Evidence for the presence of complex carbohydrates in *Candida albicans* cell wall glycoproteins

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Summary

In order to test the hypothesis that cell wall glycoproteins of *Candida albicans* contained non-mannan oligosaccharides, the sugar composition of cell wall extracts and fractions of cell wall extracts was examined by means of fluorophore-assisted carbohydrate electrophoresis (FACE). In addition to the expected mannose, glucose, and N-acetyl-glucosamine, this analysis showed the presence of galactose, N-acetyl-galactosamine, fucose, and sialic acid and two unknown sugars. These sugars are also associated with complex oligosaccharides of mammalian glycoproteins. Presence of fucosylated cell wall components was further demonstrated by lectin-blotting analysis of cell wall extracts. Besides their structural role, complex carbohydrate structures on the surface of *C. albicans* may represent additional motifs through which interactions of this fungus with host cells and tissues could be established.

Key words *Candida albicans*, Cell wall, Carbohydrate composition

Evidencia de la presencia de carbohidratos complejos en las glicoproteínas de la pared celular de *Candida albicans*

Resumen

Hemos investigado la hipótesis de que las glicoproteínas de pared celular de *Candida albicans* pudieran contener oligosacáridos no manosídicos. Para ello, analizamos la composición de azúcares en extractos de pared celular y fracciones de los mismos extractos utilizando una técnica electroforética para separacion de carbohidratos marcados con fluorescencia (FACE). Además de los esperados residuos de manosa, glucosa y N-acetil-glucosamina, el análisis detecto la presencia de galactosa, N-acetil-galactosamina, fucosa, ácido siálico y dos azúcares no identificados. Estos residuos azucarados son también importantes constituyentes de carbohidratos complejos presentes en glicoproteínas de mamíferos. La presencia de fucosa fue corroborada usando una técnica de detección con lectina en extractos de pared separados electroforéticamente y transferidos a soportes de nitrocelulosa. Además de un papel estructural, estos carbohidratos complejos en la superficie celular de *C. albicans* podrían desempe'ar importantes funciones en la interacción del hongo con las células y tejidos del huésped.

Candida albicans, Pared celular, Azúcares

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The major components of the cell wall of *C. albicans* are polymers of glucose (glucan) and N-acetyl-glucosamine (chitin), and polymers of mannose (mannan) covalently associated with proteins (mannoproteins) [1-3]. However, several observations seem to indicate that the monosaccharide composition associated with cell wall constituents may display a higher degree of complexity. First, the fact that not all proteinaceous moieties present in cell wall extracts from this fungus react with concanavalin A (a lectin that recognizes mannose and glucose residues) or with antibodies which recognize mannan epitopes, such as factor 6 and a number of monoclonal antibodies [4]. Secondly, differences in glycosylation and neuraminidase sensitivity of candidal receptors for complement fragments [5,6]. Third, the presence of sialic acid on the surface of *C. albicans* has recently been described [7].

These observations prompted us to reexamine whether other sugars are present in cell wall glycoproteins of this pathogenic fungus. In the present report, by using a highly sensitive analytical technique for the analysis of glycoproteins, we present evidence for the existence of a complex carbohydrate composition associated with cell wall glycoproteins of *C. albicans*.

MATERIALS AND METHODS

Organism and culture conditions. C. albicans strain 3153A was used in this work. It was maintained on Sabouraud medium containing 2% (w/v) agar. Yeast cells (blastoconidia) were grown in suspension culture in the medium of Lee *et al.* [8] at 22 °C in an orbital water bath shaker at 180-200 rpm. Germ tubes (germinated blastoconidia) were induced from stationary phase yeast cells that were resuspended at a concentration of 5 x 10^7 cells per ml in fresh prewarmed medium and incubated at 37 °C for 4 h with shaking.

Preparation of candidal cell wall extracts. Intact cells of each *C. albicans* morphological phase (yeast cells and germ tubes) were treated with β-mercaptoethanol (βME) and subsequently with a β-glucanase (Zymolyase 20T) to release cell wall components as previously described [4,9]. Mannan was extracted as previously described by autoclaving cells in citrate buffer and precipitated with Fehling solution [10,11]. The total sugar content in the different extracts was determined colorimetrically with mannose as the standard [12].

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions was performed basically as described by Laemmli [13] with minor modifications [14]. Fractions containing cell wall proteins of different molecular sizes from yeast cells or germ tubes were obtained by preparative SDS-PAGE [14]. About 10 mg (based on total sugar content) of the corresponding βME extract was applied to a 13 cm wide x 20 cm height, 5-15% polyacrylamide slab gradient gel. Prestained molecular weight standards (Gibco-BRL, USA) were run in parallel in a single reference well formed to one side of the resolving gel slab. Transverse sections of the gel containing moieties within different molecular size ranges (>105 kDa, 69-105 kDa, 43-49 kDa, 28-43 kDa, and <28 kDa; see below) were excised, crushed and polypeptide moieties electroeluted [14,15].

Sugar composition analysis. The Glyko-FACE (Fluorophore-Assisted-Carbohydrate-Electrophoresis) (Glyko Inc., USA) monosaccharide composition kit was used for the sugar composition analysis following the manufacturer's instructions. The neutral and amino sugars were released using 4 N trifluoroacetic acid (TFA). Sialic acid was assayed in hydrolysates obtained with 0.1 N TFA. The monosaccharides were labeled with a fluorescent tag and separated in pre-cast polyacrylamide gels provide as part of the kit. After electrophoresis the gels were examined under ultraviolet light. Sugars were identified by reference to standard monosaccharides run in parallel. The following cell wall extracts and fractions were analyzed: mannan from blastoconidia, βME and Zymolyase extracts from both blastoconidia and germ tubes, and the five size fractions obtained from βME extracts of yeast cells and germ tubes described above.

Immunoblotting and lectin-blotting analysis of components present in cell wall extracts. Proteinaceous components present in the cell wall extracts were separated by SDS-PAGE using 4%-15% gradient minigels (Bio-Rad, USA); kaleidoscope prestained standards (Bio-Rad) were run in parallel with samples for determination of molecular size of polypeptide species separated after the electrophoretic run. Electrophoretic transfer to nitrocellulose paper (Western blotting) was carried out using a semi-dry electroblotter at 0.8 mA/cm^2 for 1 h as previously reported [10,14]. Immunodetection of proteins transferred to nitrocellulose was done as previously described [11] using a pooled rabbit polyclonal antiserum raised against different *C. albicans* cell wall extracts (pPAb) [16] diluted 1:1,000 dilution in 10 mM Tris/HCl buffer (pH 7.4) plus 0.9% (w/v) NaCl (TBS), containing 0.05 % Tween-20 (TBST) and 1 % (w/v) bovine serum albumin (BSA) (TBSTB). Peroxidase-labelled goat anti-rabbit immunoglobulins diluted in TBSTB (1:2,000 dilution in TBSTB) was used as secondary antibody. Colored reactive bands were developed with H_2O_2 and 4-chloro-1-napthol as the chromogenic reagent. For detection of fucose-containing glycoproteins, the membranes were blocked in TBSTB (containing in this case 3% BSA) for 2 h at 37 °C and rinsed in TBS. Blocked filters were incubated for 30 min at room temperature in TBSTB with 10 mg per ml of peroxidase-conjugated Lotus lectin (EY Laboratories Inc., USA). The membranes were washed four times with TBST and developed as described above.

RESULTS

Sugar composition of cell wall extracts and fractions. In addition to the typical expected monosaccharides mannose, glucose, and N-acetyl-glucosamine (GlcNAc) [1-3], analysis of *C. albicans* cell wall extracts (βME, Zymolyase, and mannan) revealed a more complex carbohydrate composition with the presence of additional monnosaccharides. The material released by treatment of intact cells with βME, which removes components associated with the outermost (surface) layers of the cell wall [17], showed the greatest complexity, followed by the mannan preparation, whereas only mannose and glucose were detected in the Zymolyase extracts (Table 1).

Table 1. Sugar composition of different cell wall extracts of *C. albicans*.

Sugar	Extract[a]				
	Mannan	βME-Bl	βME-GT	Zymo-Bl	Zymo-GT
GalNAc	-	+	+	-	-
Unknown	+	+	-	-	-
Sialic acid	+	+	+	-	-
Mannose	++	++	++	++	++
Fucose	-	-	-	-	-
Glucose	+	+	++	++	++
Galactose	-	+	+	-	-
GlcNAc	-	-	-	-	-

Composition indicated by: (-), not detected; (+), detected; (++), relative or significant amount.
[a]Abbreviations: βME, 2-mercaptoethanol; Zymo, Zymolyase; Bl, blastospores; GT, germ tubes.

Analysis of fractions of different molecular mass ranges obtained by preparative electrophoresis of the βME extracts from both blastoconidia and blastoconidia-bearing germ-tubes further confirmed the complexity of the monosaccharide composition associated with these materials. Overall, these analysis revealed the presence of N-acetyl-galactosamine (GalNAc), sialic acid, fucose, galactose and two yet undetermined sugars, together with the expected mannose, glucose, and N-acetyl-glucosamine (Table 2).

Table 2. Monosaccharide composition of different fractions in the βME extracts from blastospores and germ tubes of *C. albicans.*

	Fraction[a]									
	<28 kDa		28-43 kDa		43-69 kDa		69-105 kDa		>105kDa	
Sugar	Bl	GT	Bl	GT	Bl	GT	Bl	GT	Bl	GT
GalNAc	++	++	-	-	-	-	-	+	-	+
Unknown I	++	++	++	++	+	+	+	+	-	-
Sialic acid	-	-	+/-	+/-	++	++	++	++	++	++
Mannose	+	+	+	+	+	+	+	+	+	+
Fucose	+	+	++	++	++	++	++	++	+	+
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	-	-	-	+	-	+	+/-	+
GlcNAc	+/-	+/-	+/-	-	+/-	+/-	+/-	+/-	+	+
Unknown II	-	-	+	+	-	-	-	-	-	-

Composition indicated by: (-), not detected; (+/-), questionable; (+), detected; (++), relative or significant amount. Abbreviations: βME, 2-mercaptoethanol; Zymo, Zymolyase; Bl, blastospores; GT, germ tubes. aMolecular size of fractions expressed in kDa (see Methods).

Lectin blotting analysis of cell wall extracts. Lectin-blotting analysis of cell wall extracts using Lotus lectin, that specifically recognizes fucosyl residues, further confirmed the presence of fucosylated cell wall components. The analysis revealed the presence of several low-to-medium molecular mass fucose-containing moieties present in the cell wall extracts from both morphological forms of the fungus (Figure 1B). Many of the components in the extract were unreactive or below detection limits. The complexity of the extract was revealed by Western blot analysis with a pooled polyclonal antisera (pPAb) generated against cell wall components [16]. pPAb recognized most of the protein and glycoprotein components present in the extract (Figure 1A).

DISCUSSION

In this study we present evidence in support of the contention that the surface of *C. albicans* cells may have a complex oligosaccharide composition. Thus, in addition to the expected mannose, glucose and N-acetyl-glucosamine, our analysis of the monosaccharide composition in various cell wall extracts of *C. albicans* revealed the presence of N-acetyl-galactosamine, sialic acid, fucose, galactose and two yet undetermined sugars (Tables 1 and 2). Of particular interest is the detection of N-acetyl-galactosamine, since it is usually indicative of the presence of O-linked carbohydrates. Furthermore, the presence of several fucosylated components in cell wall extracts was confirmed by lectin-blotting analysis with Lotus lectin, that specifically recognizes fucosyl residues. Overall, the analysis suggests that at least some cell wall carbohydrates contain monosaccharides other than mannose and that the oligosaccharides may have a much more complex structure than initially thought. The presence of non-mannan oligosaccharides is consistent with previous reports showing that candidal cell wall components may contain sialic acid [5-7].

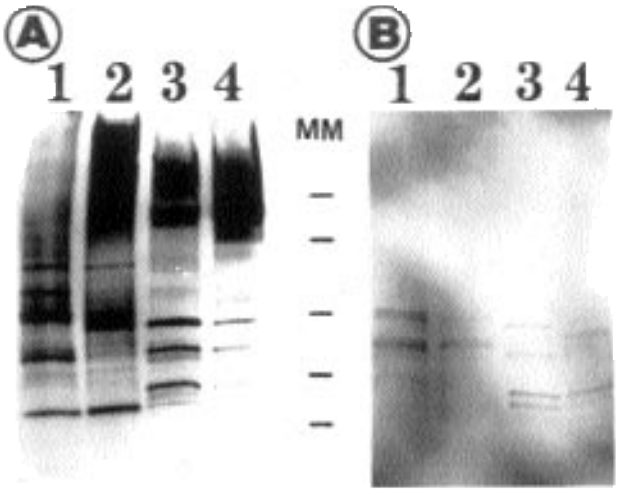

Figure 1. Reactivity of *C. albicans* wall components with the Lotus lectin. Western blots of bME (lanes 1 and 2 [containing 50 µg of material expressed as total sugar content per well]) and Zymolyase (lanes 3 and 4 [containing 100 µg of material, also expressed as total sugar content per well]) extracts from blastoconidia (lanes 1 and 3) and germinated blastoconidia (lanes 2 and 4) were reacted with the pPAb preparation (panel A) and with the Lotus lectin (panel B). Molecular masses (MM, 31, 42, 72, 130, and 217 kilodaltons from bottom to top) of standard proteins run in parallel are indicated between both panels.

Complex carbohydrates are vital constituents of living organisms. They provide energy and act as structural support for cells. There is increasing evidence that carbohydrate moieties of glycoconjugates also play important roles as recognition determinants in receptor-ligand and cell-to-cell interactions, as immunomodulators, in protein folding, and in the regulation of different protein functions. [18,19]. In *C. albicans*, carbohydrates both on the surface of the fungus and the surface of host cells have been implicated in adhesion. A role for fucosylated blood group antigens (BGRAgs) as epithelial cell ligands for lectin-like adhesins on the fungal cells has been suggested. The ABO(H) and Lewis systems are suggested as the best ligand candidates [20-25]. Also, among multiple adhesive mechanisms that have been described for the fungus are those mediated by fungal surface carbohydrates including chitin and mannooligosaccharides such as factor 6 and a β-linked mannotetraose [26-28]. In this context, presence of complex carbohydrates on the surface of *C. albicans* may be indicative of a new, yet unrecognized, mechanism by which the fungal cells interact with host structures. Also, our detection of fucose and N-acetyl-galactosamine among the monosaccharides of *Candida* cell wall, which have been also postulated as host cell ligands for *C. albicans* [25, 29], may reflect a two-way carbohydrate-mediated *Candida*-host cell interactions.

In summary, we have shown that sugars associated with complex oligosacharides are present in cell wall extracts of *C. albicans*. Thus, glycoproteins of the cell surface of this pathogenic fungi are not uniform in the structure or synthesis of the carbohydrate modification. The presence of such oligosaccharides has implications for the synthesis of cell wall proteins and the interactions of fungal surface proteins with the host.

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