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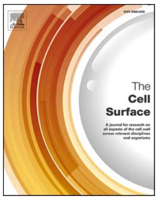

Glucan and glycogen exist as a covalently linked macromolecular complex in the cell wall of *Candida albicans* and other *Candida* species

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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. ^{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.

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 mannoprotein 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

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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 *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). *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 *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; Lenardon et al., 2020; Lowman et al., 2011a).

During the course of our studies on *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 *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 *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 *C. dubliniensis*, *C. haemulonii* and *C. auris*. To the best of our knowledge, this is a new and novel finding, which expands our understanding of the cell wall of *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

C. albicans SC5314, *C. dubliniensis, C. glabrata* and *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 dH_2O, lyophilized and stored (-20 °C) until used for extraction.

C. albicans deletion strains *fks1*+/−, *fks2*−/− and *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 *C. auris*, we cultivated a reference strain (KCTC17810) and seven clinical strains (see Table 1) as described previously (Bruno et al. 2020).

To examine the effect of different culture conditions on the glucan-glycogen complex, *C. albicans* reference strain SC5314 was grown in YPD, blood and serum as previously described by our group (Kruppa et al, 2011; Lowman et al 2011a). SC5314 was taken directly from the frozen stock and passaged onto YPD and serum (5% serum, 2% agar) plate media at 30 and 37 °C. Cells were passaged every 48 hrs for three times (Lowman et al., 2011a). The morphology of the cells was confirmed by microscopy.

Glucan-glycogen Complex Isolation

The glucan-glycogen complex was isolated from the various fungal species using the method for glucan extraction as described by our laboratory (Lowman et al., 2014) with modifications. Briefly, lyophilized fungal cells were extracted with 0.75 N NaOH (3x) and 0.5 N H_3PO_4 (2x) at 100 °C, followed by extraction with absolute ethanol (90 °C) to reduce lipids. The resulting product was a water-insoluble particulate. The product was ≥ 95% carbohydrate. The water-insoluble particulate, *i.e.*, the glucan-glycogen complex, was washed (3x) with Type I H_2O, lyophilized to dryness and stored (−20 °C) until analyzed. To confirm the reproducibility of our results, at least seven separate experiments were performed on *C. albicans* SC5314 and other fungal strains.

Glucan was isolated from *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 H_3PO_4 (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).

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

C. auris strain identification	Drug resistance[1]	Side chain length[2]	Branching frequency[3]	Glycogen (%)[4]
KCTC17810	Single	4.5	20.7	6.1%
10–03-11–60 2012	Single	6.3	39.5	3.7%
10–03-11–63 2012	Single	4.1	30.2	2.0%
10–05-12–56 2013	Double[5]	4.9	23.8	3.3%
10–05-12–63 2013	Single	4.6	17.5	6.5%
10–05-12–44 2014	Single	3.5	20.3	1.2%
10–05-12–52 2014	Single	2.5	24.7	2.5%
10–05-15–22 2014	Single	3.6	21.0	5.2%

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

[2] Glucan side chain length - The average number of glucopyranosyl repeat units in the β -(1 → 6)-linked side chain of the glucan in the glucan-glycogen complex.

[3] Glucan branching frequency – The average number of β -(1 → 3)-linked glucopyranosyl repeat units in the glucan backbone chain between each β -(1 → 6)- linked side chain. Larger numbers indicate fewer β (1 → 6)-linked side chains.

[4] The integral area of the resonance for the α -(1→4)-linked anomeric protons in glycogen relative to the total integral areas of the resonances for the anomeric protons of glycogen and β -(1→3)- and β-(1→6)-linked glucan anomeric protons expressed as a percentage.

[5] Resistant to azoles and amphotericin B.

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 H_2O, followed by lyophilization and stored (−20 °C) until analyzed.

Pronase Treatment of the glucan-glycogen complex

Pronase (Roche Mannheim, Germany) from *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 (5x) 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 ^{1}H 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-sec relaxation delay and processed with exponential apodization using a 0.3 Hz line broadening. Either trifluoroacetic acid-$_d$ (TFA) (Cambridge Isotope Laboratories) or formic acid-$_{d2}$ (Cambridge Isotope Laboratories) was added to the NMR solution to shift the exchangeable proton resonance downfield. ^{1}H 2D Diffusion Ordered SpectroscopY (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-d_6, 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 *ledbpgp2s*) (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)-β 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 anomeric protons in glycogen relative to the total integral areas of the resonances for the α -(1 →4)-linked anomeric protons of glycogen and β -(1 →3)- and β -(1 →6)-linked glucan anomeric protons. The integral area of the resonance for the α -(1 →6)-linked anomeric 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 ^{1}H NMR spectrum of the glucan portion of the glucan-glycogen complex (Fig. 1**A**) 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. 1**B**). 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 chains 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. 1**C**), 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. 1**D**).

Diffusion Ordered SpectroscopY (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. 2**A**). 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

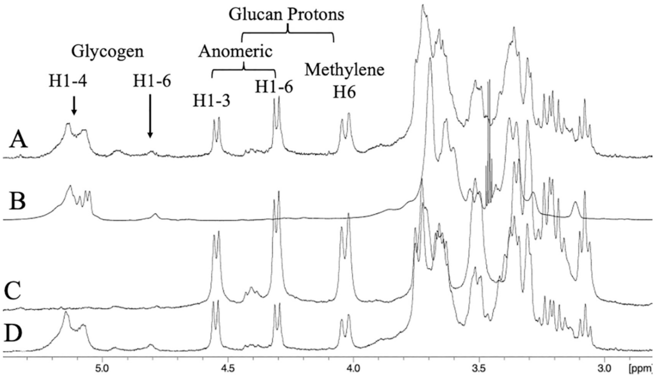

Fig. 1. Comparison of the ^{1}H NMR spectra of the glucan-glycogen complex isolated from *C. albicans.* (A) ^{1}H NMR spectrum of the glucan-glycogen macromolecular complex. The glucan portion of the complex is characteristic of β -(1 → 3,1 → 6)-glucan. (B) ^{1}H NMR spectrum of bovine glycogen. The presence of glycogen in the glucan-glycogen complex is confirmed by comparison with the bovine glycogen. In addition, the anomeric proton chemical shifts in the α -(1 → 4)- and α -(1 → 6)-glycosidic linkages of glycogen are consistent with the published literature (Zang et al., 1991). (C) ^{1}H NMR spectrum of the *C. albicans* glucan-glycogen complex after amylase treatment. Amylase reacts with α -linkages to enzymatically degrade the glycogen leaving the β -(1 → 3,1 → 6)-glucan intact, thus further confirming the identity of the glycogen. (D) ^{1}H NMR spectrum after pronase treatment does not result in the loss of glycogen from the complex, thus demonstrating that glycogen and the glucan are not connected by a peptide or small protein.

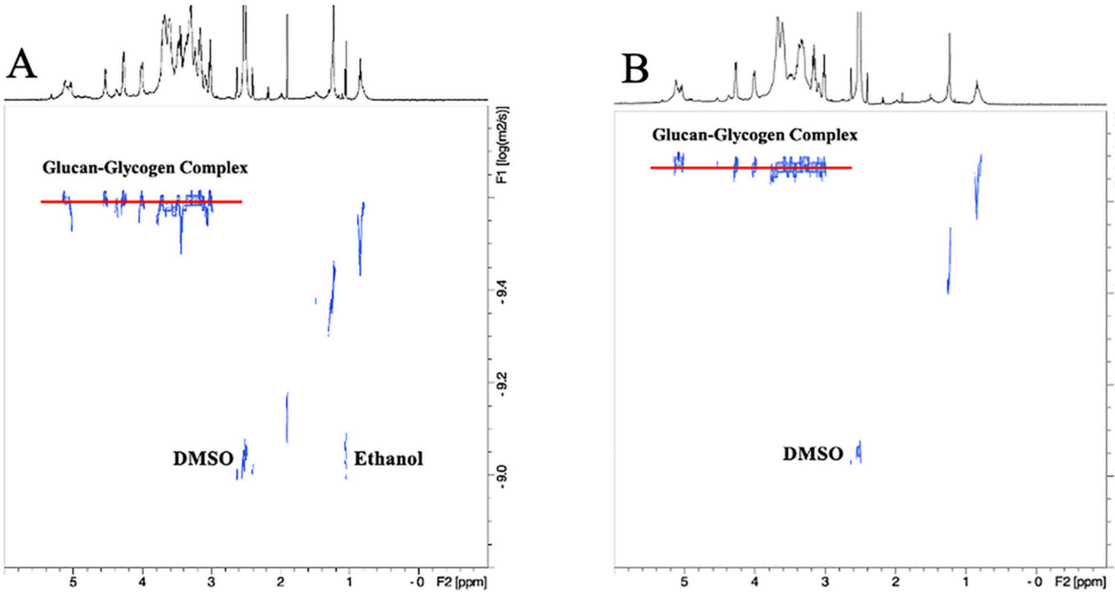

Fig. 2. Diffusion Ordered SpectroscopY (DOSY) 2D NMR spectra of the glucan-glycogen complex from *C. albicans* in DMSO$_{d6}$. (A) The resonances for the components of the complex, *i.e.*, glucan and glycogen, are in the same row (red line), thus they diffuse at the same rate, supporting the finding of an interconnected glycogen and glucan macromolecule. The dip in the diffusion row of the complex at 3.44 ppm is most likely due to the methylene group of ethanol. (B) 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. As can be seen, 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. The glucan-glycogen complexes in A and B do not diffuse at the same rate, due to differences in the hydrodynamic volumes of their macromolecular complexes following TFA hydrolysis in B. The x-axis is the NMR chemical shift dimension. The y-axis is the diffusion dimension. The presence of ethanol was also detected in A as the sample was treated with ethanol during isolation. The unlabeled signals are associated with a small amount of lipids that co-extracted with the glucan-glycogen complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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. 2**A**). 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 followed by DOSY analysis (Fig. 2**B**). The gentle TFA hydrolysis of the glucan-glycogen complex at low temperature reduced the number of β -(1 → 3) linkages in the glucan, thus enriching for β -(1 → 6) glucan. As can be seen, 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. 2**B**). This data suggests that the glucan and glycogen are linked via the β -(1 → 6)-linked side chain.

C. albicans glucan and glycogen are covalently linked via the β -(1 → 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 α -(1–4)-linked reducing terminus of glycogen and the non-reducing terminus of the β -(1 → 6)-linked glucan side chain through an α-(1 → 6)-linked glycosidic bond, -4Glcα1−6Glc β 1-. This structural fragment resembles the structure of β-isomaltose (Glcα1 -6Glc β). The chemical shift of the anomeric proton of the non-reducing terminus of β -isomaltose is 4.91 ppm in D$_2$O (Arnosti and Repeta, 1995). There is a small, broad proton resonance at 4.93 ppm in the ^{1}H NMR spectrum of the glucan-glycogen complex (Fig. 1**A and D and** Fig. 3**A and B**). This resonance declines in size in the spectrum of *C. dublinensis* (Fig. 3**B**) and is virtually non-existent in the spectrum of *C. haemulonii* (Fig. 3**D**). Therefore, due to the deshielded nature of this resonance, we assign the resonance at 4.93 ppm to the glycogen reducing terminus anomeric proton in the α-configuration (Glcα) that is attached to the non-reducing terminus of the (1 → 6)-linked glucosyl side chain of the glucan through an α-(1 → 6)-linked glycosidic bond (-4Glcα1−6Glc β 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. 3**B**) possesses the glucan-glycogen complex at a level comparable to *C. albicans* (Fig. 3**A**). In contrast, *C. glabrata* did not contain the glucan-glycogen complex (Fig. 3**C**). *C. haemulonii* showed trace amounts of the glucan-glycogen complex (Fig. 3**D**).

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. 3**D**). ^{1}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

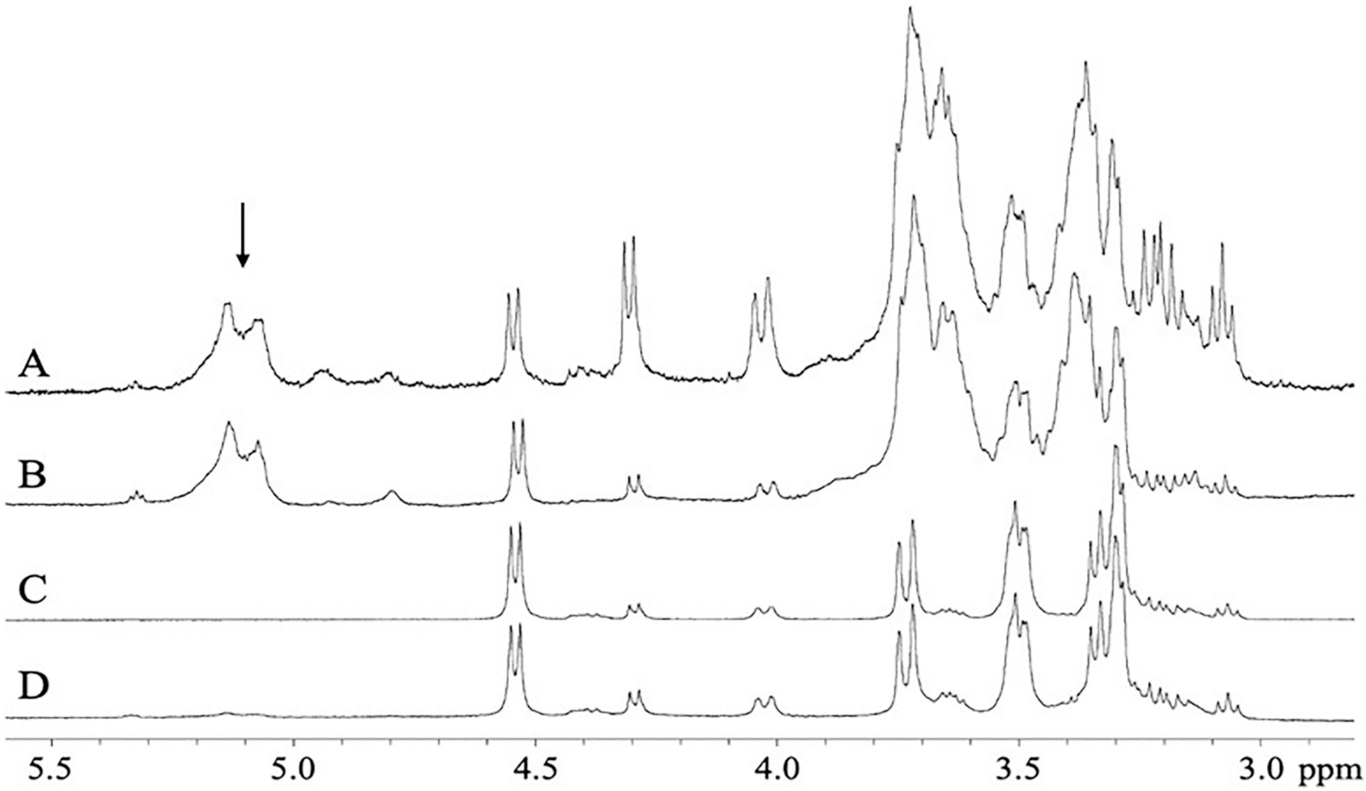

Fig. 3. ^{1}H NMR spectra of four *Candida* species demonstrating that the glucan-glycogen complex is present in some, but not all *Candida* species. *C. albicans* SC5314 (A) and *C. dubliniensis* (B) show the presence of the glucan-glycogen complex (arrow). In contrast, *C. glabrata* (C) did not show evidence of the glucan-glycogen complex. *C. haemulonii* (D) shows a trace amount of the glucan-glycogen complex. The glucan or the glucan-glycogen complex were extracted from *C. albicans* SC5314, *C. dublinensis*, *C. glabrata* and *C. haemulonii* in parallel and under identical conditions. The resonance at 4.93 ppm is assigned to the glycogen anomeric proton of the glucosyl repeat unit in the α -(1 → 6)-linked glycosidic bond −4Glcα1-6Glc β 1- connecting glycogen to the β -(1–6)-linked glucan side chain. The x-axis is the chemical shift dimension.

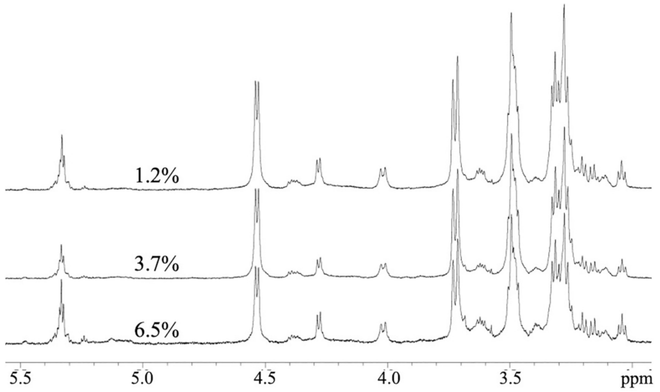

Fig. 4. Representative ^{1}H NMR spectra of the glucan-glycogen complex from three strains of *C. auris*. *C. auris* clinical strains show evidence of the glucan-glycogen macromolecular complex, albeit at much lower levels than in *C. albicans* SC4314 and *C. dublinensis*. We isolated the glucan-glycogen complex from one reference strain and seven clinical strains of *C. auris* (Table 1) in parallel and under identical conditions. The numbers above each spectrum show the relative amount of glycogen in each glucan-glycogen complex isolated from the three strains. The percent glycogen across all eight strains ranged between 1.2 and 6.5%. The x-axis is the chemical shift dimension.

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.

Table 2
Percentages of glucan and glycogen and the glucan-to-glycogen ratio in the macromolecular complex isolated from control, parental and glucan synthase mutant strains of *C. albicans*.[1]

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 fks2 −/−	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)

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.

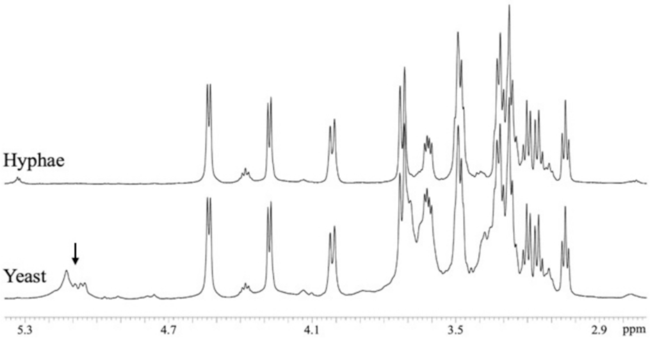

Fig. 5. *C. albicans* hyphal glucan is not complexed with glycogen. ^{1}H NMR spectrum of *C. albicans* SC5314 yeast shows the presence of the glucan-glycogen complex (arrow), but the NMR spectrum of the hyphal glucan (top) derived from *C. albicans* SC5314 does not. The yeast and hyphal cells were extracted in parallel and under identical conditions. The x-axis is the chemical shift dimension.

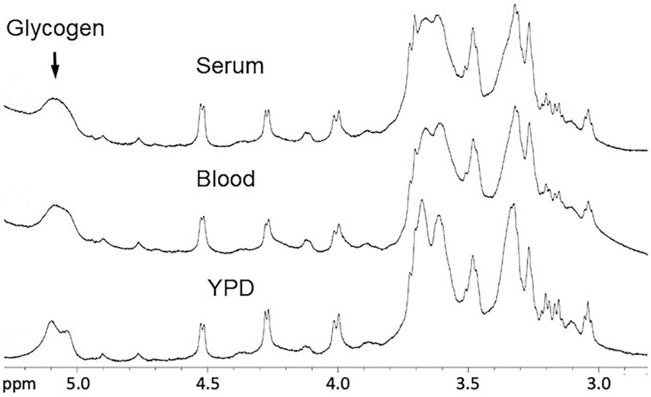

Fig. 6. Comparison of the ^{1}H NMR spectra of the glucan-glycogen complex in *C. albicans* SC5314 grown in YPD, blood or serum. As can be seen, the glucan-glycogen complex was present (arrow) in all three growth conditions. The x-axis is the chemical shift dimension.

The ratio of glucan to glycogen in the macromolecular complex is altered in C. albicans glucan synthase fks deletion strains

In this study, a *C. albicans* control strain (CAF 2–1), a parental strain (CAF 4–2) and six glucan synthase deletion strains (Table 2) were examined for the presence of the glucan-glycogen complex. ^{1}H NMR spectra for the control strain (CAF 2–1) (Fig. 7**A**) and three representative fks deletion strains, *i.e.*, *fks1*+/− (Fig. 7**B**), *fks2*−/− (Fig. 7**C**) and *fks3*−/− (Fig. 7**D**) are shown. Glycogen is clearly present at 5.1 ppm (arrow) in the stacked plots of the ^{1}H NMR spectra of the control strain and three mutant strains (Fig. 7**)**. As demonstrated in the DOSY data, glucan and glycogen are the only components in the macromolecular complex. From the NMR spectra (Fig. 7), the glucan and glycogen content of the macromolecular complex as a percentage and the ratio of glucan-to-glycogen was calculated (Table 2). The glucan and glycogen composition are essentially identical in the complexes isolated from the control and parental strains. Of great importance, the glucan-glycogen complex in the *ks1*+/*f*− strains, having half of the amount of glucan 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 either the control or parental strains (Table 2). In contrast, the fks2 and fks3 deletion strains show glucan to glycogen ratios that are approximately equivalent. It was also noted that the glucan content in *fks2*−/− and *fks3*−/− is lower than that observed in the control, parental or *fks1*+/− strains (Table 2).

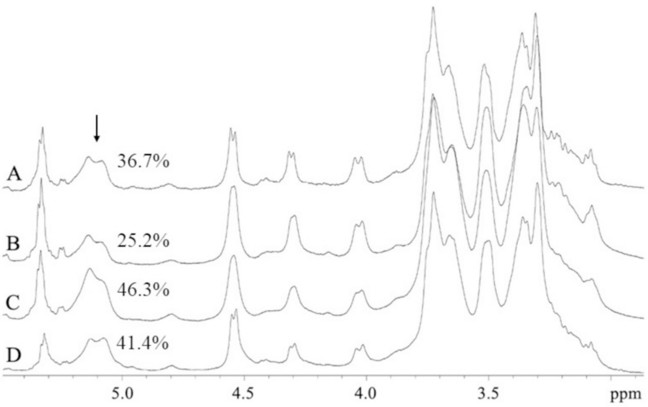

Fig. 7. Comparison of the glucan-glycogen content in a *C. albicans* parental strain and three representative *fks* mutant strains. In this study, the CAF2-1 parental strain (A) and three representative *fks* mutant strains, *i.e.*, *fks1*+/− (B), *fks2*−/− (C) and *fks3*−/− (D), were extracted to examine their glucan-glycogen complex content. ^{1}H NMR stacked plot of the carbohydrate spectral region plotted from 2.8 to 5.6 ppm of the parental and three mutant strains demonstrates the presence of the glucan-glycogen complex. We observed that the *fks1*+/− strain (B) shows a lower level of the glucan-glycogen complex 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 ^{1}H 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. 2**B**). 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 glycogen 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. auris* 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

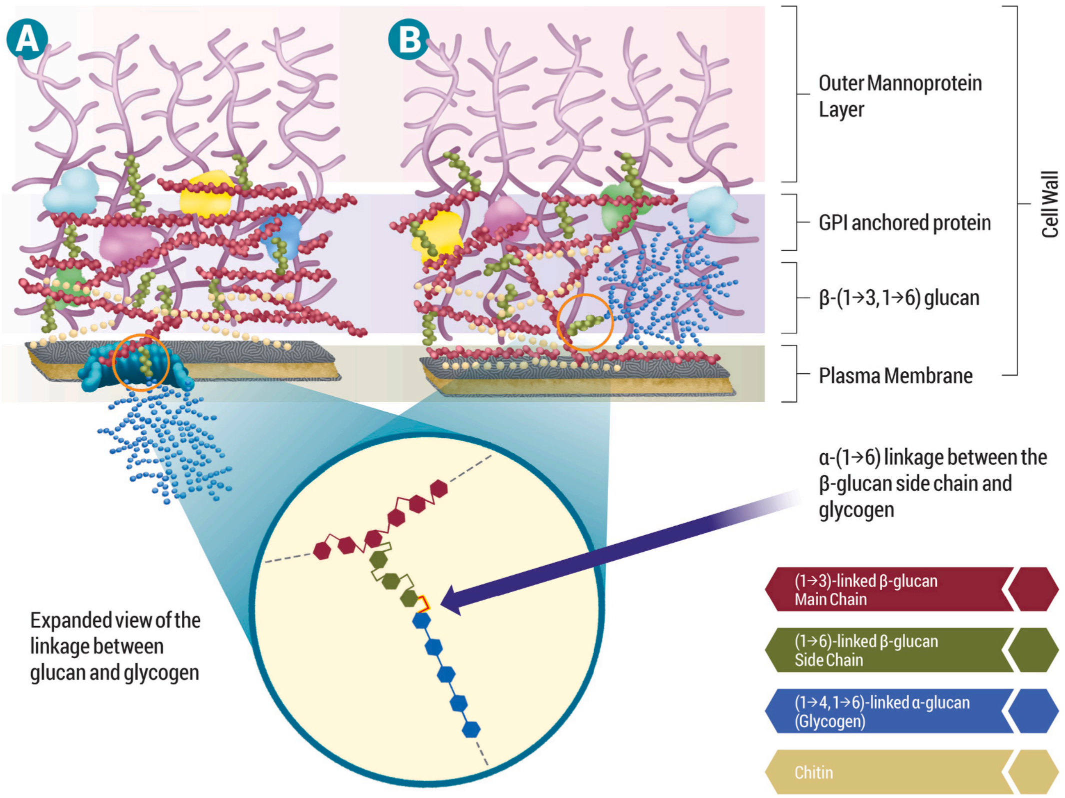

Fig. 8. Proposed models showing how the glucan-glycogen macromolecule may exist within the cell wall of *C. albicans*. Model A proposes that glycogen is present within the cell cytosol and is linked to glucan through a β -(1 → 6)-linked plasma membrane-spanning glucan side chain. The bond between glucan 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. 8**A**). 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 β- (1 → 3)-glucan synthase is located near the plasma membrane of *Candida glabrata,* where it 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 → 3,1 → 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. 8**B**). 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). Kakutani and colleagues have reported that TLR2 is essential for macrophage activation by glycogen (Kakutani 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 → 3,1 → 6)-glucan by an ∝-(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. dublinesis* 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. **M. Sameer Al-Abdul-Wahid:** Methodology, Formal analysis, Validation, Resources, Data curation, Writing – original draft, Writing - review & editing. **Zuchao Ma:** Methodology, Validation, Resources, Formal analysis, Investigation, Data curation, Writing – original draft, Writing - review & editing. **Michael D. Kruppa:** Conceptualization, Methodology, Investigation, Resources, 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.tcsw.2021.100061.

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