Evidence for different mannosylation processes involved in the association of β-1,2-linked oligomannosidic epitopes in *Candida albicans* mannan and phospholipomannan

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A monoclonal antibody specific for β-1,2-linked oligomannosides was used to study the association of these residues with *Candida albicans* mannan and phospholipomannan (PLM) in relation to growth conditions and in mannan mutant strains. Double immunofluorescence assays performed on cells grown under standard conditions indicated a highly heterogeneous cell surface expression of these epitopes in comparison with the homogeneous expression of α-linked oligomannosidic epitopes. Growth in the presence of tunicamycin, which inhibits mannan N-glycosylation, resulted in an absence of β-1,2-oligomannosidic epitopes on the cell surface, although PLM synthesis still occurred as shown by autoradiography. Similarly, growth in acidic conditions, which inhibits the incorporation of β-1,2-oligomannosides in mannan, resulted in an absence of β-1,2-oligomannosidic epitopes at the cell surface, although they still associated with PLM as shown by Western blotting. Western blots of *C. albicans* mutant strains with reduced amounts or an absence of phosphorus and acid-labile β-1,2-oligomannosides in their mannan confirmed that the association of β-1,2-linked oligomannosides with mannan and with PLM involves different mannosylation processes.

**Keywords:** β-1,2-oligomannosidic epitopes, mannan, phospholipomannan, β-mannosylation processes

**INTRODUCTION**

Homopolymers of β-1,2-linked oligomannosides were initially described in *Candida albicans* cell wall phosphopeptidomannan (PPM), also called mannan, where they correspond to the antigenic factor 5 (Shibata *et al.*, 1992). Such β-1,2-linked oligomannosidic epitopes were further observed on a *C. albicans* 14–18 kDa phospholipomannan (PLM) (Trinel *et al.*, 1992), a glycolipid that induced TNFα production by macrophages (Jouault *et al.*, 1994). Mapping of these β-1,2-oligomannosidic epitopes among glycoconjugates of *Candida* species revealed that the presence of PLM was restricted to the most pathogenic species of the genus *Candida*, *C. albicans* and *C. tropicalis* (Cantelli *et al.*, 1995) and also demonstrated that in other *Candida* species, such as *C. parapsilosis*, *C. krusei* and *C. glabrata*, β-1,2-oligomannosidic epitopes were shared by numerous mannoproteins according to species-specific profiles.

From a physiopathological point of view, recent observations increasingly suggest that β-1,2-oligomannosides are important molecules involved in the host–*Candida* interplay during pathogenic processes. β-1,2-Oligomannosides have been shown to bind to host cell membranes (Li & Cutler, 1993), to trigger the cytokine network (Jouault *et al.*, 1995) and elicit an antibody response different from those supported by α-Man residues (Poulain *et al.*, 1993) during candidosis. In contrast to these studies, very little is understood about the expression of these residues by the yeast. Previous studies with monoclonal antibodies (mAbs) (Brawner *et al.*, 1990; Fruit *et al.*, 1990; Molinari *et al.*, 1993; Li & Cutler, 1991), the specificity of which for β-1,2-oligomannosides was later established (Trinel *et al.*, 1992; Li & Cutler, 1993), have revealed a highly complex expression of the corresponding epitopes. Since it is now known that these
epitopes may be expressed in C. albicans in at least two unrelated families of carrier molecules (mannan and PLM), the study of their relative contribution to the cell wall appeared necessary to define their precise role in pathogenic processes. With this aim in mind, we first studied the synthesis of PLM-associated β-1,2-oligomannosides. A second condition consisting of growth in the absence of tunicamycin, 0.5 mCi [2-3H]Man was added after the 30 min incubation period and cells were incubated at 37 °C for 4 h. Cells were then washed and broken using a French press as described below.

**Monoclonal antibodies.** The antibody used throughout these experiments was selected according to its ability to react with acid-labile oligomannosides released from C. albicans mannan (Trinel et al., 1992). It consisted of a mouse IgM monoclonal antibody, AF1 (Cassone et al., 1988), provided by Professor A. Cassone, Istituto Superiore di Sanita, Roma, Italy.

A rat monoclonal antibody, CA1 (SanoDi Diagnostics), which has been shown to react with C. albicans α-linked mannoside residues (Trinel et al., 1992), was used as a control in IFAs.

**Preparation of cell extracts.** The extraction of both cytoplasmic and cell wall components was performed using either alkaline extraction under reducing conditions (AERC) (Hernando et al., 1993) or cell rupture with a French press (Cantelli et al., 1995). The first method, whose application led to the original description of the C. albicans PLM, was intended exclusively for Western blotting analysis. Cells were treated with 185 M NaOH/5% (v/v) 2-mercaptoethanol for 15 min on ice. Extracted proteins were then precipitated by the addition of an equal volume of 50% (w/v) trichloroacetic acid. After 15 min on ice, samples were centrifuged and washed three times in 1 M Tris, pH 11.5. Cells and precipitated proteins were centrifuged at 2500 g and treated for 5 min at 100 °C with sample buffer [62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) saccharose]. Insoluble material was then removed by centrifugation at 10000 g for 5 min.

The second method, which allowed further extraction of native antigens, was used for autoradiography after radiolabelling. Cells washed in 50 mM phosphate buffer/150 mM NaCl, pH 7.5, were broken by three consecutive runs in a French pressure cell (Aminco) at 20000 p.s.i. (138 MPa). After centrifugation at 2500 g for 15 min, the supernatant was dialysed, dried and stored at −20 °C.

**Preparation of PLM.** As previously described (Trinel et al., 1993), French press extracts were first subjected to an extraction with chloroform/methanol (2:1, v/v) for 1 h at room temperature to remove lipids. The residual pellet was then stirred twice for 1 h at room temperature in chloroform/methanol/water (10:10:3, v/v/v) to extract polar glycolipids. The chloroform/methanol/water extracts were then pooled, filtered on a GF/F membrane (Whatman), dried in a Speed Vac concentrator (Savant Instruments) and stored at −20 °C until used.

**Analysis of extracts.** Polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) for 18 h at a constant current of 4 mA on 5–15% (w/v) gradient polyacrylamide gel slabs. For Western blotting, gels loaded with AERC extracts (100 μg protein) were electrophoretically transferred onto 0.45 μm pore size nitrocellulose membranes (Schleicher & Schuell) with a semi-dry blottter apparatus (Biometra). For immunodetection, strips of nitrocellulose were cut and saturated with non-fat milk (5%, w/v) in TBS buffer (50 mM Tris/150 mM NaCl, pH 7.5) for 1 h at 37 °C. Different mAb dilutions (1/200 and 1/1000) or Con A-peroxidase (1 μg ml−1) in TBS buffer/0.05% Tween 20 (TBST) were added and incubated for 1 h at 37 °C. Alkaline phosphatase conjugates against immunoglobulins were used at a 1/2500 dilution in TBST for 1 h at 37 °C, and staining of the relevant bands was obtained with a mixture of nitro blue tetrazolium (0-165 mg ml−1) and bromochloroindolyl phosphate.
Differential β-mannosylation processes in C. albicans

(0.0825 mg ml⁻¹) in Tris/HCl buffer (100 mM Tris/5 mM MgCl₂, pH 9.5) for phosphatase alkaline conjugates and 0.05 % 3, 3'-diamino-benzidine (Sigma-Aldrich Chimie)/0.03% H₂O₂ in TBS for ConA-peroxidase.

After PAGE, gels containing radiolabelled material (French press extracts) were fixed and stained with Coomassie blue, then dried, soaked in En'Hance (Beckman) and exposed to X-omat film (Eastman Kodak) at -80 °C for fluorography.

**IFA.** Cells grown in the different conditions described above were washed in PBS (50 mM PO₄Na¹/150 mM NaCl, pH 7.5) and adjusted to a concentration of 10⁶ ml⁻¹. Drops of these suspensions were added to each well of a microslide (Biomerieux) and air-dried. They were then incubated for 1 h at 37 °C with mAb AF1 diluted 1/100 in PBS/0.001 % Tween 20 (PBST). Reactivity of the mAb with the cells was visualized with a fluorescein-conjugated goat anti-mouse immunoglobulin M (Jackson Immunoresearch Laboratories) diluted 1/75 in the same buffer.

To compare the distribution of α-mannosidic and β-mannosidic epitopes, a double indirect immunofluorescence assay was also performed. Primary incubation with mAb AF1 was followed by a second incubation with mAb CA1 diluted 1/100 in PBST. Fixation of mAbs was visualized with a fluorescein-conjugated goat anti-mouse immunoglobulin (as above) and with a Texas-red-conjugated goat anti-rat IgM (Jackson Immunoresearch Laboratories) diluted 1/50 in PBST, respectively. Readings were performed at 1000 x magnification using a Zeiss Axiophot microscope equipped with a filter combination for fluorescein (excitation 490 nm; emission 525 nm) and for Texas red (excitation 590 nm; emission 615 nm).

**RESULTS**

**Comparative analysis of cell surface distribution of α- and β-linked oligomannoside epitopes by IFA**

Fig. 1 represents an example of double immunofluorescence assay on C. albicans VW32 growth forms. It shows a mother cell which has produced variously shaped daughter cells in RPMI medium. Staining was performed with mAbs reacting with α- (Fig. 1a) or with β- (Fig. 1b) linked mannoside residues. α-Mannoside epitopes covered all cell surfaces and the fluorescence intensity seemed to be related to the wall thickness and the age of the cellular structures (a). In contrast, β-1,2-oligomannoside epitope distribution appeared discontinuous and preferentially localized on the newly generated wall elements (buds, apexes of germ tubes). However, some older structures in the vicinity of the septa were also labelled (b). No significant qualitative or quantitative differences in staining distribution were noted by changing the order of the added monoclonal antibodies or conjugates (data not shown).

**Influence of tunicamycin on β-1,2-oligomannosidic epitope surface expression and their association with PLM**

Growth of C. albicans in the presence of tunicamycin resulted in the formation of enlarged yeast cells which produced buds (Fig. 2a) although cytokinesis leading to the separation of daughter cells was not observed. When the surface antigens of these grape-shaped features were examined by IFA using mAb AF1 specific for β-1,2-oligomannosidic epitopes, the fluorescence was restricted to the cell wall of mother cells from the inoculum whereas the buds were unlabelled (Fig. 2a, arrows). Because of the low biomass increase observed under these growth conditions, it was suspected that components from blastoconidia of the inoculum (which were grown in the absence of tunicamycin) could interfere with Western blot analysis of PLM synthesis. Therefore, [³H]mannose labelling of cells was performed in the presence of tunicamycin (Fig. 2b). In comparison with control cells which revealed the usual smear of polydispersed high-molecular-mass mannoproteins (Fig. 2b, lane 1), cells grown in a medium containing 10 µg tunicamycin ml⁻¹ showed a highly modified autoradiographic profile where the smears were replaced with more resolved bands, some of which displayed lower molecular mass (Fig. 2b, lane 2). In contrast, labelling of the polydispersed 14–18 kDa components remained unaffected and, in fact, seemed to increase during the first hour of labelling in tunicamycin-containing media when lanes loaded with similar amounts of radioactive material were compared (Fig. 2b, lanes 1-3).
Expression of β-1,2-oligomannoside epitopes among glycoconjugates of different strains deficient in mannan phosphate-bound oligomannosides

Fig. 4 shows Western blots of AERC extracts from C. albicans strains KD 101 (lane 1), A4 (lane 2) and IFO 1397 (lane 3), the mannan of which has been reported to have defects in phosphorylation and/or β-1,2-oligomannoside expression. Staining of these blots with anti-β-1,2-oligomannoside antibody revealed PLM expression similar to that observed in the reference strain (Fig. 3c, lane 2).

DISCUSSION

β-1,2-Mannosidic linkages have been described to date only in the bacterial species Salmonella thompson (Lindberg et al., 1988) and Serratia marcescens (Oxley & Wilkinson, 1991) and in yeast species (Kobayashi et al., 1992b, 1994a; Shibata et al., 1985, 1993). In C. albicans, structural analysis has shown their presence either in homopolymers of the acid-labile part of mannan in both serotypes A and B (Shibata et al., 1989; Kobayashi et al., 1990) and in short sequences at the reducing end of α-1,2-linked oligomannoside side-chains in the acid-stable part of serotype A mannan (Kobayashi et al., 1992a). These two families of residues correspond to epitopes of serum factors 5 and 6, respectively (Shibata et al., 1992). By using monoclonal and polyclonal antibodies specific for homopolymers of β-1,2-oligomannosides, including factor 5 antiserum, we demonstrated that these epitopes were also present on a C. albicans 14–18 kDa glycolipid which was designated phospholipomannan (Trinel et al., 1993). Several experiments have recently indicated the biological properties of β-1,2-oligomannosides which appeared, like PLM, to be involved in cytokine induction (Jouault et al., 1994, 1995), adhesion of C. albicans cells to macrophages (Li & Cutler, 1993) and to induce an antibody response different from α-Mann residues during candidosis (Poulain et al., 1993). As these data from several groups suggest a potent pathophysiological role for β-1,2-oligomannosides, this preliminary study was undertaken to further define the respective contribution of their two main carrier molecules i.e. PLM and PPM, to their expression by C. albicans.

As shown by double IFA, the surface expression of β-1,2-oligomannosidic epitopes appeared heterogeneous when compared with α-mannosidic epitopes which covered the entire cell surface. This observation is consistent with other, previously unrelated immunocytological analyses (Brawner et al., 1990; Fruit et al., 1990; Molinari et al., 1993; Li & Cutler, 1991) performed with antibodies whose common specificity for β-1,2-oligomannosides was later established (Trinel et al., 1992; Li & Cutler, 1993). In each case, a large surface antigenic variability within a cell population was observed, although attempts have failed to correlate a given antigenic expression with a given stage of the cell cycle. The double IFA performed in this study, involving two monoclonal antibodies specific for epitopes present in the cell wall mannan, provide for the first time cytological evidence for intramolecular heterogeneity of mannan at the cell surface. Although the
uniform distribution of α-mannosidic epitopes is compatible with the general scheme of mannan structure presented by Shibata et al., 1992, the discontinuous distribution of β-1,2-oligomannosides reported here shows that this scheme (which infers that the mannan structure is the same at any point of the cell surface) must be viewed with caution. It must be made clear, for a real understanding of Candida cell surface antigen expression and its pathophysiological relevance, that this chemical scheme represents only the general average structure of a group of molecular species which are to be found in a given cell population in given growth conditions. In the same way, refinement and improvement of chemical analysis methods has begun to reveal, as demonstrated several years ago by immunochemical analysis (Brawner et al., 1990), that strong differences in polysaccharide epitopes may exist between strains of the same species or in a given strain according to growth conditions (Kobayashi et al., 1994b). With regard to β-1,2-oligomannosides, which represent a relatively homogeneous family of epitopes, the complexity of their expression at the cell surface may be due to their association with at least two unrelated carrier molecules, PPM and PLM. As a first step in the exploration of the mechanics of this expression, the objective of this preliminary study was to establish whether both biosynthetic processes were related by using growth conditions known to inhibit directly or indirectly β-mannosylation of mannan (Elorza et al., 1988; Kobayashi et al., 1994b).

We first tested the influence of tunicamycin, which specifically inhibits N-glycosylation of proteins without affecting directly their O-glycosylation (Elorza et al., 1988). This drug blocks the first step of the synthesis of the dolichol-linked oligosaccharide precursor involved in all N-glycosylations (Lehle & Tanner, 1976) which is transferred, after its synthesis, to proteins through asparaginyl residues and processed to complex or hybrid structures. Under these conditions, autoradiography of mannose-labelled extracts displayed an altered pattern of mannoproteins which corresponded to a decrease in their polydispersed character and in their relative molecular mass. By contrast, the same labelling also demonstrated an unaffected PLM synthesis, as determined by the presence of the 14–18 kDa components. The extraction by chloroform/methanol/water confirmed the PLM nature of these components and that it did not correspond to the interference of a mannoprotein displaying a lower molecular mass in the presence of tunicamycin. The more specific effects of growth at pH 2, which suppresses the transfer of mannosyl phosphate groups and β-1,2-mannose units in mannan as reported by Kobayashi et al. (1994b), was then studied. Although these growth conditions effectively led to a loss of surface expression of β-
1,2-oligomannosides epitopes, as observed by IFA, their incorporation in PLM, observed on Western blots, was still effective after 48 h of growth, during which at least a tenfold increase in cell number occurred.

The results obtained by inhibiting N-glycosylation by tunicamycin led to the conclusion that PLM synthesis does not involve the dolicholpyrophosphate-N-acetylglucosamine pathway. However, results from the experiments run at pH 2 demonstrated two independent modes of β-1,2-mannosylation for the mannan and the PLM. As both the transfer of mannosyl phosphate groups and β-1,2-linked mannopyranose units during the biosynthesis of cell wall mannan have been reported to be suppressed under the low pH environment of parent cells (Kobayashi et al., 1994b), two different hypotheses could be proposed to explain our results: (i) the existence of at least one β-1,2-mannosyltransferase specific for PLM and less sensitive to pH change than β-1,2-mannosyltransferases specific for mannan, and (ii) different susceptibility of related signal transduction pathways or cellular compartments to pH changes leading to different degrees of inactivation of β-1,2-mannosyltransferases devoted to both mannan and PLM mannosylation.

In addition to the influence of growth conditions on the phenotypic association of β-1,2-oligomannosides in mannan, some strains which are defective in the synthesis of these residues have been described in the literature. The strain IFO 1397 (formerly C. stellatoidea), used to prepare factor 6 antiserum by adsorption (Suzuki & Fukazawa, 1982; Fukazawa, 1989), has a mannan devoid of phosphorus and β-1,2-linked α-mannopyranose units. The analysis of this mannan in a representative C. stellatoidea strain first led to the conclusion that the defect was a subspecies-specific feature (Tojo et al., 1990) until further studies demonstrated that other C. stellatoidea strains displayed mannan acid-labile β-1,2-oligomannosides (Kobayashi et al., 1991a), whereas the major differences between C. stellatoidea type I and II lies in the presence of the C. albicans serotype A antigen in type II (Kobayashi et al., 1992c). Other mutant strains, for example C. albicans KD 101 and A4, have been obtained through mutagenesis and displayed reduced amounts of phosphorus and β-1,2-oligomannosides (Chaffin et al., 1993; Shimokawa & Nakayama, 1984). When analysed in Western blots, all of these strains exhibited normal PLM expression levels. Although they do not favour either of the previously suggested hypotheses, these results, as well as those obtained by varying growth conditions, suggest two different biosynthetic pathways for β-1,2-oligomannosides depending on their association with either mannan or PLM. Importantly, a preliminary characterization of C. albicans β-1,2-mannosyltransferases has been reported (Suzuki et al., 1995). These authors demonstrated a high and unexpected substrate specificity for mannan oligomannoside chain elongation. Considering the strongly different nature of mannan and PLM, these results are compatible with the conclusions of the present study. Taken together, these data show that C. albicans modulates in different ways the expression of β-1,2-oligomannosides involved in the host–parasite interface (Jouault et al., 1995; Li & Cutler, 1993).

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