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Candida albicans fibrinogen binding mannoprotein: expression in clinical strains and immunogenicity in patients with candidiasis

Summary A 58 kDa cell wall-associated fibrinogen binding mannoprotein (mp58), previously characterized by our group in a *Candida albicans* laboratory strain (ATCC 26555), was found to be also present in the cell wall of clinical isolates of this fungus. Most strains examined appear to have functional mp58 species, as detected by their ability to bind fibrinogen. Western immunoblot analysis, with a monovalent polyclonal antibody generated against the mp58 species from strain ATCC 26555, revealed differences in recognition patterns depending on the strain tested and the culture conditions used. Serum samples from normal and *Candida* infected individuals were examined for the presence of antibodies against mp58 by Western immunoblotting. None of the sera from control individuals and patients suffering from superficial candidiasis contained antibodies against mp58. However, positive reactivity with this antigen and other cell wall constituents was detected for all sera from patients with confirmed systemic candidiasis. Together, these results suggest that mp58 could play an active role during infection and may be useful as a specific antigenic marker for candidiasis.

Key words *Candida albicans* · Clinical strains · Cell wall · Fibrinogen-binding mannoprotein · Serologic response

Introduction

Candida albicans is one of the most frequent etiologic agents causing opportunistic infections so called candidiasis, a disease that in its systemic manifestation could prove fatal, and whose incidence is increasing as result of an expanding population of immunocompromised (i.e., allogenic-bone marrow transplant recipients and HIV-infected individuals) patients [2, 42]. Despite the efforts of numerous groups of investigators, the importance of various virulence factors in the pathogenicity of this microorganism remains unresolved [15]. The lack for an early and effective diagnostic procedure, the toxicity displayed by the most commonly used drugs to treat the infection, and the emergence of resistant strains are causes for the high mortality rates observed with this type of disease [39].

The cell wall is the structure that maintains the shape characteristic for each morphology (yeast and mycelium) displayed by *C. albicans*, and mediates the initial interaction with the host [46]. Different studies have revealed the presence of many protein and glyco(manno)protein components bound

to the cell wall in *C. albicans* [8, 10, 17, 43]. Among them, mannoproteins appear to play essential roles in morphogenesis, antigen presentation and immunomodulation, and adherence [12, 13, 16, 32, 34].

In the last years interest has been focused in defining specific antigenic markers for the serodiagnosis of systemic candidiasis. A number of cytosolic candidal antigens have been described in this context, such as enolase [51], a 47 kDa fragment of a 90 kDa heat-shock-protein (HSP90) [35–38], a 75 kDa HSP and a non-HSP 96 kDa antigen [14], and some glycolytic enzymes [48]. However, none of them have been proven clearly to be clinically useful. On the other hand, attempts to define cell wall-bound antigens have shown a great variability in the antigenic composition associated with the cell wall, depending on different microorganism-related and environmental factors [1, 3–5, 18, 20, 29–31, 33, 44]. Besides, serological tests used to detect anti-*Candida* antibodies in sera from patients have a low specificity and sensitivity, since they mainly recognize antibodies against *Candida* cell wall mannan (the carbohydrate moiety of mannoproteins), which seem to be ubiquitous in human sera [23, 41, 52]. Hence, *C. albicans* cell wall antigens other than

mannan could represent potential candidates as antigenic markers. One of the best characterized candidal cell wall components is a 58 kDa mannoprotein (mp58), which specifically interacts with human fibrinogen [11]. The mp58 species is expressed by *C. albicans* cells in culture and in infected human tissues [11, 33], and represents a molecular entity biochemically and functionally distinct than other *C. albicans* receptor-like components, such as the C3d-receptor and the laminin binding protein [27, 28]. In the present report we have examined the expression of this cell wall component in different *C. albicans* clinical isolates, and have investigated the presence of antibodies against the mp58 in the sera from patients with candidiasis. Results presented in this report support the contention that mp58 may be (i) a putative virulence determinant, and (ii) a potential antigenic marker for the serodiagnosis of systemic candidiasis.

Materials and methods

Organisms and culture conditions *Candida albicans* ATCC 26555 and different *C. albicans* clinical isolates were employed for this study (Table 1). Clinical strains were isolated from patients with local or invasive candidiasis (n = 6) or from individuals colonized by the fungus (n = 11) at Hospital Clínico, University of Valencia (Spain). Both biochemical and serological procedures confirmed the identity of the clinical isolates. The different strains were maintained on 1.5% agar slopes of Sabouraud-dextrose medium. They were propagated at 28°C as blastoconidia (yeast phase) or at 37°C under germination inducing conditions, following basically the procedure reported previously [9] in the minimal medium supplemented with amino acids described by Lee et al. [25].

Table 1 *Candida albicans* strains used

Number ^a	Strain identification number	Isolated from
1	ATCC 26555	CMC ^b
2	105688	Blood
3	104851	Urine
4	104682	Blood
5	36985	Urine
6	32878	Feces
7	105671	Blood
8	24248	Blood
9	104011	Glans penis swap
10	30259	Feces
11	105719	Sputum
12	24249	Urine
13	32714	Tracheal exudate
14	32216	Gastric fluid
15	105805	Blood
16	24250	Blood
17	106203	Sputum
18	106199	Sputum

^aLane number of the electrophoretic runs shown in the different panels of Figs. 1, 2, and 3.

^bCMC, chronic mucocutaneous candidiasis.

Sera from human patients Serum samples were collected and frozen at -70°C until they were assayed. Samples were collected from healthy volunteers and patients admitted to the Hospital Clínico (Valencia), and classified as follows: (i) 28 patients with confirmed systemic candidiasis, 15 of whom were intravenous drug (heroin) abusers with cutaneous candidal lesions and positive serial blood cultures (clinical group A) (9 of these patients were highly immunosuppressed, whereas the remaining individuals developed a catheter-related disseminated candidiasis); (ii) 46 non-hospitalized patients suffering from superficial candidiasis (vaginitis [n = 40] and urethritis and/or balanitis [n = 6]) (clinical group B); and (iii) normal healthy individuals (n = 31) and hospitalized patients with no evidence of immunosuppression, nor harboring indwelling intravascular catheters, and not subjected to broad spectrum antibiotics or hyperalimentation (n = 36), that is people at low risk of developing systemic candidiasis (these individuals showed no symptoms of either systemic or superficial candidiasis) (clinical group C). Patients suffering invasive candidiasis were diagnosed as such when they met one of the following criteria: (i) repeated isolation of *C. albicans* from blood (at least 3 positive blood cultures taken from separate intravenous sites at different times) or specimens from sterile sites; or (ii) histopathological and microbiological proof by autopsy (necropsy) or biopsy.

Preparation of cell wall extracts β -mercaptoethanol (β -ME) was used to solubilize protein and glycoprotein components from the walls of intact cells from each *C. albicans* strain grown at 28°C or 37°C, basically as has been previously described by our group [10, 11]. The total sugar content in the different β -ME extracts was determined colorimetrically [19] with mannose as a standard.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot techniques The cell wall-bound protein and mannoprotein components present in the β -ME extracts were separated by SDS-PAGE basically as described previously [24]. Electrophoretic transfer (Western blot) to nitrocellulose membranes (Schleicher & Schüell) was performed by conventional methods [6, 49]. Indirect staining of mannoproteins present in the membranes with Concanavalin A (Con A) was performed as previously described [21, 40]. Immunodetection of blotted polypeptides was carried out using as probe a monovalent polyclonal antibody generated against the purified mp58 species from strain ATCC 26555 (PAb anti-mp58) [11]. Colored reactive bands were developed as described elsewhere [9]. Blotted proteins were assayed for fibrinogen binding as already reported [11]. Reactivity of human sera towards proteins and mannoproteins present in the β -ME extracts from germinated blastoconidia of *C. albicans* ATCC 26555 strain in a Multiscreen device (Bio-Rad), that allow independent but simultaneous testing of up to 16 different sera samples on the same blot. Reactive bands were detected with a peroxidase-labeled goat anti-human IgG (Bio-Rad).

Results

Expression of mp58 in clinical isolates of *C. albicans*

C. albicans clinical isolates recovered from different human specimens were propagated under conditions that favor growth as blastoconidia (28°C), or blastoconidia bearing germ-tubes (37°C) in the reference strain (ATCC 26555). Cells were chemically extracted by treatment with β-ME. β-ME extracts were electrophoretically separated in 5–15% acrylamide gradient gels. Coomassie blue staining of the gels allowed the detection of a variety of medium-to-low molecular mass species present in the different samples (Fig. 1). In general, the protein patterns were similar for all strains tested independently of the growing conditions, though both qualitative (some moieties appeared to be specific for a given strain) and quantitative (some other

components were found to be more abundant in the β-ME extracts of several of the strains tested) differences were readily discernible. A major band with an apparent molecular weight of 58 kDa appeared to be common to all strains and growth conditions tested, although its level of expression appeared to vary between isolates. Indirect Con A-mediated peroxidase staining of nitrocellulose blots revealed the mannoprotein nature of the 58 kDa band and stressed the occurrence of quantitative differences in its expression depending on the strain and the in vitro conditions under the cells were grown (Fig. 2).

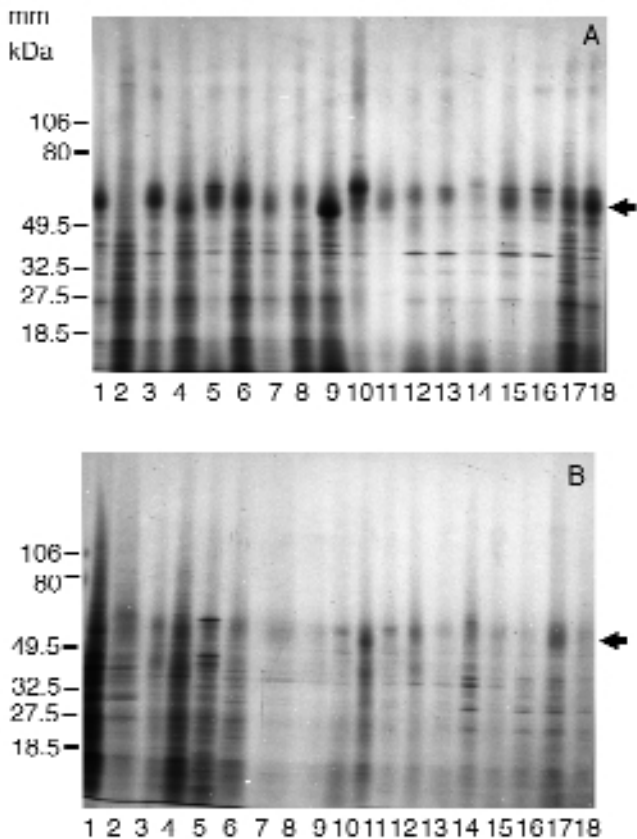


Fig. 1 Coomassie blue staining patterns of the polypeptides present in the β-ME extracts obtained from strain ATCC 26555 (lane 1) and clinical isolates of *Candida albicans* (lanes 2–18; see Table 1) grown at 28°C (panel A), or at 37°C (panel B). Samples were separated by SDS-PAGE using 5–15% polyacrylamide gradient gels. The amount of sample applied to each well was 300 μg (expressed as total sugar contain). Arrows in both panels point to a 58 kDa polypeptide species. The electrophoretic mobility of standard proteins of known molecular mass (mm) run in parallel is indicated to the left of both panels, expressed in kilodalton (kDa)

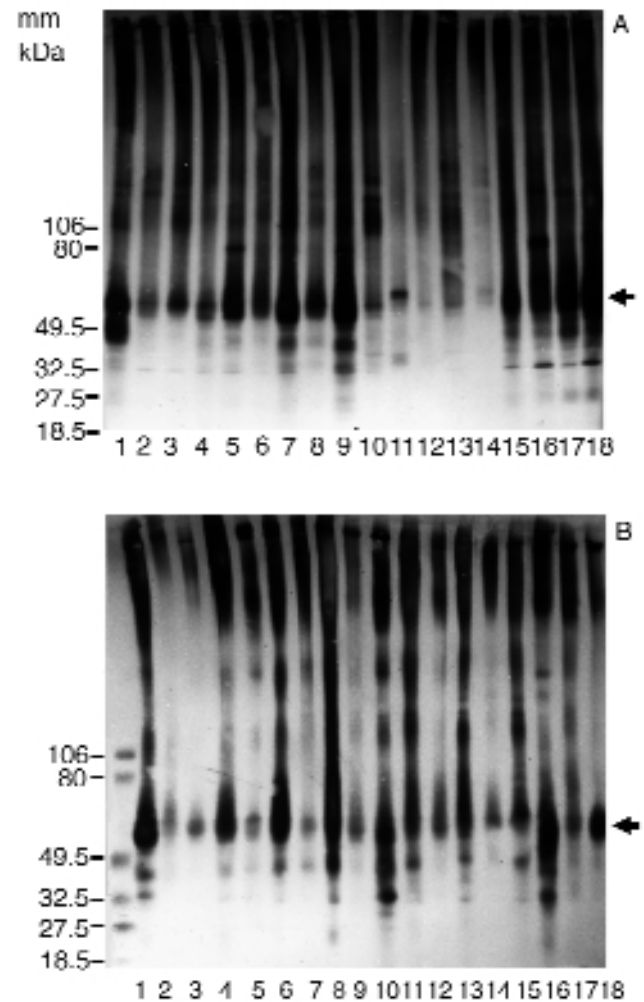


Fig. 2 Characterization of mannoproteins present in β-ME obtained from strain ATCC 26555 (lane 1) and clinical isolates of *Candida albicans* (lanes 2–18; see Table 1) grown at 28°C (panel A), or at 37°C (panel B). The amount of sample applied to each well was 100 μg (expressed as total sugar contain). Mannoproteins were separated by SDS-PAGE (5–15% slab gradient gels), transferred to nitrocellulose sheets, and detected with Con A-mediated peroxidase staining. Arrows in both panels point to a 58 kDa mannoprotein. The electrophoretic mobility (expressed in kilodalton; kDa) of standard proteins of known molecular mass (mm) run in parallel, is indicated to the left of each panel

The possible relationship of the polypeptide species exhibiting apparent molecular weights about 58 kDa present in the β -ME extracts as revealed by both Coomassie blue (Fig. 1) and Con A-mediated peroxidase (Fig. 2) staining, with the 58 kDa fibrinogen-binding mannosylated protein (mp58) previously characterized by our group [11], was investigated by Western immunoblotting using the PAb anti-mp58 preparation [11], which recognizes both sugar and protein antigenic motifs, as a probe. Cross-reactivity against PAb anti-mp58 evidenced the presence of mp58-like species in the clinical strains of *C. albicans* examined (Figs. 3A and 3C), yet differences in the reactivity towards the antiserum were observed. Thus, although 58 kDa species were consistently detected in the strains examined grown under different conditions (Figs. 1 and 2), some of these species present in β -ME extracts obtained from several strains grown at 28°C failed to react with the PAb anti-mp58 preparation, whereas reactivity was observed in β -ME extracts obtained from these same strains but grown at 37°C (compare lanes 10–14 in Fig. 3A vs. Fig. 3C). The opposite situation was also observed, since 58 kDa species in β -ME extracts from some strains cross-reacted with the antiserum when the cells were incubated at 28°C but not at 37°C (compare lanes 2 and 15 in Fig. 3A vs. Fig. 3C). Since PAb anti-mp58 recognize both sugar and protein epitopes present on mp58 [11], it is possible that subtle differences in the regulation of the expression and/or in the levels of glycosylation of the mp58-like species expressed on the surface of the *C. albicans* strains tested, as a consequence of the different growing temperatures, may account for the differences observed in reactivity towards the antiserum.

Fibrinogen binding ability of mp58-like species expressed by clinical isolates of *C. albicans* The presence of 58 kDa moieties in β -ME extracts obtained from the different clinical strains of *C. albicans* assayed in this work, exhibiting antigenic similarity with the previously characterized mp58 species [11], pruned us to assess the ability of such mp58-like molecules to bind fibrinogen by means of the ligand-affinity Western blotting assay reported elsewhere [11]. Most of the strains tested appeared to have functional mp58-like species, as concluded by their ability to interact with fibrinogen in the ligand-affinity experiments (Figs. 3B and 3D). However, the presence of bands cross-reactive with PAb anti-mp58 did not strictly correlated with the ability of mp58-like moieties to bind fibrinogen (compare Figs. 3A and 3C vs. Figs. 3B and 3D). Differences in ligand binding were also observed for a given strain depending on the in vitro growing conditions in which the cells were grown (compare Fig. 3B vs. Fig. 3D).

Western immunoblot analysis of *Candida* antigens recognized by human serum from patients suffering invasive candidiasis The fact that most *C. albicans* clinical strain examined appeared to possess mp58-like species bound to their cell walls, led us to investigate immunogenicity

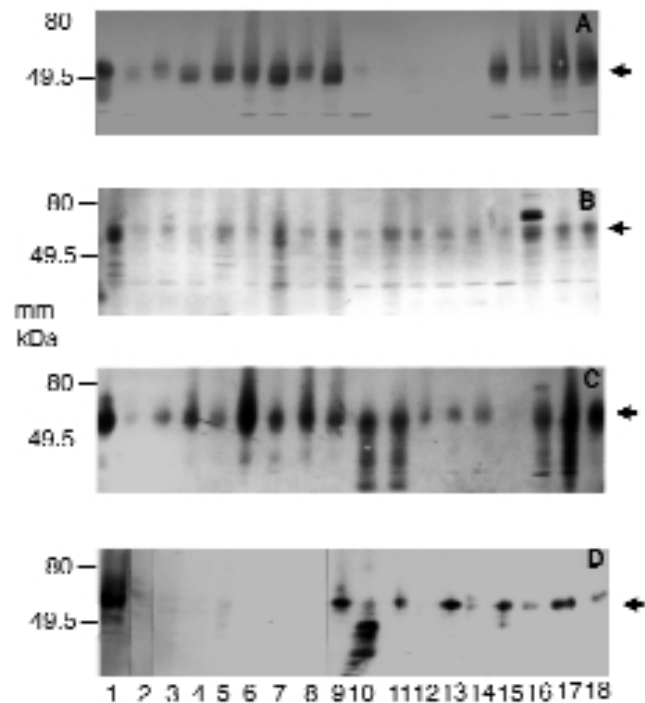


Fig. 3 Western blotting analysis of mp58-like species present in β -ME extracts. β -ME extracts obtained from strain ATCC 26555 (lane 1) and clinical isolates of *Candida albicans* (lanes 2–18; see Table 1) grown at 28°C (panels A and B), or at 37°C (panels C and D) were separated by SDS-PAGE in 5–15% slab gradient gels (the amount of sample applied to each well, expressed as total sugar content, was 200 μ g) and transferred to nitrocellulose sheets. Blots were subsequently reacted with PAb anti-mp58 (panels A and C) or probed with fibrinogen-anti-fibrinogen antibody (panels B and D) (see Materials and methods). Arrows point to 58 kDa species that exhibited reactivity following detection with PAb anti-mp58 and/or with an anti-fibrinogen antibody subsequent to incubation of the nitrocellulose sheets in purified human fibrinogen. The electrophoretic mobility (expressed in kilodalton; kDa) of prestained standard proteins of known molecular mass (mm) run in parallel is indicated to the left of each

towards this/ese molecule/s in patients affected by superficial or systemic candidiasis, to assess its potential interest as a specific marker antigen for immunodiagnosis of candidiasis. Blots of β -ME extracts from strain ATCC 26555 were probed with serum samples from individuals belonging to the different clinical groups previously established (see Materials and methods). Some features of serum samples whose reactivity in Western immunoblotting assays is shown in Fig. 4, are indicated in Table 2. Humoral immune response of patients was currently observed against *C. albicans* cell wall protein components within a molecular weight range from >140 kDa to 20 kDa (Fig. 4, bands a–i). A 58 kDa component (band e) along with two low molecular weight species (<28 kDa; bands h, i), were the antigens most frequently recognized by the sera from patients from clinical group A, followed by antigens with molecular masses of 62

kDa (band d), and >140 kDa (bands a, b, c). Strong reactivity with further antigens having apparent molecular weights of 43 kDa (band f), and 38 kDa (band g) was eventually detected with some sera (Fig. 4, lanes 1–3), yet much less frequently. A smeared immunoreaction towards polydisperse cell wall-bound antigens in the medium to high molecular weight range was observed with some sera (Fig. 4, lanes 1–3, 10, 11), which eventually made very difficult to visualize bands in the medium molecular weight range where the mp58-like species are present. Antibodies directed against carbohydrate immunodeterminants may account for the recognition of such antigens, as previous adsorption of sera with *Candida* mannan-coated polystyrene latex microspheres [41] abolished immune reaction in the high molecular weight range, yet reactivity towards the medium and low molecular weight antigens above described (Fig. 4, bands a–i) remained mostly unaffected (not shown). χ^2 tests revealed significant differences of frequencies of occurrence of major bands, between the clinical group A and the control clinical group C ($P < 0.005$) and between clinical groups A and B ($P < 0.05$) (data not shown).

Table 2 Identity and characteristics of human serum samples whose reactivity towards antigens present in β -ME extracts is shown in Fig. 4

Number ^a	Underlying condition(s)	Diagnosis of candidiasis
1	AML ^b , neutropenia	Blood culture
2	Legionellosis, IC ^c	Blood culture, necropsy
3	Diabetes, IC	Blood culture
4	HIV ^d , lymphopenia	Lung biopsy, blood culture
5	AML, neutropenia	Blood culture, necropsy
6	Mammary carcinoma, autologous BMT ^e	Necropsy
7	Glioblastoma, autologous BMT	Necropsy
8	Astrocytoma IV, autologous BMT	Necropsy
9	SCLC ^f , autologous BMT	Necropsy
10	Embryonary carcinoma, BMT	Necropsy
11	Sarcoma, autologous BMT	Necropsy
12	Superficial candidiasis	Culture
13	Superficial candidiasis	Culture
14	None (healthy)	—

^aLane number in Fig. 4; ^bacute myeloid leukemia; ^cintravascular catheter; ^dhuman immunodeficiency virus; ^ebone marrow transplantation; ^fsmall cell lung carcinoma.

Discussion

A high degree of complexity and antigenic variability in the protein and mannoprotein components present in the cell wall of *C. albicans* have been reported by several authors [10, 16, 17, 32, 43, 46]. The antigenic variability, which is considered

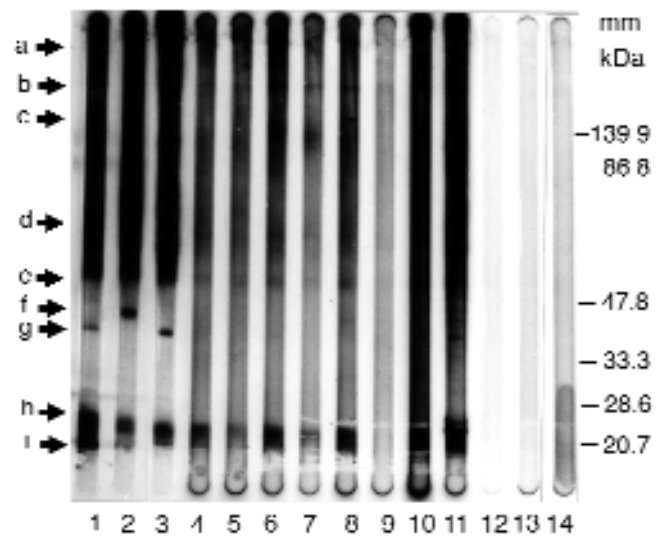


Fig. 4 Western strip blots showing representative recognition patterns of antigens present in β -ME extracts from *Candida albicans* germinated cells by sera from patients with proven or strongly suspected invasive candidiasis, superficial candidiasis, and healthy individuals (see Table 2 for further information). Immunoblot analysis was performed with diluted (1:500) sera samples. Molecular mass (mm; in kilodalton, kDa) of standard molecular weight markers are indicated to the right. Letters are used to identify the most relevant antigenic bands recognized by the different sera (see text)

to be an important virulence factor, appears to be dependent on multiple environmental- and organism-related factors [1, 3–5, 9, 16, 18, 20, 29–32, 44, 45, 47, 50].

In the present study, we have detected complex and variable protein and mannoprotein patterns in β -ME cell wall extracts obtained from different clinical isolates of *C. albicans*. However, some components in the extracts appeared to be expressed by most strains, among them a species with an apparent molecular mass of 58 kDa, although quantitative (level of expression) and qualitative (pattern of glycosylation) variations were found between isolates depending on both the strain and the incubation conditions used. Antigenic (cross-reactivity with a monovalent polyclonal antiserum; PAb anti-mp58) [11] and functional (ability to bind fibrinogen) similarities were found between the 58 kDa moieties present in the clinical isolates and the mp58 fibrinogen receptor previously characterized by our group in a *C. albicans* reference strain (ATCC 26555) [11, 33]. Most strains appeared to have functional (fibrinogen-binding) mp58-like species, although some differences in the recognition patterns for PAb anti-mp58 and in the ability to interact with the human ligand were detected among isolates depending again on both the strain and the culture conditions. Such differences could be partly attributable to the distinct nature of the probes used for detection, since we

have previously described that the PAb anti-mp58 recognizes both protein and carbohydrate antigenic motifs epitopes of the molecule, whereas fibrinogen binding seems to occur through the O-linked carbohydrate moiety present in the mp58 species [11]. Thus, differences in glycosylation of the mp58-like species among strains may be responsible for the differences observed.

Expression of mp58-like species was detected in *C. albicans* strains isolated from both simply colonized hosts and individuals affected by superficial or systemic candidiasis. Hence, the role, if any, that these molecules may play in pathogenesis of candidiasis remains to be determined, although it is conceivable that they may represent a putative fungal virulence factor. However, studies in animal models involving the use of mutant strains lacking mp58 and of genetic revertants in which expression of the molecule is restored, are required in order to establish whether mp58 and mp58-like molecules actually represent virulence determinants in *C. albicans*.

Several candidal antigens have been described as major targets for the host immune response. These include enolase, other glycolytic enzymes and heat-shock proteins [14, 32, 35, 38, 48, 51]. However, none of these have been proven clearly to be clinically useful as specific marker antigens, possibly due to the fact of the abundance and high levels of homology exhibited by glycolytic enzymes and heat-shock proteins among different pathogenic microorganisms as well as eukaryotic organisms. Several features of mp58 led us to assess its immunogenicity in patients with confirmed systemic candidiasis: (i) it is highly antigenic in animals [11], (ii) as a cell surface antigen [11] it is exposed to the host immune system without the necessity of previous cell lysis and processing, and (iii) it is expressed in *Candida* cells colonizing infected tissues [manuscript in preparation]. Besides, preliminary cloning and sequencing data suggest that mp58 does not show extensive homology with other proteins whose sequence has been deposited in data bases [1], it is present in most *C. albicans* strains (this work), and is a major component in β -ME extracts. In this context, we have previously showed that an enzyme immunoassay system in which a mp58-containing antigenic substrate bound to the solid phase was used, displayed a good performance in discriminating between patients with systemic candidiasis and individual affected by superficial infection or simply colonized by the fungus [41]. Thus, we investigated the presence of antibodies against the mp58 present in β -ME extracts from mycelial cells of *C. albicans* ATCC 26555 in the sera from patients suffering from candidiasis, by Western immunoblotting. No reactivity against the mp58 was detected when sera from either normal (control) individuals, or patients suffering from superficial candidiasis were tested. However, all sera from patients with confirmed systemic infection contained antibodies against the mp58. Proteins and mannoproteins solubilized from intact *C. albicans* cells

with reducing agents, i.e., dithiothreitol (DTT) or β -ME, appear to be located in the most external layers of the cell wall [7, 10, 16, 26] and seem to be involved in virulence- and pathogenicity-related aspects such as adhesion to animal tissues, interaction with serum proteins, proteolytic activities, and cell surface hydrophobicity [7, 13, 15, 22, 26, 27]. Hence, some of the protein and mannoprotein species present in the β -ME extract could be expected to act as strong inductors of the host immune response. In this context, components of 58 and 62 kDa were two of the antigens recognized by the sera of patients suffering invasive candidiasis in the β -ME extracts. Besides, cell surface proteins of 60–62 kDa that exhibited affinity for laminin and C3d have been identified in *C. albicans* [7] and fibrinogen-binding mannoprotein (characterized by our group in β -ME extracts from intact cells of both growth forms of this fungus) has, as already stated, a molecular mass of 58 kDa [11]. Further characterization of other low molecular weight antigens (<28 kDa) that were recognized by most sera samples from patients belonging to clinical group A (i.e., h and i in Fig. 4), is currently being carried out in our laboratory. In any case, the results presented here suggest that antibodies towards mp58 might represent a specific marker of fungal invasiveness.

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