Secretion, interaction and assembly of two O-glycosylated cell wall antigens from Candida albicans

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The mechanisms of incorporation of two antigens have been determined using a monoclonal antibody (3A10) raised against the material released from the mycelial cell wall by zymolyase digestion and retained on a concanavalin A column. One of the hybridomas secreted an IgG that reacted with two bands in Western blots. Indirect immunofluorescence showed that the antigens were located on the surfaces of mycelial cells, but within the cell walls of yeasts. These antigens were detected in a membrane preparation, in the SDS-soluble material and in the material released by a 1,3-β-glucanase and chitinase from the cell walls of yeast and mycelial cells. In the latter three samples, an additional high-molecular-mass, highly polydispersed band was also detected. Beta-elimination of each fraction resulted in the disappearance of all antigen bands, suggesting that they are highly O-glycosylated. In addition, the electrophoretic mobility of the high-molecular-mass, highly polydispersed bands increased after digestion with endoglycosidase H, indicating that they are also N-glycosylated. New antigen bands were released when remnants of the cell walls extracted with 1,3-β-glucanase or chitinase were digested with chitinase or 1,3-β-glucanase. These results are consistent with the notion that, after secretion, parts of the O-glycosylated antigen molecules are transferred to an N-glycosylated protein(s). This molecular complex, as well as the remaining original 70 and 80 kDa antigen molecules, next bind to 1,3-β-glucan or chitin, probably via 1,6-β-glucan, and, in an additional step, to chitin or 1,3-β-glucan. This process results in the final molecular product of each antigen, and their distribution in the cell walls.

Keywords: monoclonal antibodies, cell wall biosynthesis, lysis of yeast wall, N-glycosylated proteins, cell wall architecture

INTRODUCTION

The cell walls of Candida albicans and other fungal cells are composed of β-glucans and mannoproteins plus small amounts of chitin. Whilst great progress has been made in understanding the formation of the polysaccharide framework, the wall proteins have been studied far less. Wall proteins can be divided into two groups: proteins that are solubilized by ionic detergents or chaotropic agents, and those that are solubilized by degradation with hydrolases such as β-glucanases or chitinase (Elorza et al., 1985; Kapteyn et al., 1995). Wall biogenesis is the result of several steps that are initiated with the synthesis of the proteins at the level of the endoplasmic reticulum, and their glycosylation in the secretory pathway (Golgi apparatus and secretory vesicles), followed by their secretion and interaction with the nascent wall polysaccharides. The process ends with bond formation among the different components, producing the final architecture of the wall structure (Valentin et al., 2000). Though some of the protein–polysaccharide bonds are fairly well known, the enzymes catalysing them are practically unknown. Bond formation between proteins and with the polysaccharides occurs outside the plasma membrane and may involve different reactions. In prokaryotic and eukaryotic cells,

Abbreviations: Endo-H, N-acetylglucosaminidase H; GPI, glycosylphosphatidylinositol; IIF, indirect immunofluorescence.
some proteins are retained in the plasma membrane by means of glycosylphosphatidylinositol (GPI) anchors. In *Saccharomyces cerevisiae*, some wall proteins also carry GPI residues, suggesting that these anchors allow transport to the plasma membrane (Kapteyn et al., 1999). From here, the proteins may find their way to the wall by forming glycosidic linkages with branched 1,6-β-glucan which is linked to 1,3-β-glucan–chitin complexes (Kollár et al., 1997). Other cell wall proteins that do not carry GPI anchors have also been found in *S. cerevisiae* (Kapteyn et al., 2000). The mechanism of their incorporation into the cell wall is unknown. In this respect, some wall proteins have the capacity, once the detergents or chaotropic agents used in their solubilization have been removed, to interact, forming aggregates of increasing complexity (Glee & Hazen, 1995; Aguado et al., 1998). Other proteins bind to the structural polysaccharides, suggesting that this may be important for the formation of covalent bonds (Aguado et al., 1998). An additional understanding of the biosynthetic pathway of the cell wall will require the identification of the ways in which specific proteins are secreted and incorporated into the cell wall, and the catalytic activities implicated in these processes. In this work, we have identified the process by which two proteins reacting with the monoclonal antibody 3A10 are incorporated into the cell wall. The results show that the antigen recognized by this mAb is synthesized as two O-glycosylated molecules of 60 and 70 kDa, which, after secretion, find their way to the cell wall by binding to an N-glycosylated protein. This molecular complex and the remaining original antigens are linked to 1,3-β-glucan, chitin, or both.

**METHODS**

**Organism and culture conditions.** *C. albicans* ATCC 26555 was used throughout this work. It was maintained by subculturing every 2–3 weeks on Sabouraud dextrose agar slants. *C. albicans* was propagated as a yeast in minimal medium supplemented with amino acids as described by Lee et al. (1975). Erlenmeyer flasks (500 ml) containing 150 ml Lee medium were each inoculated with a loopful of cells from a 12–18 h culture and incubated in a gyratory incubator (200 r.p.m.) at 28 °C for 12–18 h (exponential phase). Cells were collected from the culture medium by centrifugation at 3000 g (10 min), washed twice with sterile glass-distilled water, and suspended at a concentration of approximately 1 mg cells (10 min), washed twice with sterile glass-distilled water, and finally washed four times with chilled buffer A, boiled for 10 min with 2% SDS in glass-distilled water and finally washed four more times with chilled 0.001 M PMSF in glass-distilled water. The supernatant resulting from isolation of the cell walls was further centrifuged at 40000 g for 40 min, the pellet (P40) was washed three times in buffer A and maintained at −20 °C.

**Solubilization of wall proteins by digestion with hydrolytic enzymes.** Proteins were solubilized from purified walls (yeast or mycelium) by treatment with laminarinase, a 1,3-β-glucanase (0–125 U ml⁻¹ per 100–150 µg purified freeze-dried cell walls in 0.010 M phosphate buffer, pH 6.3, containing 0.001 M sodium azide and 0.001 M PMSF in 0.1 M acetate buffer, pH 5.5, at 35 °C for 4 h) or chitinase (1 mg ml⁻¹, specific activity 570 U g⁻¹, 30 °C for 3 h). In both cases, suspensions were supplemented with pepstatin (0.7 µg ml⁻¹) and leupeptin (0.5 µg ml⁻¹). After treatment, the wall residue was recovered by centrifugation (12000 g, 15 min) and digested with the chitinase or with 1,3-β-glucanase or zymolase 20T (10 µg enzyme complex per 100–150 µl purified cell walls in 0.001 M sodium azide and 0.001 M PMSF in 0.101 M Tris/HCl buffer, pH 7.4) at 28 °C for 2 h. The solubilized material was concentrated by freeze-drying. Beta-elimination of samples was carried out in 0.1 M NaOH at room temperature for 12 h. N-Acetylglucosaminidase H (Endo-H) digestion was performed as described previously (Pastor et al., 1984). Digestion with 1,6-β-glucanase was carried out in 0.100 M sodium acetate, pH 5.5, at 37 °C for 20 h. The total sugar and protein contents in the lyophilized material were determined by the methods of Dubois et al. (1956) and Lowry, respectively.

**PAGE.** Proteins were separated by SDS-PAGE, performed basically as described by Laemmli (1970). Polyacrylamide slab gradient gels (5–15%; ratio of acrylamide to bisacrylamide, 30:0:2) with 3.5–4% polyacrylamide stacking gel were used. Two volumes of the sample were mixed with one volume of electrophoresis sample buffer (33% glycerol, 14% 2-mercaptoethanol, 7% SDS, and 0.035% bromophenol blue as a tracking dye, in 0.5 M Tris/HCl, pH 6.8) and heated for 5 min in a boiling water bath prior to application onto the gels. Electrophoresis was performed at a constant current of 25 mA.

**Western blot techniques.** After SDS-PAGE, the proteins were transferred to nitrocellulose paper by using an electrotoblot (a Bio-Rad Trans-Blot cell with an LKB 2197 power supply) essentially as described by Burnette (1981) except that the transfer buffer used was 0.025 M Tris/0.192 M glycine, pH 8.3, plus 20% (v/v) methanol and the electrophoretic transfer was accomplished at 6–8 V cm⁻¹ for 15–17 h at 4 °C. Proteins were immunodetected according to the protocol described in the Bio-Rad Immun-Blot (GAR-HRP) assay kit, based on the procedure of Burnette (1981) and Towbin et al. (1979), using mAb 3A10 at a final dilution of 1:6000 in 0.100 M Tris/HCl buffer (pH 7.4) containing 3% BSA as the blocking agent, 0.9% NaCl and 0.05% Tween 20. Diluted (1:10000) peroxidase–goat anti-mouse IgG (Bio-Rad) was used as the indicator antibody in the Western blot techniques.

**Preparation of pustulan, 1,3-β-glucan and colloidal chitin.** Pustulan was prepared from the lichen *Umbilicaria papullosa* according to the method of Lindberg & McPherson (1954), as modified by Reese et al. (1962). The lichen (100 g; Calbiochem) was pulverized and boiled for 15 min in 500 ml [5 mg beads (mg cells)⁻¹]; cell breakage was assessed by examining the preparations under a phase-contrast microscope, and complete breakage was obtained. The cell walls were sedimented (12000 g, 10 min) from the cell-free homogenate, washed four times with chilled buffer A, boiled for 10 min with 2% SDS in glass-distilled water and finally washed four more times with chilled 0.001 M PMSF in glass-distilled water. The supernatant resulting from isolation of the cell walls was further centrifuged at 40000 g for 40 min, the pellet (P40) was washed three times in buffer A and maintained at −20 °C.
Colloidal chitin was prepared as described by Pegg (1988). Alkaline-insoluble glucan was prepared by extracting whole yeast with 2 M NaOH at 100 °C for 2 h. The insoluble residue (mainly 1,3-β-glucan) was collected by centrifugation at 3000 g for 10 min and washed three times with glass-distilled water.

**Concanavalin A affinity chromatography.** The zymolase extract obtained from yeast or mycelial cells was dissolved in 40 mM Tris/HCl, pH 7.4, containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, and 0.5 M NaCl (ConA buffer) and applied to a concanavalin A/Sepharose 4B (Pharmacia) column equilibrated with the same buffer. The column was washed with three bed volumes of ConA buffer, and the material bound by the concanavalin A was eluted with ConA buffer containing 0.5 M methyl α-D-manno-pyranoside. The eluted fractions were desalted by dialysis against distilled water and then lyophilized.

**Production of mAbs.** For the production of mAbs, female BALB/c mice were inoculated intraperitoneally with 100 µl (50 mg protein) ConA-retained zymolase-extracted material from *C. albicans* cell walls in PBS, emulsified with an equal volume of complete Freund’s adjuvant. Two weeks later, the mice received a booster intraperitoneal injection with the same amount of antigen emulsified with incomplete Freund’s adjuvant. One week after this immunization, the mice were tail-bled and the sera were tested for anti-antigen antibody titre by using an ELISA. Selected animals received two boosts with the same immunogen, and, 3 d after the last injection, the splenocytes from the immunized mice and P3-X63 Ag 8.653 murine myeloma cells were fused at a 5:1 ratio by using polyethylene glycol 1500 as the fusing agent. Cell fusion and selection of hybrids were performed as described by Galfre & Milstein (1981). The fused cells were distributed in 96-well culture plates at an approximate density of 10⁶ cells per well. Ten days after the fusion, the culture supernatants were screened for the presence of antibodies against the antigen (zymolase-extracted material from *C. albicans* cell walls) by using an indirect ELISA. Positive hybridomas were cloned by limiting dilution in 96-well Nunclon plates containing feeder cells. Ten days later, supernatants from growing hybridomas were tested by using an ELISA, and hybridomas from positive wells were subcloned. The mAbs were purified from ascites by precipitation with 50% ammonium sulfate. The immunoglobulin class of antibodies was determined with a mouse monoclonal antibody isotyping kit (Sigma Immuno Chemicals).

**ELISA.** These assays were performed as described by Voller et al. (1980), but with the following modifications. Wells of Nunc-Immunoplate I (A/S Nunc) were coated with 25 µg (expressed as total sugar) antigen (the soluble material released from yeast cell walls by zymolase digestion and retained by a concanavalin A affinity column) in 50 µl 0.030 M sodium carbonate buffer (pH 9.6) and incubated at 4 °C for 18 h. The plate was rinsed three times with Tris-buffered saline (TBS), 0.05% Tween 20 (TBST), then filled with 3% BSA in TBS and incubated for 2 h at 37 °C.

The mAbs from the ascitic fluid (see above) were diluted with PBST and added to the appropriate well, the plate being incubated at 37 °C for 1 h in a moist chamber. The wells were then rinsed three times with PBST, and 50 µl of a 1:100 dilution in BST of goat anti-mouse immunoglobulins conjugated to peroxidase (Sigma) was added to each well. After incubation for 1 h at 37 °C, the wells were rinsed with PBST.

The addition of substrate mixture containing o-phenylenediamine, the plate was incubated in the dark for 10 min and the reaction was stopped by the addition of 25 µl 3 M H₂SO₄ to each well. The colour intensity was determined at 492 nm with an automated plate reader (Easy Reader EAR 400; Institut Pasteur Laboratories).

**Indirect immunofluorescence for detection of cell-surface antigens.** The immunofluorescence assay was carried out basically as described by Sundstrom & Kenny (1985). Organisms were washed twice in PBS, fixed for 10–20 min with 0.5% formalin in PBS, and stored in this suspension until used. The cell concentration was adjusted to 10⁶ cells ml⁻¹ by the addition of PBS, and drops from this suspension were placed on the wells of Microslides (BioMerieux), then allowed to air-dry. Drops (10 µl) from different antiserum dilutions in PBS (dilutions of ascitic fluid in the case of mAb (alternatively, undiluted culture supernatants were also used), or dilutions of the yeast-adsorbed polyclonal antibodies against cell walls (from mycelium) were dropped over the antigen. Microslides were placed in a moist chamber at 37 °C for 30 min and washed in two changes of PBS for a total of 15 min. FITC-conjugated goat anti-rabbit or anti-mouse IgG (10 µl; Ortho-Diagnostics Systems) was added to the slides, which were washed again at 37 °C for 30 min in a moist chamber, washed with PBS as described above, rinsed with glass-distilled water, and mounted in PBS containing 90% glycerol. The indirect immunofluorescence (IF) of insoluble polysaccharides was investigated in a similar way, but omitting the fixation step. The cells (or polysaccharides) were examined under a Zeiss Photomicroscope III equipped for epi-fluorescence (UV filter no. 487702; excitation line 365/366 nm). The fluorescence was dependent upon the reaction of the cells with the mAb, since no fluorescent cells were observed if they were incubated only with the second antibody.

**Miscellaneous.** Reagents used for gel electrophoresis and blotting were from Bio-Rad. The SDS molecular mass markers were from Sigma and Pharmacia. Culture media compounds and complete and incomplete Freund’s adjuvants were purchased from Difco. Laminaranase (EC 3.2.1.6), chitinase (EC 1.2.3.14) from *Serratia marcescens*, pepstatin, antipain and the antiprotease cocktail were from Sigma. Unless specifically indicated otherwise, all other chemicals were from Sigma. The 1,6-β-glucanase from *Trichoderma harzianum* was a gift from T. Benitez of the University of Seville (De la Cruz et al., 1995).

**RESULTS**

**Preparation and evaluation of mAbs.**

Mice were immunized with the soluble material released from yeast or mycelial cell walls by zymolase digestion and retained by a concanavalin A affinity column. Seven positive hybridomas secreting antibodies were obtained, and ascitic fluid was collected from three of them (3A10, 5B1 and 5D8). The first two were shown to be IgG, whereas 5D8 is an IgM. Antibody determination was carried out by using an ELISA with a mixed membrane preparation (P40) and materials solubilized by hot SDS, zymolase or chitinase from isolated yeast walls. The results showed that 3A10 reacted strongly with the latter two preparations, whereas reactivity towards P40 and SDS-soluble materials was low. mAb 5D8 did not react with zymolase- or chitinase-solubilized material but
gave some reactivity against SDS and P40 materials. Finally, mAb SB1 reacted with low intensity against all of the extracted materials.

Further experiments were carried out with mAb 3A10. To determine whether mAb 3A10 reacts with a sugar or a protein epitope, the antibody was reacted against insoluble preparations of pustulan (1,6-β-glucan), colloidal chitin and alkali-insoluble wall polysaccharide (mainly 1,3-β-glucan). IIF was negative with each of these three preparations, demonstrating that the mAb did not recognize these polysaccharides (IIF not included) and, as a consequence, mAb 3A10 probably recognized a mannoprotein antigen(s). The three polysaccharides (1,6-β-glucan, 1,3-β-glucan and chitin) are stable under the mild alkali conditions used in the β-elimination.

IIF was also used to determine whether the 3A10 antigen was located on the surface of cells. Exponentially growing yeast and mycelial cells were collected and reacted with the mAb. Yeast cells showed no fluorescence (Fig. 1a, panel 2) whereas mycelia were stained (but not the original parent yeast cells) (Fig. 1b, panel 2). This result suggested that the epitope recognized by mAb 3A10 was absent or differently located in yeast and mycelial cell walls. The latter possibility was confirmed when we found that yeast cells extracted with

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**Fig. 1.** Phase-contrast microscopy (panel 1) and immunofluorescence (panel 2) of intact yeast (a) and yeast extracted with SDS (c), and intact mycelium (b) and mycelium extracted with SDS (d) stained with mAb 3A10 (1:150 dilution) raised against concanavalin-A-retained zymolyase-extracted material from *C. albicans* cell walls. Bars, 5 µm.
hot SDS were stained with mAb 3A10 (Fig. 1c, panel 2) and the mycelia appeared brighter (Fig. 1b, d, panel 2).

Mechanism of covalent incorporation of wall mannoproteins

As the interactions between bulk proteins, and with wall structural polysaccharides, have been described previously (Aguado et al., 1998; Glee & Hazen, 1995), our next goal was to determine the mechanism of incorporation of specific proteins into the wall. For these studies, we used mAb 3A10 since it recognizes, as described above, a mannoprotein epitope present in both yeast and mycelial cell walls. With this aim, we used Western blotting to analyse a mixed membrane preparation containing particles from organelles involved in the synthesis and secretion of proteins (P40), and extracts from the cell walls obtained with hot SDS and by digestion with chitinase, laminarinase (a 1,3-β-glucanase) or zymolyase (an enzymic complex rich in 1,3-β-glucanase). Two bands of 70 and 80 kDa were detected in P40 (Fig. 2a, lane 1); these were also found in the material extracted with hot SDS from the cell walls, along with a polydispersed band with a molecular mass greater than 220 kDa (Fig. 2a, lane 2). Bands of 70 and 80 kDa, a highly polydispersed band with an apparent mass between 200 and 280 kDa, plus two possible additional bands of 15 and 16 kDa were detected from the walls digested with chitinase (Fig. 2a, lane 3). With laminarinase, in addition to the 70 and 80 kDa bands, a highly polydispersed band with an apparent molecular mass between 160 and 260 kDa, and small amounts of a protein of 120 kDa, were also found (Fig. 2a, lane 4). Zymolyase extract from yeast yielded the 70 and 80 kDa bands along with another of 15 kDa (Fig. 2, lane 5). Similar results were obtained when mycelial cell walls were analysed (Fig. 2b).

Additional information about the chemical characteristics of the bands detected in the previous experiment was obtained when each of the extracted materials was digested subsequently with Endo-H, laminarinase or 1,6-β-glucanase, or treated with small amounts of alkali (β-elimination). No change in mobility in Western blots was detected for the 70 and 80 kDa bands from the P40 fraction after treatment with Endo-H, laminarinase or 1,6-β-glucanase digestion (lanes 3, 4) and 1,6-β-glucanase digestion (lane 5), stained with mAb 3A10 (1:6000 dilution). Molecular masses of proteins are indicated to the right of the panel.
moieties or any other sugar moiety, as their original electrophoretic mobility did not change after digestion with the Endo-H, laminarinase or 1,6-β-glucanase (Fig. 3).

The results obtained with the SDS-solubilized material were similar, in that β-elimination also led to the disappearance of all antigenic bands (Fig. 4, lane 3). The release of this carbohydrate moiety(ies) by Endo-H could be responsible for the observed increase in electrophoretic mobility.

Furthermore, the bands extracted with laminarinase also disappeared after β-elimination (Fig. 5, lane 3), and, again, treatment with Endo-H resulted in a significant increase in the electrophoretic mobility of the highly polydispersed material. The polydispersed band was also resolved into two bands of 140 and 160 kDa (Fig. 5, lane 2). Digestion with either 1,3-β-glucanase or 1,6-β-glucanase produced no changes in the mobilities of the bands.

Similarly, when the SDS-extracted walls were digested with chitinase and the solubilized materials were β-eliminated, no antigenic bands were detected, demonstrating once more that the antigen(s) that was covalently linked to chitin was O-glycosylated (Fig. 6). Endo-H treatment again resulted in the disappearance of the highly polydispersed band and the appearance of two new ones with a significant increase in electrophoretic mobility (Fig. 6, lane 2). These results show

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**Fig. 4.** Western blot of 5–15% slab gradient gels loaded with material from SDS-extracted yeast cell walls (lane 1, untreated), and after Endo-H treatment (lane 2), β-elimination (lane 3), laminarinase digestion (lane 4) and 1,6-β-glucanase digestion (lane 5), stained as described in Fig. 3. Molecular masses of proteins are indicated to the right of the panel.

**Fig. 5.** Western blot of 5–15% slab gradient gels loaded with laminarinase-released material (lane 1, untreated) from isolated yeast cell walls, and after Endo-H treatment (lane 2), β-elimination (lane 3), laminarinase digestion (lane 4) and 1,6-β-glucanase digestion (lane 5), stained with mAb 3A10 (1:6000 dilution). Molecular masses of proteins are indicated to the right of the panel.

**Fig. 6.** Western blot of 5–15% slab gradient gels loaded with chitinase-released material (lane 1, untreated) from isolated yeast cell walls, and after Endo-H treatment (lane 2), β-elimination (lane 3), laminarinase digestion (lane 4) and 1,6-β-glucanase digestion (lane 5), stained with mAb 3A10 (1:6000 dilution). Molecular masses of proteins are indicated to the right of the panel.
that the original protein extracted by chitinase, as was the case with laminarinase, was N-mannosylated. No change in the original electrophoretic pattern was detected after an additional digestion with 1,6-β-glucanase or 1,3-β-glucanase (Fig. 6).

In additional experiments, laminarinase-treated cell walls were extracted with chitinase (Fig. 7), and chitinase-treated walls were extracted with laminarinase. In the first case, bands of 70 and 80 kDa were released together with another one of very low molecular mass (15 kDa; Fig. 7, lane 1). In the latter treatment, in addition to the 70 and 80 kDa bands, a 220 kDa band of polydispersed material was also seen (Fig. 7, lane 2).

**DISCUSSION**

Wall proteins are modified during the secretory process and then delivered to the cell surface, where they interact with polysaccharides during their incorporation into the cell wall. The mechanisms associated with these interactions are not fully understood. In this paper, we demonstrate that information of this nature can be obtained with the help of monoclonal antibodies that specifically label wall antigens. With this aim, we raised monoclonal antibodies directly against cell wall proteins by immunizing mice with zymolyase-solubilized manno-proteins of SDS-extracted walls. One of these antibodies (3A10) reacted strongly with the material solubilized by zymolyase, but did not react with 1,3-β-glucan, 1,6-β-glucan or chitin. Reactivity disappeared after digestion with trypsin, hinting that the antigen was of a manno-protein nature (results not shown). By IIF, the antigen was located on the external surfaces of mycelial cells but not yeast cells. Nevertheless, the antigen was detected in yeasts after removal of the external protein layer by SDS, confirming that the epitope is also present in yeast walls but is masked by the manno-proteins that cover the outer surface of the wall (Eroles et al., 1997).

As expected of a secreted antigen, material reacting with mAb 3A10 was found in a mixed membrane preparation containing membranous fragments belonging to the secretory pathway (Fig. 2). Two bands with apparent molecular masses of 70 and 80 kDa, whose mobilities were not affected by treatment with Endo-H (an enzyme that releases N-linked mannose residues), 1,3-β-glucanase or 1,6-β-glucanase, were detected in these particles. Treatment with alkali (β-elimination) destroyed the reacting material. It is known that under these conditions the sugar residues linked to threonine or serine in a manno-protein (O-linkages) are released and the protein degraded. This behaviour suggests that the antigen molecules do not contain 1,3-β-glucan, 1,6-β-glucan or N-linked mannan moieties, but contain only O-glycosidically linked mannose residues. The detection of two bands with apparent molecular masses of 70 and 80 kDa might also indicate that the smaller one is a degradation product, or that the larger one carries an additional repeat (if it is a protein with internal repeats; Kapteyn et al., 2000). As the reactivities of the two bands are similar in all preparations, the second hypothesis would appear to be the correct one. The two bands were also solubilized from the walls by SDS treatment (Fig. 2), suggesting that non-covalent linkages retained them. SDS-PAGE demonstrated that the material extracted with the detergent contained, in addition to the two bands of 70 and 80 kDa, polydispersed high-molecular-mass material. The electrophoretic mobilities of the two first bands were not modified by Endo-H treatment, which, interestingly, increased the mobility of the third band. The three bands disappeared after β-elimination, but their mobilities did not change when laminarinase or 1,6-β-glucanase treatments were used. Therefore, it may be concluded that the polydispersed band (in reality two bands; Figs 4, 5 and 6) is N-glycosylated in addition to being O-glycosylated. Although we initially considered the possibility that the high-molecular-mass highly polydispersed antigen(s) was a totally distinct cell wall protein, we rejected this hypothesis, as the antigen would also have been detected at the level of the mixed membrane preparation. It is therefore possible that the two O-glycosylated antigens
found in the mixed membrane preparation are the precursors of the N-glycosylated, high-molecular-mass mannoproteins, given that they are the only antigens found in the mixed membrane preparation and are not covalently linked to the wall (SDS-extractable). The possibility of high-molecular-mass mannan residues being transferred to asparagine residues of the antigen molecules, giving rise to N-glycosidic linkages, is unlikely since this process occurs only intracellularly. The alternative possibility, whereby the antigens of 70 and 80 kDa are transferred to an N-glycosylated mannoprotein(s), seems more likely. We have no information about the nature of the putative N-glycosylated mannan linked to the antigens, about how this molecular complex(es) might be formed, or about the enzymic activity (or activities) that catalyse this reaction(s). A transglutaminase activity was detected in the cell walls of *C. albicans*, *S. cerevisiae* and *Yarrowia lipolytica* (Ruiz-Herrera et al., 1995). This enzyme (EC 2.3.2.13) catalyses the formation of inter-peptidic cross-links between the carboxamide group of a glutamine residue in one protein and the ε-amine group of a lysine residue of another peptide. Participation of an N-glycosylated protein in the incorporation of an O-glycosylated protein has been found in the case of a mycelial specific protein (Elorza et al., 1989) and with Ywp1 of *Y. lipolytica* (Ramón et al., 1999). In this case, the protein was also retained in the wall by disulfide bridges to lattice proteins, as in *S. cerevisiae* (Cappellaro et al., 1998).

Three bands were also detected in the materials released by laminarinase (a 1,3-β-glucanase) and four in those released by chitinase. This result was expected, as in *C. albicans* 90% of the GPl proteins are attached via 1,6-β-glucan to 1,3-β-glucan and the remaining ones are attached through 1,6-β-glucan to chitin (Kapteyn et al., 2000). Similarly in *S. cerevisiae*, 1–2% of cell wall proteins are attached directly to chitin through a 1,6-β-glucan moiety (Kapteyn et al., 1997), and Cwp1p is found as a 1,6-β-glucanase-extractable and non-extractable, chitin-linked fraction (Van der Vaart et al., 1996; Kapteyn et al., 1997). Although the mobility of the high-molecular-mass band was different, the behaviour of the three antigens towards additional digestions or β-elimination was similar to that found in the material released by SDS. Therefore, it is hypothesized that part of each antigen molecule is covalently linked to a high-molecular-mass N-glycosylated molecule, and, in successive steps, associates with the wall structure by forming covalent bonds with either 1,3-β-glucan or chitin. The observation that part of the antigen remaining in the wall after extraction with either 1,3-β-glucanase or chitinase was released by further digestion with chitinase or 1,3-β-glucanase suggests that parts of the antigens are cross-linked to both 1,3-β-glucan and chitin. These results are consistent with the idea that, after the formation of covalent linkages between the antigens and the 1,3-β-glucan or chitin, these molecules bound to the 1,3-β-glucan are cross-linked to chitin, and that those initially bound to chitin are now linked to 1,3-β-glucan. If that were the case, formation of these new linkages would lead to the transformation of the cell wall from an elastic structure to a rigid one. In *S. cerevisiae*, in agreement with our observations, nascent wall GPl proteins carrying 1,6-β-glucan moieties are retained initially at the level of the plasma membrane (Kapteyn et al., 2000), and later are transferred to a 1,3-β-glucan molecule that finally appears cross-linked to chitin (Kollár et al., 1997). Enzymic activities that participate in the formation of these linkages are the Gas1p of *S. cerevisiae* (Ram et al., 1998) and Phr1p and Phr2p of *C. albicans* (Popolo & Vai, 1998; Fonzi, 1999). The expression of Phr1p and Phr2p is regulated by ambient pH; these enzymes process 1,3-β-glucans by making acceptor sites available for the attachment of mannoproteins carrying 1,6-β-glucans. In null PHR1 and PHR2 mutants, and at the non-permissible pH, there is an increase in the amounts of proteins in the spent media, and mannoproteins cross-linked to chitin are detected. We are currently trying to determine the fate of the 3A10 antigens in these mutants.

In conclusion, the results reported in this paper indicate that some mannoproteins in *C. albicans* are incorporated into the wall architecture directly, or after interacting with an N-glycosylated protein(s), by binding to 1,3-β-glucan or chitin – probably via 1,6-β-glucan (Kapteyn et al., 2000). In a second step, they are cross-linked to the alternative polysaccharide (chitin or 1,3-β-glucan).

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