degraded the β-(1→3) linkages in the glucan, thus enriching for β-(1→6) glucan. The β-(1→6) glucan enriched material diffuses at the same rate as the glycogen, indicating that the enriched β-(1→6) glucan and glycogen exist as a complex (Fig. 2B). There is the possibility that the glucan and glycogen could exist as “free” or non-complexed moieties and that they diffuse at exactly the same rate in the DOSY experiment. We consider this to be a very remote possibility because the glucan and glycogen would have to have identical hydrodynamic volumes and diffusion coefficients under the NMR conditions employed. When considered as a whole, the evidence strongly supports the conclusion that glucan and glycogen exist as a macromolecular complex covalently linked via the β-(1→6) glucan side chains in C. albicans.

Our data suggest that the majority of the glucan isolated from C. albicans SC5314 is covalently linked to glycogen. This conclusion is
based on several observations. First, the isolation methodology that we employ is optimized for the extraction of glucans from fungal cell walls (Lowman et al., 2003, 2014). Second, the extraction method we employ should degrade or solubilize glycogen, but in this case the glucogen covalently linked to glucan appears to be more resistant to the degradation. Third, the DOSY data clearly shows that all of the glucan diffuses at the same rate as the glycogen, thus there does not appear to be any free glucan or free glycogen in our isolate. Since our methodology is designed to extract the majority of the glucan from the fungal cell, it is reasonable to conclude that the majority of the β-(1 → 3, 1 → 6)-glucan in the C. albicans SC5314 cell wall exists as a macromolecular complex with glycogen. It has been reported that there is a pool of branched β-(1 → 6)-linked glucan with β-(1 → 3)-linked glucan side chains in C. albicans (Iorio et al., 2008). In this study, we did not observe a glucan pool comparable to that described by Iorio and colleagues. Thus, we cannot comment on whether this glucan pool is covalently linked to glycogen.

We also found that the glucan-glycogen complex is present in some Candida species, but not all. Specifically, it is present in C. albicans and C. dublinensis at comparable levels and in C. albicans at lower levels. Trace amounts of the complex were found in C. haemulonii. In contrast, C. glabrata showed no evidence of the glucan-glycogen complex. We also discovered that C. albicans hyphal glucan does not appear to be complexed to glycogen. Our studies also showed that the glucan-glycogen complex was present in C. albicans SC5314 cultivated in YPD, human blood or human serum.

We also addressed the question “does drug resistance alter the glucan-glycogen complex?” To answer this question, we examined the glucan-glycogen complex in eight C. auris strains, all of which were single or double resistant to standard anti-fungal therapies. The glucan-glycogen complex was present in all eight C. auris strains, albeit at much lower levels than in C. albicans. We found no significant relationship between drug resistance and the glucan-glycogen complex in C. auris.

Arvindekar and Patil proposed that glycogen resides in two pools in S. cerevisiae – one soluble and the other insoluble (Arvindekar and Patil, 2002). The glucan-glycogen complex isolated in our studies is a water insoluble. To determine if any soluble glycogen was present, we examined the supernatants from the alkaline and acidic aqueous extraction phases. We identified soluble glycogen in the acidic phase extraction supernatant (data not shown). We cannot be certain if this is a separate pool of acid-soluble glycogen or whether this represents a degradation product of the glycogen in the glucan-glycogen complex. Additional studies will be required to resolve this question.

Additional support for the glucan and glycogen complex is provided by the studies with glucan synthase mutants. We utilized a series of C. albicans strains which are deficient in the glucan synthase pathway, i.e., FKS1, FKS2 and FKS3. We found the glucan-glycogen complex in all of the strains examined. Most importantly, the fks1+/− strain showed less glycogen content in the complex, when compared to the parental strains and fks2−/− and fks3−/− (Table 1). This result is in line with an earlier report that the function of FKS1 in the biosynthesis of the cell wall is different from that of FKS2 and FKS3 (Suwunnakorn et al., 2018). Depletion of one copy of FKS1 results in a substantial decrease of FKS1 transcripts and cell wall glucan, which is consistent with the role of Fks1p as a catalytic subunit of the glucan synthase complex. In contrast, FKS2 and FKS3 play regulatory roles by serving as negative regulators of FKS1 despite containing a conserved catalytic domain (Suwunnakorn et al., 2018). The decreased amount of glucan in the fks+/− strain is consistent with decreased glycogen content in the complex.

Based on the data, we propose two possible structural models for the glucan-glycogen complex (Fig. 8). First, the glucan-glycogen complex may exist as a membrane-spanning moiety in which the glycogen is

**Fig. 8.** Proposed models showing how the glucan-glycogen macromolecule may exist within the cell wall of C. albicans. Model A proposes that glycogen is present within the cell cytosol and is linked to glucan through a β-(1 → 6)-linked plasma membrane-spanning glucan side chain. The bond between glucan and glycogen is a unique α-(1 → 6) linkage (arrow). Model B proposes that glycogen may be present within the cell wall and is linked with the β-(1 → 6) glucan side chain via the α-(1 → 6) linkage. Our models are not mutually exclusive; thus, glycogen could be both intracellular and within the Candida cell wall.
intracellular and the glucan is located in the inner cell wall external to the plasma membrane (Fig. 8A). This model would require that the glucan-glycogen complex extends through the plasma membrane. In this model, we propose that the β-(1→6)-glucan side chain is part of a membrane spanning component of the complex which links the intracellular glycogen with the cell wall glucan. It is also possible that part of the glycogen molecule spans the plasma membrane. The membrane-spanning model presents some intriguing possibilities. It has been reported that the fungal biosynthetic enzyme α-L-arabinofuranosidase catalyzes the biosynthesis of cell wall glucan (Jimenez-Ortigosa et al., 2021). Glycogen is an α-linked glucose polymer that could serve as a monosaccharide reservoir for the synthesis of β-(1→3, 3→6)-glucan. If the glucan-glycogen complex spans the plasma membrane in proximity to β-(1→3)-glucan synthase, then it is possible that the discovery of the glucan-glycogen complex may provide important insights into fungal cell wall glucan biosynthesis. The fact that echinocandin drugs, which inhibit β-(1→3)-glucan synthase, are front-line therapies for fungal sepsis adds further relevance to the discovery of the glucan-glycogen complex (Eschenauer et al., 2007).

It is also possible that the glycogen exists within the fungal cell wall exterior to the plasma membrane, where it is covalently linked to cell wall glucan (Fig. 8B). While we are not aware of any evidence for glycogen in the Candida cell wall, our data do not exclude this possibility. If the glucan and glycogen complex exists in the cell wall architecture, this may have implications for anti-fungal innate immune responsiveness. Glucan is a Dectin-1 agonist, but it has also been implicated in TLR2 activation (Brown et al., 2003). Nakatani and colleagues have reported that TLR2 is essential for macrophage activation by glycogen (Nakatani et al., 2012). Thus, a glucan-glycogen complex in the cell wall could serve as a fungal PAMP. It is also important to note that our proposed models are not mutually exclusive. Thus, it is possible that glycogen, covalently linked to glucan, is present both intracellularly and in the cell wall.

Conclusion

This study provides insight into a previously unknown glucan-glycogen macromolecule that is present in the cell wall of C. albicans and other Candida species. We propose that the reducing terminus of glycogen is linked to the non-reducing terminus of the β-(1→6)-linked side chain of β-(1→3, 3→6)-glucan by an α-(1→6)-linkage. To the best of our knowledge, this is the first demonstration of a glucan-glycogen macromolecular complex in the cell wall of opportunistic fungal pathogens, such as C. albicans, C. dubliniensis and C. auris. These data add to our knowledge of the complexity to Candida cell wall architecture.

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CRediT authorship contribution statement

Douglas W. Lowman: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing - review & editing, Visualization, Funding acquisition. Elena Rustchenko: Resources, Writing - review & editing, Funding acquisition. David L. Williams: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tcrm.2021.100061.

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