Identification of glucan–mannoprotein complexes in the cell wall of *Candida albicans* using a monoclonal antibody that reacts with a (1,6)- β -glucan epitope

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The use of a novel monoclonal antibody (mAb) that reacts with (1,6)- β -glucan has permitted the study of the different covalent linkages between glucan and mannoproteins in the cell wall of *Candida albicans*. The mAb JRR1 was originally raised by immunization with Zymolyase extracts from *C. albicans* cell walls, but it soon became apparent that it reacted with a (1,6)- β -glucan epitope. By using this antibody, we show the existence of glucan–mannoprotein complexes between the (1,6)- β -glucan epitope recognized by the antibody and cell wall mannoproteins. The topology of the (1,6)- β -glucan in the cell wall of *C. albicans* has also been studied.

Keywords: *Candida albicans*, cell wall, monoclonal antibody, (1,6)- β -glucan assembly and topology

INTRODUCTION

The components of the walls of *Candida albicans* are β -glucans, chitin and mannoproteins, and although some aspects of their biosynthesis have been studied, the molecular organization of the cell walls is largely unknown, owing to the difficulties in identifying individual macromolecules. Glucans and chitin form a skeleton for the mannoproteins, which are localized mainly at the outer surface, although they are also found throughout the cell wall structure (Elorza *et al.*, 1989; Calderone & Braun, 1991; Shepherd & Gopal, 1993; Klis, 1994). Several chitin-synthase-coding genes have been cloned from both yeast and mycelial gene libraries of *C. albicans* (Chen-Wu *et al.*, 1992), and wheat germ agglutinin has been used to show that chitin, although concentrated in the cross-walls between mother and daughter cells, is also randomly distributed throughout the whole cell wall (Marcilla *et al.*, 1991). In the case of *Saccharomyces cerevisiae* mannoproteins, biochemical (Schekman & Novick, 1982) and genetic (Ballou, 1990; Schreuder *et al.*, 1993) approaches have facilitated the study of their synthesis and secretion, whilst the distribution of mannoproteins in

the walls of *C. albicans* has been studied using concanavalin A (ConA) lectin (Marcilla *et al.*, 1991).

The study of the cytological distribution of glucans has been hampered by lack of specific lectins or antibodies, as no lectin reacts exclusively with glucans and because glucans are not immunogenic by themselves. Only when coupled to a protein does a small oligosaccharide, such as laminaribiose (Horisberger & Rouvet-Vauthey, 1985) or pustulan (Montijn *et al.*, 1994), become immunogenic. Nonetheless, the biosynthesis of both (1,3)- β - and (1,6)- β -glucans has been studied in *S. cerevisiae* using biochemical and genetic methods. In the case of (1,3)- β -glucan, its formation takes place at the plasma membrane (Shematek *et al.*, 1980). (1,6)- β -Glucan synthesis is apparently initiated at the endoplasmic reticulum and the nascent molecules are elongated along the secretory pathway (Roemer *et al.*, 1993).

All cell wall components, once secreted or extruded from the plasma membrane, could interact to produce different types of bonds, including hydrophobic, ionic and covalent bonds which give rise to the final molecular organization of the cell wall. Glucan and chitin are connected by covalent bridges in *Schizophyllum commune* (Sonnenberg *et al.*, 1982; Wessels *et al.*, 1983); linkages between glucan and mannoproteins have been reported in *S. cerevisiae* (Van Rinsum *et al.*, 1991; Montijn *et al.*, 1994). In *C.*

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Abbreviations: ConA, concanavalin A; mAb, monoclonal antibody.

albicans, linkages between a (1,6)- β -glucan and chitin (Surarit *et al.*, 1988), and between mannoproteins and both glucans and chitin (Elorza *et al.*, 1985; Marcilla *et al.*, 1991, 1993), have also been described.

The development of monoclonal antibodies (mAbs) has opened new ways to identify specific molecules (Casanova *et al.*, 1989; Sundstrom *et al.*, 1986) and to investigate the molecular processes involved in their formation, as well as the types of bridges connecting them in the fungal cell walls (Elorza *et al.*, 1989; Marcilla *et al.*, 1991; Torosantucci *et al.*, 1990; Trinel *et al.*, 1992).

In this paper we present what to our knowledge is the first mAb (JRR1) that reacts with a (1,6)- β -glucan epitope, although a polyclonal antibody reacting against the same epitope has recently been described (Montijn *et al.*, 1994). Using this mAb we have studied the linkages connecting (1,6)- β -glucan to the mannoproteins, the presence of (1,6)- β -glucan in the mannoproteins secreted by regenerating protoplasts, and also the topology of the distribution of (1,6)- β -glucan in the cell walls of *C. albicans*.

METHODS

Strains and conditions of growth. *Candida albicans* ATCC 26555 serotype A was grown according to the method of Elorza *et al.* (1989). Cells incubated at 28 °C in Lee medium (Lee *et al.*, 1975) were collected after 12–18 h, when they were at the exponential growth phase. For the formation of mycelium, yeast cells stored in water at 4 °C for 24–42 h were incubated at 37 °C for 6 h in Lee medium and then collected. To analyse the effect of tunicamycin on the localization of the mAb JRR1 epitope in the cell surface, cells were grown in Lee medium at 28 and 37 °C for 2 h, and then the antibiotic was added at a concentration of 20 $\mu\text{g ml}^{-1}$. After 4 h incubation cells were harvested and indirect immunofluorescence was performed.

Preparation of different materials from cell walls. Cell walls were obtained as described by Marcilla *et al.* (1993) and extracted with 2% (w/v) SDS for 10 min at 100 °C to remove non-covalently-retained molecules.

The wall material was solubilized by Zymolyase 20T, and mannan from the walls of different fungi was obtained as described by Marcilla *et al.* (1993). Walls previously treated with 2% SDS from 30 mg (dry wt) of cells were suspended in 1 mM PMSF containing Zymolyase 20T (1 mg ml^{-1}) and incubated at 30 °C for 3 h. The suspension was then centrifuged (1200 g, 15 min), the wall residues discarded and the solubilized material concentrated by lyophilization.

In order to obtain mannan preparations, walls previously treated with 2% SDS were boiled in 2% (w/v) NaOH for 2 h. Solubilized mannan was purified by precipitation with Fehling solution (Edwards, 1965), washed with hot water and re-dissolved by addition of 0.5 M HCl until the blue colour disappeared. The mannan was precipitated by 75% (v/v) ethanol in water at 20 °C for 18 h and after washing twice with 75% ethanol, it was dissolved in distilled water.

The total sugar content of the different materials was determined by the method of Dubois *et al.* (1956), and the total protein content was determined by the Lowry method.

Preparation of mannan from mannoproteins secreted by regenerating spheroplasts. Spheroplasts of *C. albicans* were obtained and regenerated as previously described (Elorza *et al.*,

1983). After 6 h incubation at 28 °C, the regenerating medium was separated from cells by centrifugation. The medium which contained the mannoproteins secreted by the spheroplasts was dialysed against distilled water at 4 °C and concentrated by lyophilization. To obtain the mannan, this concentrated material was treated with 2 M NaOH for 2 h at 100 °C, and then processed in the same way as described above for the material extracted from cell walls.

Indirect immunofluorescence for the detection of cell surface antigens. The immunofluorescence assay was performed basically as described by Casanova *et al.* (1989). Cells were washed three times with PBS (0.145 M NaCl, 0.15 M sodium phosphate) and incubated at 37 °C for 1 h with the appropriate dilution in PBS of the antibodies (1:25 for mAb JRR1 and 1:50 for mAb 1B12). After three washes with PBS the cells were incubated with fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (Boehringer Mannheim), diluted 1:10 in PBS at 37 °C for 1 h. After three washes with PBS the cells were then examined with a Zeiss Photo Microscope III equipped for epifluorescence. Fluorescence was dependent on the reaction of the cells with the mAbs, since no fluorescence was detected when the cells were incubated with the second antibody only.

ConA-affinity chromatography. The mannan or Zymolyase extract obtained from yeast walls of *C. albicans* was dissolved in 40 mM Tris/HCl, pH 7.4, containing 1 mM CaCl_2 , 1 mM MgCl_2 , 1 mM MnCl_2 and 0.5 M NaCl (ConA buffer) and applied on a ConA-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 ConA was eluted with ConA buffer containing 0.5 M methyl α -D-mannopyranoside. The non-bound and the eluted mannan or Zymolyase-extracted fractions were desalted by dialysis against distilled water and lyophilized.

Paper chromatography. The mannan fractions were hydrolysed in 4 M HCl for 4 h at 105 °C and lyophilized to eliminate the HCl. Partial acid hydrolysis was carried out in 2 M HCl for 2 h followed by lyophilization. Sugars were separated by descending paper chromatography in ethyl acetate/pyridine/water (8:2:1, by vol.). After 54 h, the chromatography paper was developed by silver staining.

Production of mAbs. For the production of mAb JRR1, female BALB/c mice were inoculated intraperitoneally (i.p.) with 100 μl (50 μg protein) Zymolyase-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 i.p. 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 tested for anti-antigen antibody titre by ELISA. Selected animals received two boosts with the same immunogen, and 3 d following the last injection, the splenocytes from the immunized mouse and P3-X63/Ag 8.653 murine myeloma cells were fused at a 5:1 ratio using PEG 1.500 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 10^5 cells per well. Ten days after the fusion, the culture supernatants were screened for the presence of antibodies against the antigen (Zymolyase-extracted material from *C. albicans* cell walls) by 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 ELISA and hybridomas from positive wells were subcloned. Ascitic fluid was obtained as described by Hoogenraad & Wraight (1986). mAbs were purified from ascites by precipitation with 50%

(w/v) ammonium sulfate. The immunoglobulin class of antibodies determined with a Mouse Monoclonal Antibody Iso-typing Kit (Sigma Immuno Chemicals).

Preparation of pustulan. Pustulan was prepared from the lichen *Umbilicaria papulosa* according to the method of Lindberg & McPherson (1954), and modified by Reese *et al.* (1962). The lichen (100 g) (Calbiochem) was pulverized and boiled for 15 min in 500 ml 0.165 M H_2SO_4 . After this, ethanol was added until the concentration reached 13% (v/v) and then the mixture was centrifuged. The supernatant was collected and ethanol was added until reaching 50% (v/v); after centrifuging, the white pellet was washed three times with acetone and dried at 37 °C.

ELISA. These assays were performed as described previously by Voller & Bidwell (1986) with the following modifications. Nunc-Immunoplate I (A/S Nunc) plates were coated with appropriate amounts of different materials and incubated overnight at 4 °C. In the case of mannan, previous experiments have shown that similar results were obtained either when directly coating with the polymer or after initial coating with the antibody (Marcilla *et al.*, 1993). The wells were rinsed three times with 0.05% Tween 20 in Tris-buffered saline (TBS) (TBST) then filled with 3% (w/v) BSA in TBS and incubated for 2 h at 37 °C. Each plate was washed three times with TBST. The appropriate diluted antibody (1:1000 for mAb JRR1 and 1:8000 for mAb 1B12) in TBST was added to the wells and the plate incubated at 37 °C for 2 h. After washing three times with TBST, an alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin (Bio-Rad) was diluted 1:500 in TBST and 50 μ l was added to the wells. After 2 h at 37 °C they were rinsed again and the substrate mixture (50 μ l *p*-nitro-phenylphosphate, 1 mg ml⁻¹, in diethanolamine buffer) was added to each well and developed for 45 min. The colour intensity was read at 405 nm with a plate reader (Titertek Multiscan Plus MKII; Labsystems).

The antigen competition ELISA was carried out on plates coated with 10 μ g (valued as sugar), per well, of material solubilized by Zymolyase from *C. albicans* yeast cell walls. The mAb JRR1 diluted 1:10000 was incubated with laminarin (Sigma) (a 1,3- β -glucose polymer) or pustulan (a 1,6- β -glucose polymer) solutions of different concentrations for 2 h at 37 °C before being added to the wells of the ELISA plates.

RESULTS

Characterization of the mAb JRR1

JRR1 was raised by injecting mice with Zymolyase extracts from *C. albicans* cell walls, but its reactivity with similar extracts from different fungi, together with its property of being immunoabsorbed by glucan (data not shown), led us to think that the epitope recognized could be either (1,3)- β - or (1,6)- β -glucan. To determine which of the two epitopes was recognized by the mAb, a competition ELISA assay between pustulan or laminarin and an antigen recognized by the mAb was devised. The mAb diluted 1:10000 was pre-incubated with increasing amounts of either laminarin or pustulan for 2 h at 37 °C, to promote formation of the antigen-antibody complexes. The free mAb present in the solutions after the pre-incubation was titrated by ELISA, coating the wells of the microtitre plates with Zymolyase extracts. When the pre-incubation had taken place at low concentration of the polysaccharides, no decrease of mAb titre was detected, but when pustulan was present at a concentration of 0.11 mg ml⁻¹ and higher, significant drops in mAb titre

Fig. 1. Laminarin (Δ) and pustulan ($\square$) competition assay. mAb JRR1 was preincubated with increasing concentrations of laminarin or pustulan solutions and used afterwards to detect the JRR1 epitopes present in the Zymolyase extract from *C. albicans* yeast cell walls. Wells were coated with 10 μ g (valued as sugar) Zymolyase-extracted material. The values shown correspond to the residual soluble antibody left behind and are the mean of at least three separate experiments, the standard deviation values of which were never higher than 10% of the mean.

were detected (Fig. 1). Laminarin did not affect the titre of the mAb, regardless of how high the concentrations used in the pre-incubation were.

Fractionation of mannan in a ConA-Sepharose column

To study the relationships of (1,6)- β -glucan and mannan, mannan from *C. albicans* cell walls was passed through a ConA-Sepharose column and two fractions, corresponding to non-retained material (17% of the original carbohydrate) and to material retained and eluted by addition of methyl α -D-mannopyranoside (85% of the original carbohydrate), were obtained. The carbohydrate content of the fractions was determined and equivalent amounts were analysed by ELISA using the mAb JRR1. The non-retained material, which contained only 17% of the original carbohydrate, nevertheless exhibited three times more immunoreactivity against the mAb JRR1 than the retained material ($A_{405} = 1.25$ vs $A_{405} = 0.40$). Analysis by paper chromatography of the total acid hydrolysate of the retained material showed it to consist mainly of mannose and of a small amount of glucose, whilst partial hydrolysis also revealed the presence of gentiobiose (6-O- β -D-glucopyranosyl-D-glucose), a (1,6)- β -disaccharide. These results show the existence of (1,6)- β -glucan that is covalently bound to mannan and is consequently retained by the ConA-Sepharose. Total and partial acid hydrolysis of the non-retained material showed that it consisted mainly of glucose and gentiobiose, respectively.

Fractionation of Zymolyase-extracted material in a ConA-Sepharose column

In order to test the possible existence of bonds between the glucan epitope recognized by the mAb JRR1 and the protein moiety of cell wall mannoproteins, mannoproteins released by Zymolyase from *C. albicans* yeast cell walls

were passed through a ConA-Sepharose column. In this case the JRR1-epitope-containing material, as detected by ELISA, was mostly retained by the column ($A_{405} = 1.36$ vs $A_{405} = 0.46$), indicating that the epitope was bound to cell wall mannoproteins. The material retained and eluted with methyl α -D-mannopyranoside was dialysed, lyophilized, dissolved in 2 M NaOH and heated at 100 °C for 2 h to degrade the protein moiety of mannoproteins. The aim of this treatment was to release the oligosaccharides and possible glucan chains that were directly bound to amino acids in the mannoproteins. After neutralizing and diluting with ConA buffer, the sample was passed again through the ConA-Sepharose column, and the presence of the JRR1 epitope in the retained and non-retained fractions was determined by ELISA. The result showed that after degradation of the protein moiety, an important percentage of the JRR1 epitope was no longer retained by the column ($A_{405} = 1.22$ vs $A_{405} = 0.61$), pointing to the possibility that it was bound either directly to amino acids in the mannoproteins or through O-linked oligomannosides (see later). The remaining JRR1 epitope that was still retained by the ConA column might represent an epitope that is bound to the mannan moiety of mannoproteins, as has been shown above, and whose retention by ConA is not affected by the degradation of the protein moiety of mannoproteins.

mAb JRR1 does not react with the mannoproteins secreted by regenerating spheroplasts

In an attempt to determine whether (1,6)- β -glucan is added to mannoproteins during their synthesis or after their secretion to the periplasmic space and incorporation to the extending wall, microtitre plates were coated with mannan preparations obtained either from the mannoproteins secreted by spheroplasts, or from isolated walls of yeast and mycelial cells of *C. albicans*. The results showed that the mAb JRR1 reacted with the mannan preparations obtained from the cell walls of either yeast ($A_{405} = 1.37$) or mycelial ($A_{405} = 0.73$) cells, indicating that they carried the corresponding epitope. On the other hand, mAb JRR1 did not react with the mannan preparations obtained from the mannoproteins secreted by regenerating spheroplasts after 6 h ($A_{405} = 0.11$).

The JRR1 antigen is located in an inner layer of the cell wall

The location of the antigen recognized by mAb JRR1 on the cell wall of *C. albicans* was determined by indirect immunofluorescence of cells growing either normally or in the presence of tunicamycin, an inhibitor of N-glycosylation of proteins.

Control cells were not labelled by the antibody (data not shown). Conversely, cells that had been incubated with tunicamycin were labelled, but the labelling was found only on the areas of the cell wall that had presumably been synthesized in the presence of the antibiotic (i.e. buds in the yeast form and apical tips in the mycelial form) (Fig. 2) and, as a consequence, their proteins were devoid of the mannan moieties.

DISCUSSION

The nature of the epitope recognized by the mAb JRR1 was deduced from the fact that pustulan, a (1,6)- β -glucan polymer, competitively inhibits the binding of the mAb to material containing a JRR1 epitope. The isolation of a mAb against (1,6)- β -glucan, a material that is not immunogenic by itself, can be explained by the use of Zymolyase extracts, in which short (1,6)- β -glucan chains may still remain attached to mannoproteins, as antigens.

An obvious application of the mAb JRR1 is the study of the relationships between mannoproteins and glucan. A direct linkage between (1,6)- β -glucan and chitin has been reported by Surarit *et al.* (1988) and between glucan and mannan by Van Rinsum *et al.* (1991), both in *S. cerevisiae*. Direct linkages between glucan and the protein moiety have been described in halobacteria (Wieland *et al.*, 1983), in *Prototheca zopfii* (Rivas & Pont-Lezica, 1987), and also recently in *S. cerevisiae* (Montijn *et al.*, 1994).

In the present study, existence of linkages between the glucan-epitope recognized by the mAb JRR1 and the mannan moiety of mannoproteins was studied in ConA-Sepharose-retained mannan from *C. albicans* yeast cell walls. ConA specifically binds materials that contain α -mannose or α -glucose residues, which can then be released by competition with methyl α -D-mannopyranoside (Goldstein *et al.*, 1965). The JRR1 epitope is present in the retained fraction, and this fact, together with the data on the composition of this material, as determined by acid hydrolysis and paper chromatography, showing that it includes both mannose and glucose, support the existence of direct links between the mannan moiety of mannoproteins and glucan in the cell walls of *C. albicans*.

The existence of direct linkages between the (1,6)- β -glucan epitope recognized by the mAb JRR1 and the protein moiety of mannoproteins was investigated using the Zymolyase extract from *C. albicans* yeast cell walls. The cell wall mannoproteins in the extract were purified by a first round of ConA-Sepharose chromatography and, after degradation of the protein moiety, the resulting material was submitted to a second round of ConA-Sepharose chromatography, and both retained and non-retained fractions were obtained. After the first chromatography most of the JRR1 epitope was in the retained fraction, that is, bound to the intact mannoproteins. Following the degradation of the protein moiety the behaviour of the epitope changed and became, in a high percentage, non-retainable by the ConA, indicating the possibility of a direct linkage to the protein moiety of cell wall mannoproteins.

Alternatively, two other explanations can be offered for this result. The first one would be the existence of a linkage between (1,6)- β -glucan and the terminal mannose of the O-linked oligomannosides of mannoproteins. This glucan-oligomannoside would be released after the degradation of the protein moiety of mannoproteins, and it would not be retained by the ConA because of the interaction between ConA and the oligomannoside being hampered by the presence of the (1,6)- β -glucan. This

Fig. 2. Analysis by immunofluorescence of the distribution of the JRR1 epitope on the surface of *C. albicans* yeast (a, b) and mycelial (c, d) cells grown in the presence of tunicamycin. (a, c) Phase contrast micrographs; (b, d) fluorescence micrographs. Arrows point to fluorescent surfaces.

interpretation of our results would be in agreement with the fact that a mutant of *S. cerevisiae* (*kre2*), resistant to the killer toxin k1, is defective in the elongation of *O*-linked oligomannobiose (Hill *et al.*, 1992; Häusler *et al.*, 1992). As (1,6)- β -glucan is the receptor for the killer toxin, the defect in *O*-linked oligomannoside completion would prevent the attachment of (1,6)- β -glucan to the manno-protein and hence prevent the formation of the k1 receptor. The second explanation would be the existence, as has been recently suggested by de Nobel & Lipke (1994), of a direct linkage between the C-terminal glycan (derived from a glycosyl-phosphatidyl-inositol anchor) from a manno-protein and (1,6)- β -glucan. In this case, the degradation of the protein moiety would equally lead to the non-retention of the epitope by the ConA-Sepharose column.

Meaden *et al.* (1990) and Roemer *et al.* (1993), using a genetic approach in *S. cerevisiae*, have shown that synthesis of (1,6)- β -glucan begins early in the secretory pathway; accordingly it would be reasonable to assume that cell wall manno-proteins, as secreted by regenerating spheroplasts, would contain (1,6)- β -glucan residues. Neverthe-

less, our results show otherwise, since the mAb reacted neither with manno-proteins secreted by regenerating spheroplasts, nor with mannan obtained from secreted manno-proteins. This indicates that either (1,6)- β -glucan is incorporated at a later stage, or that manno-proteins that contain this epitope are only secreted if there is a preformed cell wall structure to which they are directly incorporated.

Finally, the topology of the distribution of (1,6)- β -glucan was studied by indirect immunofluorescence, and the results confirm previous reports indicating that it is located within an inner layer of the cell wall (Kopecká *et al.*, 1974) which is not accessible unless the outer layer, being formed by manno-proteins (Horisberger & Vonlanthen, 1977; Koch & Rademacher, 1980), is disturbed by the effect of tunicamycin.

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