Mapping of β -1,2-linked oligomannosidic epitopes among glycoconjugates of *Candida* species

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The distribution of β -1,2-linked oligomannosides among glycoconjugates of various *Candida* species was investigated by Western blotting, using monoclonal and polyclonal antibodies which react with these epitopes. Expression of β -1,2-linked oligomannosidic epitopes on a 14–18 kDa polydisperse antigen nonreactive with concanavalin A (ConA), previously identified as a *C. albicans* serotype A phospholipomannan (PLM), appeared to be restricted to *C. albicans* serotypes A and B (including var. *C. stellatoidea* types I and II) and *C. tropicalis*. In *C. albicans*, β -1,2-linked oligomannosidic epitopes also appeared to be slightly associated with high molecular mass (> 100 kDa) polydisperse ConA-reactive mannoproteins. For all the other *Candida* strains investigated, belonging to the species *C. parapsilosis*, *C. krusei*, *C. glabrata* and *C. robusta* (*S. cerevisiae*), β -1,2-linked oligomannosidic epitopes were found to be present in association with medium molecular mass (18–100 kDa) and high molecular mass ConA-reactive mannoproteins, giving reproducible labelling profiles that varied between species.

Keywords: *Candida*, glycoconjugates, mannoproteins, phospholipomannan, β -1,2-linked oligomannosides

INTRODUCTION

Since the relatively recent discovery of β -1,2-linked oligomannosides (Shibata *et al.*, 1985), their presence in mannans from *Candida* species has been extensively studied both chemically (Kobayashi *et al.*, 1991, 1992a, b, 1994) and immunologically: they have been proved to correspond to the antigenic factor 5 (Shibata *et al.*, 1992) and to support the specificity of numerous anti-*Candida* polyclonal or monoclonal antibodies (Li & Cutler, 1993; Trinel *et al.*, 1992, 1993). More recently, β -1,2-linked oligomannosides have also been shown to be involved in *C. albicans* adhesion (Li & Cutler, 1993) and cytokine induction (Jouault *et al.*, 1995). In *C. albicans*, β -1,2-linked oligomannosidic epitopes have been found in mannan and in a 14–18 kDa antigen, a TNF α -inducing phospholipomannan (PLM) (Jouault *et al.*, 1994; Trinel *et al.*, 1993). Since the presence of PLM has, to date, been demonstrated in only a single *C. albicans* strain, we

investigated the distribution of β -1,2-linked oligomannosides epitopes in various strains of the genus *Candida*. For this purpose, we used Western blots of yeast cell extracts probed with monoclonal and polyclonal antibodies known to react with both *C. albicans* PLM and β -1,2-linked oligomannosides released from *C. albicans* mannan by mild acid hydrolysis and converted into neoglycolipids (Trinel *et al.*, 1992, 1993).

METHODS

Organisms and culture conditions. *C. albicans* VW 32 cloned strain (serotype A), was originally isolated from a case of human renal candidosis, and is used as reference strain in our laboratory (Poulain *et al.*, 1985). *C. albicans* strains 348 A and 330 B were respectively serotype A and B clinical isolates from AIDS patients with oesophageal candidosis. *C. stellatoidea* strains B:4404 and B:4405 (respectively type I and II) were kindly provided by Dr J. Kwong-Chung, Bethesda, MD, USA (Kwong-Chung *et al.*, 1988). *C. tropicalis* U42Ct1, *C. krusei* U42Ck1, *C. parapsilosis* U42Cp1 and *C. glabrata* U42Cg1 strains corresponded to clinical isolates identified on the basis of auxanography with the API 20 C test system. *Saccharomyces cerevisiae* CBS 1315 was a reference strain purchased from the

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Abbreviations: AERC, alkali extraction in reducing conditions; ConA, concanavalin A; PLM, phospholipomannan; TNF, tumour necrosis factor.

Centraalbureau voor Schimmelcultures, Baarn, the Netherlands. These strains were maintained in our collection by subcultures on Sabouraud Dextrose Agar (SDA) stored at 4 °C.

Germ tubes were obtained following incubation for 4 h at 37 °C with constant shaking in synthetic medium RPMI 1640 (Boehringer, Mannheim) from a standard inoculum of 5×10^6 yeast cells ml^{-1} . Yeast cells in stationary phase were obtained on SDA after 24 h at 37 °C. Some experiments involved *C. albicans* VW 32 cells grown at 28 °C in a bioreactor as previously described (Faille *et al.*, 1990).

Monoclonal and polyclonal antibodies. The antibodies used throughout these experiments were selected according to their ability to react with *C. albicans* mannan-released acid-labile oligomannosides (Trinel *et al.*, 1992, 1993). They consisted of two mouse IgM monoclonal antibodies, mAb AF1 and mAb DF9-3, respectively furnished by Professor A. Cassone (Cassone *et al.*, 1988) and Dr M. Borg-von-Zepelin (Borg-von-Zepelin & Grunes, 1993), and serum factor 5, a rabbit polyclonal IgG, purchased from Iatron Laboratory (Tokyo, Japan).

Preparation of whole-cell extracts. Extractions of cytoplasmic and cell wall components were performed either by alkali extraction in reducing conditions (AERC) as previously described (Hernando *et al.*, 1993) or with a French press. For the latter procedure, cells were washed three times in PBS for 10 min, centrifuged and resuspended in PBS. Cells were then broken at a pressure of 20000 p.s.i. (138 MPa) in a French pressure cell (Aminco). After centrifugation at 4000 r.p.m., supernatants were dialysed against water and dried in a Speed Vac concentrator (Savant Instruments).

PAGE. This 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. Each lane was loaded with 150 µg protein.

Western blotting. Upon completion of electrophoresis, proteins were electrophoretically transferred to 0.45 µm pore size nitrocellulose membrane (Schleicher & Schuell) with a semi-dry blotter apparatus (Biometra). For immunodetection, blots were saturated with non-fat milk (5%, w/v) in TNT buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h at 37 °C. Different mAbs diluted (1/100 and 1/500) in TNT were incubated for 1 h at 37 °C.

Alkaline phosphatase-goat anti-mouse IgM or goat anti-rabbit immunoglobulins conjugates (Zymed Laboratories) were used diluted 1/2500 in TNT. Staining was done with a mixture of nitro blue tetrazolium (0.165 mg ml^{-1}) and bromochloroindolyl phosphate (0.0825 mg ml^{-1}) in 100 mM Tris/HCl, 100 mM NaCl, 5 mM MgCl_2 , pH 9.5.

RESULTS

Influence of extraction method and antibodies on the detection of β -1,2-oligomannosides in Western blots of *C. albicans* VW 32 germ tubes

Fig. 1 shows representative results of Western blots of AERC (panel a) or French press (panel b) extracts of *C. albicans* VW 32 germ tubes stained with ConA (lane 1) or antibodies known to react with β -1,2-linked oligomannosides (lanes 2, 3 and 4). Application of both methods allowed the detection of a 14–18 kDa antigen on the basis of its unreactivity with ConA and reactivity with antibodies. Regardless of the antibody concentration and extraction method used, the ability of the antibodies to reveal the polydisperse character of the 14–18 kDa antigen

Fig. 1. Western blots of an AERC extract (a) and French press extract (b) of *C. albicans* VW 32 germ tubes. Nitrocellulose strips were stained with ConA (lane 1), mAb AF1 (lane 2), mAb DF9-3 (lane 3) or serum factor 5 (lane 4).

decreased in the order mAb AF1 > mAb DF9-3 > serum factor 5. Only a faint reactivity was generally observed for these antibodies with polydisperse high molecular mass (> 80 kDa) ConA-reactive mannoproteins. Since no qualitative differences in the immunodetection of glycoconjugates were observed in relation to the extraction method, later experiments were done using the AERC method as an easy single-step procedure allowing direct electrophoretic screening of strains and growth conditions.

Influence of growth conditions on the detection of β -1,2-linked oligomannosides in Western blots of *C. albicans* VW 32

When yeast cells of *C. albicans* VW 32 grown on SDA were tested (Fig. 2), the ConA (lane 1) and antibody reactivity patterns with serum factor 5 (lane 2), mAb DF9-3 (lane 3) and mAb AF1 (lane 4) were similar to those obtained with germ tube extracts (Fig. 1). Samples from cells grown for 48 h at 28 °C in bioreactors (presenting as a mixture of a majority of yeast forms with pseudomycelium) gave the same reactivity pattern (lane 5), demonstrating the expression of PLM in all these growth conditions. As the culture conditions tested seemed to have no influence on the detection of β -1,2-linked oligomannosides in Western blots, cells grown on SDA were used for further experiments.

Fig. 2. Western blots of AERC extracts of yeast cells of *C. albicans* VW 32 stained with ConA (lane 1), serum factor 5 (lane 2), mAb DF9-3 (lane 3) or mAb AF1 (lanes 4 and 5). Yeast cells were grown either on SDA (lanes 1–4) or in yeast extract broth in a bioreactor (lane 5).

Expression of the β -1,2-oligomannoside epitopes in various *C. albicans* serotypes and other yeast species

Fig. 3 shows the patterns observed when Western blots of AERC extracts from yeast strains, grown on SDA, were probed either with ConA or with mAbs DF9-3 and AF1. The strains presenting a pattern with the characteristics of the 14–18 kDa antigen belonged to the species *C. albicans*, *C. stellatoidea* and *C. tropicalis*. Among the two *C. albicans* clinical isolates tested, only a faint 14–18 kDa antigen band was observed for the serotype A strain (Fig. 3a, lane 2). Similarly, the intensity of the 14–18 kDa band differed between the two strains of *C. stellatoidea* tested (Fig. 3b, lanes 2, 3).

The distribution of other molecular species reactive with the anti- β -1,2-linked-oligomannoside mAbs differed greatly between the species tested. For *C. albicans* and *C. stellatoidea* strains, trace or a lack of reactivity was associated with ConA-reactive high molecular mass mannoproteins, whereas medium molecular mass mannoproteins (< 80 kDa) were consistently unreactive. In contrast, all the other yeast species tested displayed a reactivity for medium molecular mass mannoproteins whose number and staining intensity decreased in the

following order: *C. parapsilosis* U42Cp1 > *C. tropicalis* U42Ct1 > *C. krusei* U42Ck1 > *S. cerevisiae* CBS 1315 > *C. glabrata* U42Cg1. In addition, *C. tropicalis* and *C. parapsilosis* strains displayed large amounts of β -1,2-linked oligomannosidic epitopes associated with high molecular mass mannoproteins. With the exception of the 14–18 kDa antigen, irrespective of the strain or species tested, all glycoconjugates reactive with anti- β -1,2-linked-oligomannoside mAbs also displayed α -Man residues, as evidenced by their reactivity with ConA.

DISCUSSION

The demonstration that the *C. albicans* 14–18 kDa PLM (Trinel *et al.*, 1993) can trigger the cells of animal and human immune systems to induce TNF α (Jouault *et al.*, 1994) led to consideration of this molecule as important in host–parasite relationships. As the initial demonstration of the existence of the PLM concerned a single *C. albicans* strain, the present work was undertaken to explore its expression among different yeast strains grown under various conditions. Initial experimental evidence for the PLM nature of the 14–18 kDa antigen was obtained by protease digestion, metabolic radiolabelling with phosphorus, mannose and palmitic acid and glycolipid extraction (Trinel *et al.*, 1993). Because it would be difficult to obtain evidence for the presence of phosphorus and lipids associated with antigenic activity easily for a large number of strains by radioactive metabolic labelling, in this study the presence of the PLM was assessed in relation to its other specific properties: molecular mass from 14 to 18 kDa, polydisperse character, nonreactivity with ConA and reactivity with antibodies specific for β -1,2-linked oligomannosides.

The extraction procedure used had only a small effect on the detection of the 14–18 kDa antigen. The slight differences observed in the ability of the different antibodies to reveal the polydisperse character of the 14–18 kDa antigen may be related to differences in the chain length of the β -1,2-linked oligomannosides (degree of polymerization), which condition the specificity of each antibody (P. M. Jacquinet and others, unpublished).

The 14–18 kDa antigen was found in all growth forms of *C. albicans* strain VW 32 (serotype A). Further testing of a *C. albicans* serotype B strain demonstrated that the 14–18 kDa antigen could be expressed by both *C. albicans* serotypes. Despite the non-quantitative character of the Western blotting method, it was possible to show variability in the expression of the 14–18 kDa antigen: some *C. albicans* (and *C. stellatoidea*) strains displayed a very faint labelling in this molecular range whatever the growth form and the technical procedures. However, the 14–18 kDa antigen appeared to be a component common to all *C. albicans* and *C. stellatoidea* strains. Among the other yeast species tested, the 14–18 kDa antigen was found only in *C. tropicalis*. For the species *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and even *C. robusta* (*S. cerevisiae*), numerous molecules reacted in Western blotting with anti- β -1,2-linked-oligomannoside antibodies. Reactivity patterns involved high and/or medium mol-

Fig. 3. Western blots of AERC extracts of yeast cells of various *Candida* species grown to stationary phase on SDA for 24 h at 37 °C. (a) *C. albicans* strains 348 A (lanes 1 and 2) and 330 B (lane 3) stained with ConA (lane 1) and mAb AF1 (lanes 2 and 3). (b) *C. stellatoidea* strains B 4404 (lanes 1 and 2) and B 4405 (lane 3) stained with ConA (lane 1) and mAb AF1 (lanes 2 and 3). (c–f) *C. tropicalis* U42Ct1 (c), *C. parapsilosis* U42Cp1 (d), *C. krusei* U42Ck1 (e) and *C. glabrata* U42Cg1 (f) stained with ConA (lanes 1), mAb AF1 (lanes 2) and mAb DF9-3 (lanes 3). (g) *S. cerevisiae* CBS 1315 stained with ConA (lane 1) and mAb DF9-3 (lane 2).

ecular mass molecules, depending on the species. The labelling of these bands, which was strikingly identical whatever the antibody used, was generally superimposed upon ConA reactivity. The distribution of β -1,2-linked mannosyl residues among a wide variety of mannoproteins from these yeast species may seem surprising in view of the literature, where, apart from *C. albicans*, structural evidence for their presence has only been gained in *C. glabrata* (Kobayashi *et al.*, 1992a) and *C. tropicalis* (Kobayashi *et al.*, 1994) mannans. Although firm conclusions can only be drawn from NMR analysis, this observation raises the question of the unobserved presence of β -1,2-linked oligomannosides in yeast glycoconjugates, although suggested during a study on *S. cerevisiae* secretory mutants (Ataoglu *et al.*, 1993). The recent demonstration that β -1,2-linked oligomannosides act as TNF α inducers (Jonault *et al.*, 1995) has led us to further investigate the role of their association with different yeast carrier molecules. It remains to be determined whether the association of the β -1,2-linked oligomannosides with the PLM, restricted to *C. albicans* (*C. stellatoidea*) and *C. tropicalis*, is related to their more pathogenic character among the *Candida* species.

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