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Covalent Association of β -1,3-Glucan with β -1,6-Glucosylated Mannoproteins in Cell Walls of *Candida albicans*

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Yeast and hyphal walls of *Candida albicans* were extracted with sodium dodecyl sulfate (SDS). Some of the extracted proteins reacted with a specific β -1,6-glucan antiserum but not with a β -1,3-glucan antiserum. They lost their β -1,6-glucan epitope after treatment with ice-cold aqueous hydrofluoric acid, suggesting that β -1,6-glucan was linked to the protein through a phosphodiester bridge. When yeast and hyphal walls extracted with SDS were subsequently extracted with a pure β -1,3-glucanase, several mannoproteins that were recognized by both the β -1,6-glucan antiserum and the β -1,3-glucan antiserum were released. Both epitopes were sensitive to aqueous hydrofluoric acid treatment, suggesting that β -1,3-glucan and β -1,6-glucan are linked to proteins by phosphodiester linkages. The possible role of β -glucans in the retention of cell wall proteins is discussed.

Candida albicans is an opportunistic, dimorphic fungal pathogen that grows in either yeast or hyphal form, depending on the environmental conditions. It is generally assumed that the morphological transition from the yeast to the hyphal form is a prerequisite for successful tissue invasion by *C. albicans*. This transition is correlated with changes in the composition of cell wall mannoproteins (5, 8, 9). Cell wall mannoproteins are also involved in other pathogenicity-related aspects, such as adhesion of *Candida* cells to mammalian tissues and immunogenicity (3, 4). It is, therefore, of interest to obtain more knowledge of the mechanisms by which mannoproteins are incorporated into the fungal cell wall. Some of the cell wall-associated proteins are extractable with hot sodium dodecyl sulfate (SDS). These proteins are mainly O-mannosylated and have relatively low molecular masses (8, 9). The remaining mannoproteins, which might have both N- and O-linked carbohydrate chains, can be extracted by β -1,3-glucanase digestion, suggesting that these proteins are linked to the β -1,3-glucan skeleton of the cell wall (8, 14, 17, 23). However, the exact nature of the bonds between these mannoproteins and the β -1,3-glucan skeleton is unknown. Recently, it was shown that the glucanase-extractable mannoproteins of *C. albicans* and *Saccharomyces cerevisiae* contain a novel type of side chain, carrying β -1,6-linked glucose residues, that was proposed to be involved in cell wall anchorage (13, 16, 19, 22, 23). Evidence that the β -1,6-glucan side chains of *C. albicans* are phosphodiester linked to the mannoproteins was presented (13). Interestingly, it has been shown that the alkali-soluble glucan of *C. albicans* cell walls consists of a β -1,6-glucan- β -1,3-glucan heteropolymer (1, 25). This raises the question whether the protein-bound β -1,6-glucan might also be part of such a heteropolymer in vivo.

Here, by immunological means, we show that the β -1,6-glucosylated cell wall proteins contain a β -1,3-glucan epitope. Both the β -1,6-glucan and β -1,3-glucan epitopes are presumably phosphodiester linked to the protein. We propose that the incorporation of proteins into the cell wall is accomplished by

extracellular cross-linkage between a β -1,6-glucan- β -1,3-glucan heteropolymer and cell wall proteins.

MATERIALS AND METHODS

Strain. *C. albicans* C3153 was donated by G. W. Gooday (Department of Molecular and Cell Biology, Marischal College, University of Aberdeen, United Kingdom). Cells were grown at 28°C in succinate-buffered (0.25% [wt/vol]; pH 4.5) synthetic growth (SG) medium containing 0.17% (wt/vol) yeast nitrogen base (Difco, Detroit, Mich.), 0.5% (wt/vol) $(\text{NH}_4)_2\text{SO}_4$, and 2% (wt/vol) glucose. Cells were harvested in the exponential growth phase.

Germ tube induction. *C. albicans* cells were rinsed three times with double-distilled water and starved by shaking in water for 1 h at 28°C. Starved cells were resuspended in prewarmed, morpholineethanesulfonic acid-buffered (0.25% [wt/vol]; pH 6.7) SG medium to which 0.22% (wt/vol) *N*-acetylglucosamine was added and were shaken (200 rpm) at 37°C for 6 h to develop germ tubes (hyphae).

Isolation of glucomannoproteins. Cells were harvested by centrifugation, washed three times with 10 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, and subsequently disrupted five times for 1 min each time (with 30-s intervals) at 0°C with glass beads in a Bead-Beater. The glass beads were separated by filtration over a nylon filter, and the cell walls were collected by centrifugation at $3,000 \times g$ for 10 min. The resulting pellet was rinsed five times with 1 M NaCl and three times with 1 mM phenylmethylsulfonyl fluoride. Isolated walls were boiled in the presence of SDS, EDTA, and β -mercaptoethanol to extract noncovalently linked mannoproteins, and they were subsequently digested with mollusc laminarinase (Sigma, St. Louis, Mo.) (0.5 U g^{-1} [wet weight of cell walls]) to obtain covalently linked wall mannoproteins (13, 19). Cell walls were also digested at 37°C for 17 h with Quantazyme ylg (Quantum Biotechnologies Inc., Montreal, Canada) (600 U g^{-1} [wet weight] of cell walls), a recombinant β -1,3-glucanase, in 50 mM Tris-HCl (pH 7.5) containing 40 mM β -mercaptoethanol.

Treatment with proteolytic enzymes. To analyze the nature of the SDS-extracted and Quantazyme-liberated *C. albicans* cell wall material, it was treated for 24 h at 37°C with pronase E (Sigma) in 50 mM Tris-HCl buffer (pH 8.2) containing 2 mM CaCl_2 .

Aqueous hydrofluoric acid (HF) treatment. Quantazyme-released cell wall mannoproteins and yeast invertase (Sigma) were treated with ice-cold aqueous HF (50% [vol/vol]) according to a modified procedure described by Ferguson (10). After aqueous HF treatment, the proteins were evaporated under a constant nitrogen flow, washed with 90% (vol/vol) ice-cold methanol, and taken up in sample buffer. This treatment removes the β -1,6-glucan side chains from wall mannoproteins but does not lead to significant protein breakdown or degradation of N and O chains (13; see also Fig. 2).

Analysis of cell wall proteins. Cell wall proteins were separated by linear gradient (2.2 to 20%) polyacrylamide gel electrophoresis (PAGE) and were visualized by silver staining (6), by staining with the periodic acid-Schiff (PAS) reagent according to the method of Zacharius et al. (26), and by blotting by electrophoretic transfer onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) for Western (immunoblot) analysis with affinity-purified polyclonal antisera directed against β -1,6-glucan and β -1,3-glucan. The PAS reagent specifically stains glycoproteins. The β -1,6-glucan-specific antibodies were raised in rabbits by using a conjugate of pustulan oligosaccharides

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FIG. 1. Characterization of the SDS-solubilized cell wall proteins from hyphal cells of *C. albicans*. Proteins were separated by SDS-PAGE (2.2 to 20%) and visualized by (A) silver staining, (B) PAS staining, and (C) Western blotting with the affinity-purified β -1,6-glucan antiserum. Lanes 1, 3, and 5, before aqueous HF treatment; lanes 2, 4, and 6, after aqueous HF treatment; lane 7, after pronase treatment; lane 8, Western blotting in the presence of pustulan; lane 9, Western blotting after treatment with periodate. Sizes of standard molecular mass markers are shown at the left in kilodaltons. The molecular masses of the wall proteins mentioned in the text are indicated with arrows.

(β -1,6-glucan) and bovine serum albumin (BSA) as antigen (19). This antiserum was purified by affinity chromatography on a pustulan-Epoxy-Sepharose 6B column. Similarly, the β -1,3-glucan-specific antibodies were raised by using a conjugate of BSA and laminarin (β -1,3-glucan). Before conjugation with BSA, laminarin was oxidized in 0.25 M NaIO₄ at 20°C for 60 min and subsequently desalted on a Sephadex G-25 column. The β -1,3-glucan antiserum was purified on a laminarin (preoxidized)-Epoxy-Sepharose 6B column. Western analyses with 1:5,000 dilutions of affinity-purified antisera were carried out as described previously (19). However, instead of being determined with a solution of aminoethylcarbazole as the chromogenic substrate, the binding of the antisera was determined with an enhanced chemiluminescence detection reagent (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom) because of its superior sensitivity.

RESULTS

Immunological identification of β -1,6-glucosylated, SDS-extractable cell wall proteins. As reported before (9, 13, 17), SDS extracts of yeast and hyphal cell walls contained many proteins with low molecular masses (<120 kDa) in addition to a poly-disperse smear with an average molecular mass of about 500 kDa (Fig. 1, lane 1). The PAS reagent stained only the high-molecular-mass smear (Fig. 1, lane 3). Western analysis with the β -1,3-glucan antiserum showed that none of the SDS-extractable proteins were β -1,3-glucosylated (data not shown). With the β -1,6-glucan antiserum being used for the analysis, the 500-kDa smear was shown to contain a β -1,6-glucan epitope (Fig. 1, lane 5). The antiserum also reacted with a band with an apparent molecular mass of 205 kDa (Fig. 1, lane 5), which was not seen with silver and PAS staining (Fig. 1, lanes 1 and 3). After periodate oxidation, which destroys β -1,6- but not β -1,3-linked glucose residues, or in the presence of pustulan (β -1,6-glucan), the epitope of the SDS-solubilized proteins was not recognized (Fig. 1, lanes 8 and 9), confirming that it contained β -1,6-glucose residues. Upon pronase digestion, the β -1,6-glucan signal disappeared, confirming that the epitope was protein linked (Fig. 1, lane 6). The β -1,6-glucosylated proteins were not affected by endo- β -N-acetylglucosaminidase H treatment (data not shown). This is consistent with the observation in *S. cerevisiae* that β -1,6-glucan-containing side chains are not modified N chains (19). The epitope was lost upon aqueous HF treatment (Fig. 1, lane 7). This treatment is used to specifically cleave phosphodiester bridges in glycosyl phosphatidylinositol (GPI) anchors (10). This result, therefore, indicates that the β -1,6-glucan epitope is linked to the protein through a phosphodiester bridge. Silver and PAS staining showed that after removal of the β -1,6-glucan epitope, the

FIG. 2. Effect of aqueous HF on SDS-PAGE of yeast invertase. Lane 1, untreated (control); lanes 2 and 3, treated with ice-cold aqueous HF for 0 and 24 h, respectively. The sizes of standard molecular mass markers are shown at the left in kilodaltons. The molecular masses of the mono-, di-, and trimeric forms of invertase are shown at the right.

500-kDa material had a lower mobility (Fig. 1; compare lanes 1 with 2 and 3 with 4). It is possible that this is due to aggregation of the dephosphorylated material, since a similar phenomenon was seen when yeast invertase was treated with aqueous HF, leading to di- and trimerization of this enzyme (Fig. 2, lane 3). This experiment also shows that aqueous HF did not significantly affect glycosidic or peptide linkages. The apparent resistance of the glycosidic linkages in invertase to aqueous HF is consistent with its being routinely used to isolate the glycan core structure of GPI anchors (Man α -1,2-Man α -1,6-Man α -1,4-GlcNAc-1,6-*myo*-inositol) (10).

Immunological identification of β -1,3- and β -1,6-glucosylated cell wall proteins. SDS-extracted cell walls were digested with laminarinase to release covalently bound mannoproteins. The extracts were analyzed by SDS-PAGE and Western blot analysis with β -1,3- and β -1,6-glucan antisera. Laminarinase was found to release β -1,6-glucosylated mannoproteins from yeast and hyphal cell walls. Digestion of yeast cell walls resulted in bands with apparent molecular masses of 680, 460, 245, 125, and 80 kDa (Fig. 3, lane 1), while in digests of hyphal walls, the β -1,6-glucan antiserum recognized three bands with apparent molecular masses of 460, 125, and 80 kDa (Fig. 4, lane 1). None of the proteins released by laminarinase was recognized by the β -1,3-glucan antiserum (data not shown).

In addition to its main β -1,3-glucanase activity, laminarinase contains small amounts of β -1,6-glucanase (7%) and α -mannanase (6%) activity (23). These contaminations might partially digest the β -1,6-glucosylated mannoproteins released. For this reason, a recombinant β -1,3-glucanase (Quantazyme ylg) was used to digest yeast and hyphal cell walls. Indeed, much more high-molecular-mass material reacting with the β -1,6-glucan antiserum was now seen in the upper parts of the gels (Fig. 3 and 4; compare lanes 1 and 2). Three β -1,6-glucosylated yeast bands with apparent molecular masses of 220, 450, and 680 kDa could be distinguished (Fig. 3, lane 2). No obvious differences were found between the materials released from yeast and hyphal cell walls, except that the 450- and 680-kDa bands in hyphal extracts could not be discriminated from each other and migrated as one zone with an average molecular mass of 550 kDa (compare Fig. 3, lane 2, and Fig. 4, lane 2). Pronase treatment resulted in the disappearance of the β -1,6-glucosylated yeast bands, demonstrating that the β -1,6-glucan is bound to protein (Fig. 3, lane 3). These bands did not lose their antigenicity by endo- β -N-acetylglucosaminidase H

FIG. 3. Characterization of the laminarinase- (A) and Quantazyme- (B) released cell wall proteins of *C. albicans* yeast cells by Western analysis with the affinity-purified β -1,6-glucan (lanes 1 to 6) and β -1,3-glucan (lanes 7 to 11) antisera or by PAS staining (lanes 12 and 13). Lanes 3 and 8, immunodetection after pronase digestion; lanes 4 and 9, immunodetection after aqueous HF treatment; lanes 5 and 10, immunodetection in the presence of pustulan (β -1,6-glucan) and laminarin (β -1,3-glucan), respectively; lanes 6 and 11, immunodetection after periodate treatment; lanes 12 and 13, PAS staining before and after aqueous HF treatment. The molecular masses of the cell wall proteins mentioned in the text are indicated with arrows. Laminarinase itself did not interact with the β -1,6-glucan antiserum. Silver staining of the laminarinase-released glucosylated proteins is not shown here, since the laminarinase preparation itself produced a large smear and thereby masked any cell wall proteins.

treatment (data not shown), indicating that the β -1,6-glucan epitope is not linked to an N chain. Cold aqueous HF removed the β -1,6-glucan epitope from the Quantazyme-released yeast and hyphal wall proteins (Fig. 3, lane 4, and Fig. 4, lane 3), suggesting that the β -1,6-glucan epitope is attached to the protein by a phosphodiester linkage. PAS staining of the aque-

ous HF-treated Quantazyme digest of yeast and hyphal cell walls resulted in a heavy high-molecular-mass smear which had a slightly lower mobility than that of untreated released material (Fig. 3, lanes 12 and 13, and Fig. 4, lanes 6 and 7). Silver staining of the Quantazyme-released wall proteins led to the same protein profiles as those revealed by PAS staining and showed further that no significant protein breakdown had occurred upon aqueous HF treatment (unpublished results).

Heteropolymers of β -1,6- and β -1,3-glucan have been demonstrated in the cell walls of *C. albicans* (1, 11, 25). Therefore, the Quantazyme-released, β -1,6-glucosylated cell wall proteins were also subjected to Western analysis with the β -1,3-glucan antiserum. The β -1,6-glucosylated bands derived from the yeast form that ran at 220 and 680 kDa were also recognized by the β -1,3-glucan antiserum (Fig. 3, lane 7). With respect to the hyphal form, the material released bound to both glucan antisera (Fig. 4, lanes 2 and 4). After pronase digestion, the β -1,3-glucan-containing bands were not observed (Fig. 3, lane 8), whereas their antigenicity towards the β -1,3-glucan antiserum was retained after endo- β -N-acetylglucosaminidase H treatment (data not shown), indicating that in a case similar to that of the β -1,6-glucan epitope, the β -1,3-glucan epitope is linked to protein but probably not via N chains. Loss of antigenicity was, however, observed after aqueous HF treatment (Fig. 3, lane 9, and Fig. 4, lane 5), suggesting that the epitope is linked through a phosphodiester bridge.

Control experiments with periodate-oxidized, Quantazyme-released cell wall proteins and competitive Western analyses with pustulan (β -1,6-glucan) and laminarin (β -1,3-glucan) demonstrated that the epitopes recognized by the β -1,6-glucan and β -1,3-glucan antisera contained β -1,6- and β -1,3-linked glucose residues, respectively. Pustulan (100 μ M) completely inhibited binding of the β -1,6-glucan antibodies to the Quan-

FIG. 4. Characterization of the laminarinase- (A) and Quantazyme- (B) released cell wall proteins of *C. albicans* hyphal cells by Western analysis with the affinity-purified β -1,6-glucan (lanes 1 to 3) and β -1,3-glucan (lanes 4 and 5) antisera or by PAS staining (lanes 6 and 7). Lanes 1, 2, 4, and 6, before aqueous HF treatment; lanes 3, 5, and 7, after aqueous HF treatment. The molecular masses of the wall proteins mentioned in the text are indicated with arrows. Laminarinase itself did not interact with the β -1,6-glucan antiserum. Silver staining of the laminarinase-released glucosylated proteins is not shown here, since the laminarinase preparation itself produced a large smear and thereby masked any cell wall proteins.

tazyme-extractable, glucosylated wall proteins (Fig. 3, lane 5). In addition, the β -1,6-glucan epitope was sensitive to oxidation by periodate, consistent with the presence of β -1,6-linked glucose residues (Fig. 3, lane 6). With regard to the β -1,3-glucan antiserum, the signals obtained could be fully inhibited with 200 μ M laminarin (Fig. 3, lane 10), whereas periodate did not abolish but rather enhanced recognition of the Quantazyme-released yeast cell wall proteins by the β -1,3-glucan antibodies (Fig. 3, lane 11). Most probably, the oxidation of the β -1,6-glucan and the N- and O-linked mannan by periodate enhanced the accessibility of the β -1,3-glucan epitope of these proteins to the antiserum.

DISCUSSION

In a previous paper (13), we reported the identification of 125-kDa and 80-kDa β -1,6-glucosylated protein bands in laminarinase extracts of yeast and hyphal cell walls of *C. albicans* by Western analysis with the affinity-purified β -1,6-glucan antiserum. Because of the more sensitive enhanced chemiluminescence detection method used in this study, we were able to identify some additional β -1,6-glucosylated, laminarinase-extractable proteins in both yeast and hyphal cell walls.

In the experiments for this report, we also used Quantazyme ylg, a recombinant β -1,3-glucanase, which unlike laminarinase does not contain α -mannanase and β -1,6-glucanase activities, to digest the yeast and hyphal cell walls of *C. albicans*. As expected, the β -1,6-glucosylated proteins that were released by Quantazyme had higher molecular masses than those liberated by laminarinase (Fig. 3 and 4; compare lanes 1 and 2). Interestingly, most Quantazyme-released, β -1,6-glucosylated protein bands contained a β -1,3-glucan epitope (Fig. 3, lane 7, and Fig. 4, lane 4). Presumably, Quantazyme had difficulty in passing β -1,6 branch points in the β -1,3-glucan polymers present in the cell wall, thereby leaving some undigested β -1,3-glucan attached to the β -1,6-glucosylated mannoproteins. It is tempting to speculate that the protein-bound β -1,6-glucan- β -1,3-glucan demonstrated here is related to the β -1,6-glucan- β -1,3-glucan heteropolymer isolated from the alkali-soluble wall fraction of *C. albicans* (1, 11, 25).

Montijn et al. (19) have shown that the β -1,6-glucose-containing side chains of glucanase-extracted cell wall proteins of *S. cerevisiae* are not modified or extended N chains and are also not O linked. Van Berkel et al. (22) demonstrated that a secretory reporter protein extended with the GPI anchor addition sequence of the cell wall protein α -agglutinin became covalently associated with the cell wall of *S. cerevisiae* and contained β -1,6-glucan, whereas the nonextended and secreted form was not glucosylated. Moreover, a C-terminal truncated form of α -agglutinin, which is not GPI anchored and is secreted into the medium (24), did not react with the β -1,6-glucan antiserum (16). In combination with our observation that aqueous HF removed both the β -1,6-glucan and β -1,3-glucan epitopes from the Quantazyme-released proteins (Fig. 3, lanes 4 and 9, and Fig. 4, lanes 3 and 5), these data indicate that the β -1,6-glucan moiety and probably also the β -1,3-glucan moiety might be connected to a GPI anchor, which is known to be phosphodiester linked to the C-terminal amino acid of the mature protein. This is consistent with the hypothesis put forward by De Nobel and Lipke (7) that proteins destined to be incorporated in the cell wall become linked to β -glucan through the glycan part of their GPI anchors. However, according to their model in its most simple form, SDS-extractable intermediate forms of cell wall proteins are not supposed to have a β -1,6-glucan epitope. Indeed, a nonglucosylated, SDS-extractable intermediate form of the cell wall

protein α -agglutinin has recently been identified (16). How then can the presence of β -1,6-glucosylated proteins in SDS extracts of cell walls (Fig. 1C) be explained? A possible interpretation is that the SDS-extractable, β -1,6-glucosylated cell wall proteins represent degradation products of wall proteins caused by the action of endogenous β -1,3-glucanases. These enzyme activities have been found in the walls of *C. albicans* (12, 18, 20, 21). However, the possibility that the SDS-extractable, β -1,6-glucosylated cell wall proteins represent an additional intermediate step and that the final incorporation in the cell wall requires the extension of β -1,6-glucan with a β -1,3-glucan chain cannot yet be excluded.

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