INFECTION AND IMMUNITY, Oct. 1992, p. 4221–4229
0019-9567/92/104221-09$02.00/0
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Vol. 60, No. 10

Identification of a 58-Kilodalton Cell Surface Fibrinogen-Binding Mannoprotein from *Candida albicans*

MANUEL CASANOVA,[1] JOSE L. LOPEZ-RIBOT,[1] CARLOS MONTEAGUDO,[2] ANTONIO LLOMBART-BOSCH,[2] RAFAEL SENTANDREU,[1] AND JOSE P. MARTINEZ[1]*

Sección Departamental de Microbiología, Facultad de Farmacia,[1] and Departamento de Patología, Facultad de Medicina,[2] Universitat de Valencia, Avenida Blasco Ibáñez, 13-17, 46010-Valencia, Spain

Received 3 February 1992/Accepted 1 August 1992

Treatment of both yeast (blastoconidia) and hyphal (blastoconidia with germ tubes) cells of *Candida albicans* with β-mercaptoethanol (βME) releases a complex array of cell wall-bound proteins and glycoproteins. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting with fibrinogen–anti-fibrinogen antibody allowed the identification of a 58-kDa mannoprotein (mp58) in both extracts which specifically interacts with human fibrinogen. Treatment of intact cells with low concentrations of β-glucanase (Zymolyase 20T) for short periods or with βME abolished or significantly reduced binding of fibrinogen. A rabbit polyclonal antiserum was raised against the purified mp58 species released by βME from germinated blastoconidia (PAb anti-mp58). By Western blotting, the antiserum cross-reacted with the homologous 58-kDa fibrinogen-binding mannoprotein present in βME extracts from blastoconidia, and by indirect immunofluorescence, the antiserum labelled both yeast cells and hyphae, yet reactivity was found primarily on the cell surface of filamentous forms. Immunostaining of human infected tissue sections with PAb anti-mp58 showed that the mp58 species is also expressed in vivo; in this case, the species is in the forms of both yeast and hyphal elements similarly labelled by the antiserum. Purified immunoglobulin G fraction from the antiserum did not alter the binding of fibrinogen as determined by a modified enzyme-linked immunosorbent assay and Western blotting. The N- and O-glycosidically linked carbohydrates represent 18 to 20% and 3 to 4%, respectively, of the molecular mass of the mp58. O-linked sugar residues may be involved in the interaction of the molecule with fibrinogen.

The opportunistic pathogen *Candida albicans* is a dimorphic fungus capable of reproducing by budding, which leads to the formation of blastoconidia (yeast cells), or by producing germ tubes, which give rise to septate hyphae (34). The yeast form of this organism is associated with its presence as a commensal in the normal host, while the hyphal form is found additionally in infected tissues (34).

Adhesion of *C. albicans* to host tissues seems to be an essential factor for the establishment of candidiasis. Attachment may involve binding between complementary molecules on both host and parasite cell surfaces. Although studies on the mechanisms underlying the specificities of attachment of fungal parasites to their hosts are still scant, remarkable progress has been made in recent years toward a better understanding of the nature of this phenomenon (3, 31).

In this context, the ability of *C. albicans* cells to bind serum proteins such as fibrinogen has been described by different authors (1, 35). Coagulation proteins may play an important role in pathogenicity, as fibrin appears to be the receptor for *C. albicans* cells that adhere to blood clots in vivo (1), and disseminated intravascular coagulation has been found to occur in parallel, in some instances, with severe *Candida* septicemia (36). Besides, binding to fibronectin, which is believed to be one of the host receptors for *Candida* adhesion (5, 39), appears to be mediated by fibrinogen (44). On the other hand, as fibrinogen appears to be the dominant plasma mediator of *Staphylococcus aureus* adherence to intravascular catheters (13, 21), it may also be likely that attachment of *C. albicans* to plastic indwelling devices and prostheses, which is a phenomenon that facilitates the dissemination of the fungal cells by the vascular system of the host (39), among some other factors, such as cell surface hydrophobicity (25), could be mediated by this blood component.

By indirect immunofluorescence with an anti-fibrinogen antibody subsequent to incubation of the cells in fibrinogen, it was observed previously that the surfaces of hyphae bind fibrinogen, whereas those of yeast cells basically do not (1); in addition, by using ^{125}I-fibrinogen, binding was found to be from 7 to 12 times higher for germ tubes than for yeast forms (1), although the nature of the fungal cell surface fibrinogen-binding components was not identified in these studies.

The cell wall of *C. albicans* is the structure that maintains the shape characteristic for each growth form and is also the site of the initial interactions between the organism and the environment. Different studies have previously shown the essential role that proteinaceous constituents of the wall, most of them glycoproteins containing N- and O-linked mannose polymers (mannoproteins), appear to play in the regulation of wall morphogenesis (9, 17, 32, 33, 41) and in the modulation of several cell surface properties that can be survival and/or virulence factors in *C. albicans* (e.g., antigenicity, adherence to inert surfaces or to animal cells, exocellular proteolytic activities, and cell surface hydrophobicity [10, 12, 14, 19, 20, 29, 37]).

In this report, we describe our findings on the characterization of a cell wall-bound mannoprotein that appears to be a specific receptor for human fibrinogen in *C. albicans*. The

* Corresponding author.

results presented will eventually lead to the deciphering of some of the mechanisms by which the fungus interacts with host ligands, and they may help us to understand the role that binding of fibrinogen plays in the pathogenicity of *C. albicans*.

MATERIALS AND METHODS

Organism and culture conditions. *C. albicans* ATCC 26555 (serotype A) was employed throughout this study. It was maintained by subculturing every 2 to 3 weeks on 1.5% Bacto-Agar slopes of Sabouraud dextrose medium.

The organism was propagated as blastoconidia (yeast phase) or blastoconidia with germ tubes (also defined here as germinated blastoconidia or mycelium) by basically following a procedure described previously (7, 29) and using the minimal medium supplemented with amino acids described by Lee et al. (27) that has been recommended for studies of the cell surface properties of *C. albicans* because it is chemically defined and produces *C. albicans* cells that are highly adhesive (24).

Assay of fibrinogen binding to cells by immunofluorescence. Fungal cells (blastoconidia and germinated blastoconidia) were washed twice with glass-distilled water and resuspended at a concentration of ca. 10^6 cells per ml in 10 mM phosphate-buffered saline (0.9% NaCl) (PBS) (pH 7.4) containing 4 mg of essentially plasminogen-free fibrinogen per ml (Sigma), a concentration similar to that occurring naturally in human blood (38). After being incubated for 2 h at 37°C with gentle agitation in a gyratory incubator, the cells were washed four times with PBS, resuspended in fluorescein isothiocyanate-conjugated rabbit anti-human fibrinogen serum (Organon Teknika Cappel, Malvern, Pa.) (1:10 dilution) in PBSB buffer (PBS plus 1% bovine serum albumin [BSA]), and incubated for 1 h at 37°C. Finally, the cells were washed again with PBS as described above and resuspended in a small volume (0.1 ml) of PBS. Drops (10 to 15 μl) of these suspensions were placed on the wells of Microslides (Biomerieux) and examined (wet mountings) with a Zeiss Photomicroscope III equipped for epifluorescence (UV filter no. 487702; excitation line, 365/366 nm).

Chemical and enzymatic treatments of cells: preparation of cell wall extracts. Protein and glycoprotein components of the walls were released from intact cells by treatment with β-mercaptoethanol (βME) and with Zymolyase after treatment with βME. This procedure leads to the solubilization of the overall protein and glycoprotein wall components and minimizes the possibility of incomplete solubilization, the loss of certain wall constituents, or other masking effects that may lead to erroneous findings (6, 8).

Blastoconidia and blastoconidia with germ tubes were harvested, washed twice with sterile glass-distilled water, resuspended in 10 mM phosphate buffer (PB) (pH 7.4) containing 1% (vol/vol) βME (the cells were resuspended in a volume of buffer equivalent to 1/10 of the original volume of the culture medium in which they were grown), and incubated for 30 min at 37°C in a rotary incubator. After being treated, the cells were sedimented, and the supernatant fluid was recovered, dialyzed against distilled water (four changes) for 48 h at 4°C, and concentrated by freeze-drying (βME extract). βME-extracted cells were washed twice with 0.6 M KCl in distilled water, resuspended in this same solution (in this case, the volume used was 1/20 of the volume of the culture medium in which the cells were grown) containing 0.5 mg of Zymolyase 20T (a 1,3-β-glucanase complex) per ml, and incubated at 28°C with gentle agitation to obtain protoplasts. Protoplast formation, which usually occurred within 60 to 90 min of incubation under the conditions mentioned above, was checked microscopically. Protoplasts were sedimented at 3,000 × *g* (10 min), and the supernatant was carefully removed and recentrifuged at 27,500 × *g* for 30 min. The supernatant was recovered, dialyzed as described above, and lyophilized (Zymolyase extract). Finally, protoplasts were washed twice with 0.6 M KCl plus 3 mM phenylmethylsulfonyl fluoride; resuspended in PB containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM phenylmethylsulfonyl fluoride, and 200 mM *N*-octylglucoside; and lysed by being stirred in a Vortex mixer. The protoplast homogenate obtained was centrifuged at 40,000 × *g* for 30 min, and the supernatant (lysate) was carefully removed and stored at −30°C. The βME and Zymolyase extracts were resuspended in small volumes of PB and also stored at −30°C. The different samples (βME and Zymolyase extracts and lysate) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted to nitrocellulose paper as described below. The total sugar and protein contents in the different samples were determined colorimetrically (15, 30) with glucose and BSA used as standards. Eventually, samples of the βME extract from germinated blastoconidia were subjected to digestion with endo-β-*N*-acetylglucosaminidase H (endo H) (0.01 U/50 μg of protein in the sample) by following the procedure described elsewhere (11) or to β-elimination by the method of Elorza et al. (16) to release N- or O-linked carbohydrate moieties, respectively. Deglycosylated materials were also analyzed by SDS-PAGE and Western blotting (immunoblotting).

PAGE and Western blot techniques. SDS-PAGE was performed basically as described by Laemmli (26) with minor modifications (7). Electrophoretic transfer (Western blot) to nitrocellulose paper (Bio-Rad) was carried out as described previously (7).

Blotted proteins were assayed for fibrinogen binding as follows. The nitrocellulose membranes were blocked with 3% BSA in 10 mM Tris hydrochloride buffer (pH 7.4) containing 0.9% NaCl (TBS buffer) for 1 h at 37°C and then incubated for 4 h at room temperature with agitation in 10 mM PB (pH 7.4) containing 1% nonfat dry milk, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Tween 20, and fibrinogen (0.3 mg/ml). After being washed four times (10 min per wash) with TBS containing 0.05% Tween 20 (TBST) (the NaCl concentration in the buffer solution was increased to 1 M for the second wash), the nitrocellulose sheets were incubated with peroxidase-labelled rabbit anti-fibrinogen antiserum (Organon-Teknika Cappel) at a 1:500 dilution in TBST plus 1% BSA (TBSTB). After agitation for 1 h at room temperature, blots were washed as described above, and reactive bands were developed with hydrogen peroxide and 4-chloro-1-naphthol as the chromogenic reagent.

Immunodetection of proteins transferred to nitrocellulose was done as previously described (7) by using the specific antibody (PAb anti-mp58; see below) at a final concentration of 1:1,000 in TBSTB. Diluted (1:2,000), peroxidase-labelled goat anti-rabbit immunoglobulin G (IgG) (Bio-Rad) in TBSTB was used as the indicator antibody. Colored reactive bands were developed as described above.

Indirect concanavalin A (ConA)-mediated peroxidase staining of mannoproteins in nitrocellulose blots was conducted as reported elsewhere (7).

Generation of polyclonal antibodies. The material solubilized by βME treatment of germinated blastoconidia as described above was concentrated by freeze-drying, sus-

pended in 1 ml of glass-distilled water, mixed with 0.5 ml of electrophoresis sample buffer (7), heated for 5 min at 100°C, and subjected to SDS-PAGE on slab gradient gels (5 to 15% acrylamide, 1.5 mm thick) with a 3.5% acrylamide stacking gel according to a previously described procedure (7, 26). The stacking gel had a large well (11.5 by 3 cm) which permitted loading of up to 4 ml of sample solution and a small reference well (0.8 by 3 cm) in one side of the resolving slab. The sample solution (1.5 ml), containing ca. 20 mg (expressed as the total sugar content) of βME-solubilized material, was loaded into the large well of the stacking gel, and 30 μl of the prestained molecular weight standard mixture was added to the reference well. After being subjected to SDS-PAGE, the proteins were transferred to nitrocellulose paper and stained with 0.2% Ponceau dye in 3% trichloroacetic acid in double glass-distilled water. A transverse section of the nitrocellulose sheet, corresponding to the polypeptide that exhibited the property of interacting specifically with fibrinogen (see Fig. 2C, arrow) (M_r, 58 × 10^3), was cut out, washed with glass-distilled water to remove the dye, dried, and dissolved in 0.4 ml of dimethyl sulfoxide. The suspension obtained was emulsified with an equal volume of complete Freund adjuvant (Difco) and injected subcutaneously into adult female New Zealand White rabbits. This procedure was repeated three times at 2-week intervals, with incomplete Freund adjuvant (Difco) used each of these times. Seven days after their third immunization, the rabbits were bled. The Ig fraction was separated from the crude serum by precipitation with 40% ammonium sulfate. The precipitate was recovered by centrifugation (12,000 × *g*, 15 min), dissolved with 10 mM Tris hydrochloride buffer (pH 7.4), and dialyzed against this same buffer containing 1 mM sodium azide. The dialyzed fluid was routinely used as antiserum (PAb anti-mp58) in most experiments. Eventually, following ammonium sulfate precipitation, the IgG fraction was purified by chromatography on protein A-agarose (Sigma). The different antiserum preparations were tested by immunoblotting and enzyme-linked immunosorbent assay (ELISA) (see below) against material extracted by βME from both blastoconidia and germinated blastoconidia.

IIF and immunohistochemistry. The reactivities of PAb anti-mp58 toward intact cells were additionally assayed by indirect immunofluorescence (IIF) and immunohistochemistry. IIF assays were performed on blastoconidia and germinated blastoconidia by basically following the procedure described elsewhere (7) with a 1:50 dilution of the antiserum in PBSB buffer. Cells were examined with a Zeiss Photomicroscope III equipped for epifluorescence as indicated above. Human tissue specimens (kidney) obtained at the times of autopsy from patients with confirmed systemic candidiasis and biopsy samples from individuals with superficial cutaneous and urethral candidiasis were processed for immunohistochemical staining by using the avidin-biotin immunoperoxidase technique basically according to Hsu et al. (22). In this case, a 1:200 dilution of PAb anti-mp58 in PBS buffer was used. Preimmune rabbit serum diluted in PBS buffer at a similar protein concentration was used in control IIF and immunohistochemistry preparations.

ELISA. The reactivity of PAb anti-mp58 toward the cell wall extracts obtained as described above (βME extracts from both blastoconidia and germinated blastoconidia and deglycosylated βME extracts from germinated blastoconidia) was assayed by ELISA according to the procedure described previously (7), with the following modifications. Nunc-Immunoplate I (A/S Nunc) wells were coated with appropriate amounts of the different materials at 4°C for 12 to 16 h. After coating, free binding sites that might have still existed in the wells were blocked with PBS buffer containing 5% nonfat dry milk (PBSM) (2 h at 37°C in a moist chamber). The plate was rinsed five times with PBS plus 0.05% Tween 20 (PBST). PAb anti-mp58 was diluted (1:2,000) in PBST containing 1% BSA (PBSTB) and added to the wells, and the plate was incubated at 37°C for 45 min as indicated above. The wells were then rinsed five times with PBST, and 50 μl of a 1:3,000 dilution in PBSTB of peroxidase-conjugated goat anti-rabbit polyvalent Igs (Bio-Rad) was added to each well. After being incubated for 45 min at 37°C, the wells were rinsed again with PBST. After a substrate mixture containing *o*-phenylenediamine was added, the plate was incubated in the dark for 10 min, and the reaction was stopped by the addition of 25 μl of 3 M H_2SO_4 to each well. The color intensity was determined at 492 nm (A_{492}) with an automated plate reader (Titertek Multiscan Plus MKII; Labsystems).

A modified ELISA was used to determine the effect of PAb anti-mp58 on the binding of fibrinogen in either a noncompetitive or a competitive assay. In both cases, the wells were coated with different amounts of βME extract from germinated blastoconidia in 200 μl of PB, blocked with 200 μl of PBSM buffer per well (2 h at 37°C in a moist chamber), and rinsed once with PBST.

For the non-competition ELISA, the purified IgG fraction from PAb anti-mp58 was diluted in PBST containing 1% nonfat dry milk (PBSTM), and suitable volumes of this dilution (100 μl containing 1 μg of protein per μl) were added to each well of the plate, which was incubated at 37°C for 2 h. The plate was washed five times with PBST, and 100 μl of a fibrinogen solution in PBSTM (0.1 mg of fibrinogen per ml) was added to each well. Subsequent incubation and rinsing were done as described for the previous step. Finally, 50 μl of a 1:1,000 dilution of peroxidase-conjugated rabbit anti-fibrinogen IgG in PBSTM was added to the appropriate wells.

For the competition ELISA, the experimental protocol used was as follows. One-hundred-microliter volumes of PBSTM buffer containing both the purified IgG fraction from PAb anti-mp58 and fibrinogen (10 μg of each reagent, expressed as the total protein content) were added to series of wells previously coated with βME-released material from germinated blastoconidia (see above). After being incubated (2 h, 37°C), the wells were washed five times with PBST, and 100 μl of a 1:1,000 dilution in PBSTM buffer of peroxidase-conjugated rabbit anti-fibrinogen antibodies or peroxidase-conjugated goat anti-rabbit IgG serum was added to the corresponding wells (see the legned to Fig. 4 for further details).

In all cases, the plates were incubated at 37°C for 1 h following the addition of the different peroxidase-conjugated marker antibodies and rinsed with PBST (five times). Finally, the colored reaction was developed and measured as described above for the regular ELISA.

Miscellaneous. Gel electrophoresis and blotting reagents were from Bio-Rad. Endo H and Zymolyase 20T were from Miles Laboratories. Prestained molecular weight markers were from Bio-Rad and Sigma Chemical Co. Unless otherwise indicated, all other chemicals used were purchased from Sigma Chemical Co.

RESULTS

Binding of fibrinogen to *C. albicans* cells. The strong fluorescence observed for hyphal filaments of *C. albicans*

ATCC 26555 (a common laboratory strain) on all of their surfaces indicated their interaction with fibrinogen, yet a patched fluorescent pattern rather than the homogeneous confluent fluorescence reported for other *C. albicans* strains (1) was observed (Fig. 1D). Cell-to-cell variations in fibrinogen binding were also detected. Thus, some hyphal elements that represented a low percentage (less than 15%) of the filamentous forms in the microscopic fields examined showed only a weak fluorescence (Fig. 1B, arrow). Most of the mother blastoconidia from which germ tubes originate (Fig. 1B and D) and nongerminating yeast cells (Fig. 1A to J, stars) were not labelled or exhibited a faint fluorescence (compare panels E and F and I and J in Fig. 1). However, a relatively low percentage (15 to 20%) of nongerminating blastoconidia showed an intensely homogeneous (Fig. 1F, arrows) or a punctate fluorescent pattern (Fig. 1H and J, arrows). Fluorescence was dependent on the reaction of the cells with fibrinogen, since no fluorescent cells were observed when incubation was performed with the fluorescein isothiocyanate anti-fibrinogen antibody only. Binding of fibrinogen to cells did not vary significantly when cells of both growth forms previously fixed with 0.5% formalin in PBS for 10 to 20 min were used instead of living cells. βME treatments or short-term (5-min) digestions with β-glucanase (Zymolyase 20T) of blastoconidia and blastoconidia with germ tubes, respectively, reduced or abolished binding of fibrinogen to cells as determined by IIF.

Identification of surface (cell wall) components that bind fibrinogen. Wall components that could promote binding of fibrinogen to the cell surface were extracted by βME treatment followed by Zymolyase digestion of intact cells. When analyzed by SDS-PAGE under reducing conditions on slab gradient gels (5 to 15% acrylamide) and then stained with Coomassie blue, βME extracts from blastoconidia (Fig. 2A, lane 1) and germinated blastoconidia (Fig. 2A, lane 2) were seen to contain about 15 polypeptide chains with molecular masses ranging from 78 to 17 kDa. Proteins solubilized with Zymolyase from both yeast (Fig. 2A, lane 3) and mycelial (Fig. 2A, lane 4) forms also represented a complex array of medium- to low-molecular-mass species (from 60 to 17 kDa), though these polypeptide patterns were qualitatively distinct from those that correspond to βME extracts of both growth forms (compare lanes 3 and 4 with lanes 1 and 2 in Fig. 2A). βME treatment and Zymolyase digestion also released several high-molecular-mass (>120-kDa) polydisperse mannoprotein species that were not detected by Coomassie blue staining of gels, being visualized only after ConA-peroxidase staining of nitrocellulose blots (Fig. 2B, lanes 1 to 4). In contrast, most of the medium- and low-molecular-mass species (<70 kDa) showed no reactivities toward the lectin (Fig. 2B, lanes 1 to 4). Among the proteins or polypeptides released with βME, only a 58-kDa component, present in extracts from both growth forms (Fig. 2A, lanes 1 and 2, arrow), was revealed by incubation of the blots with fibrinogen (Fig. 2C, lanes 1 and 2, arrow). No reactive bands were observed when the nitrocellulose sheet was incubated with the anti-fibrinogen conjugate alone, thus indicating that the reaction was dependent on the previous interaction of blotted polypeptides with fibrinogen. The 58-kDa species is a mannoprotein (mp58), as can be concluded from its reactivity against ConA (Fig. 2B, lanes 1 and 2, arrow). Zymolyase digestion of βME-treated blastoconidia and germinated blastoconidia released two weakly reactive, fibrinogen-binding polypeptides with molecular masses of approximately 58 and 40 kDa (Fig. 2C, lanes 3 and 4). The 58-kDa component exhibited no reactivity against ConA (Fig. 2B, lanes 3 and 4), while the 40-kDa species is a mannoprotein (Fig. 2B, lanes 3 and 4, open arrowhead). Neither high-molecular-mass polydisperse mannoproteins (Fig. 2B, lanes 5 and 6) nor fibrinogen-binding proteins (Fig. 2C, lanes 5 and 6) were detected in protoplast homogenates.

Characterization of the antiserum for the fibrinogen-binding species. Specificity of the rabbit polyclonal antiserum generated against the purified mp58 molecule from germinated blastoconidia (PAb anti-mp58) was examined by Western blotting. Strong reactivity with a major band of 58 kDa in βME extracts from germinated cells was observed (Fig. 3, lane 2). Cross-reactivity, along with slight reactivities with 78-, 70-, 45-, 43-, and 37-kDa bands, was observed with the mp58 species (the major band) solubilized from blastoconidia by the sulfhydryl compound (Fig. 3, lane 1). Minor reactions of PAb anti-mp58 with several bands in the 60- to 40-kDa range present in the Zymolyase digests from both blastoconidia (Fig. 3, lane 3) and germinated blastoconidia (Fig. 3, lane 4) were also noticed. No reactive bands in homogenates from yeast (Fig. 3, lane 5) and hyphal (Fig. 3, lane 6) protoplasts were observed.

The reactivity of PAb anti-mp58 against βME extracts from both growth forms of *C. albicans* was also assayed by ELISA. Although the antiserum reacted with both extracts at a titer of 1:2,000 or even higher, the intensity of the reaction was systematically about 100% higher (A_{492}, 2.15 versus 1.05) with the βME extract from germinated blastoconidia than with the equivalent material from blastoconidia; in both cases, the same amounts (at various concentrations) of antigen (expressed as total sugar) against a 1:2,000 dilution of the PAb anti-mp58 serum were used.

The removal of (i) N-linked carbohydrate by treatment with endo H, (ii) O-linked sugar residues by β-elimination, or (iii) endo H digestion followed by β-elimination treatment, which apparently caused complete deglycosylation of the mp58 species (see below), did not significantly affect the reactivity of PAb anti-mp58 against the material released with βME from germinated cells. Thus, the reduction in the intensity of the reaction (A_{492}) ranged from 15 to 25%, depending on the deglycosylated material considered, of the reactivity observed for the untreated material assayed in parallel under the same conditions.

The purified IgG fraction from PAb anti-mp58 exhibited reactivities against the βME extract and the mp58 species similar to those of the whole antiserum in ELISA and Western blotting (not shown), yet purified IgGs apparently did not inhibit the binding of fibrinogen to material released by βME from germinated blastoconidia, as determined by a modified non-competition (Fig. 4A) or competition (Fig. 4B) ELISA.

Investigation of the surface expression of the fibrinogen-binding mannoprotein (mp58) by IIF with PAb anti-mp58 as the probe revealed a pattern of cell-bound fluorescence very similar to that observed when both growth forms of *C. albicans* were assayed for fibrinogen binding (Fig. 1). Thus, reactivity was found primarily on the cell surface of hyphal elements induced in the liquid medium described by Lee et al. (27), although ca. 15% of the yeast cells grown in the same medium exhibited confluent or patched fluorescence on their surfaces (data not shown). Immunochemical detection of mp58 expression in fungal cells infecting human tissues with PAb anti-mp58 showed that this mannoprotein is also expressed in vivo; in this case, it was in the forms of both yeast cells and pseudohyphae specifically and similarly labelled by the antiserum (data not shown).

Glyco(manno)protein nature of mp58 and effect of its degly-

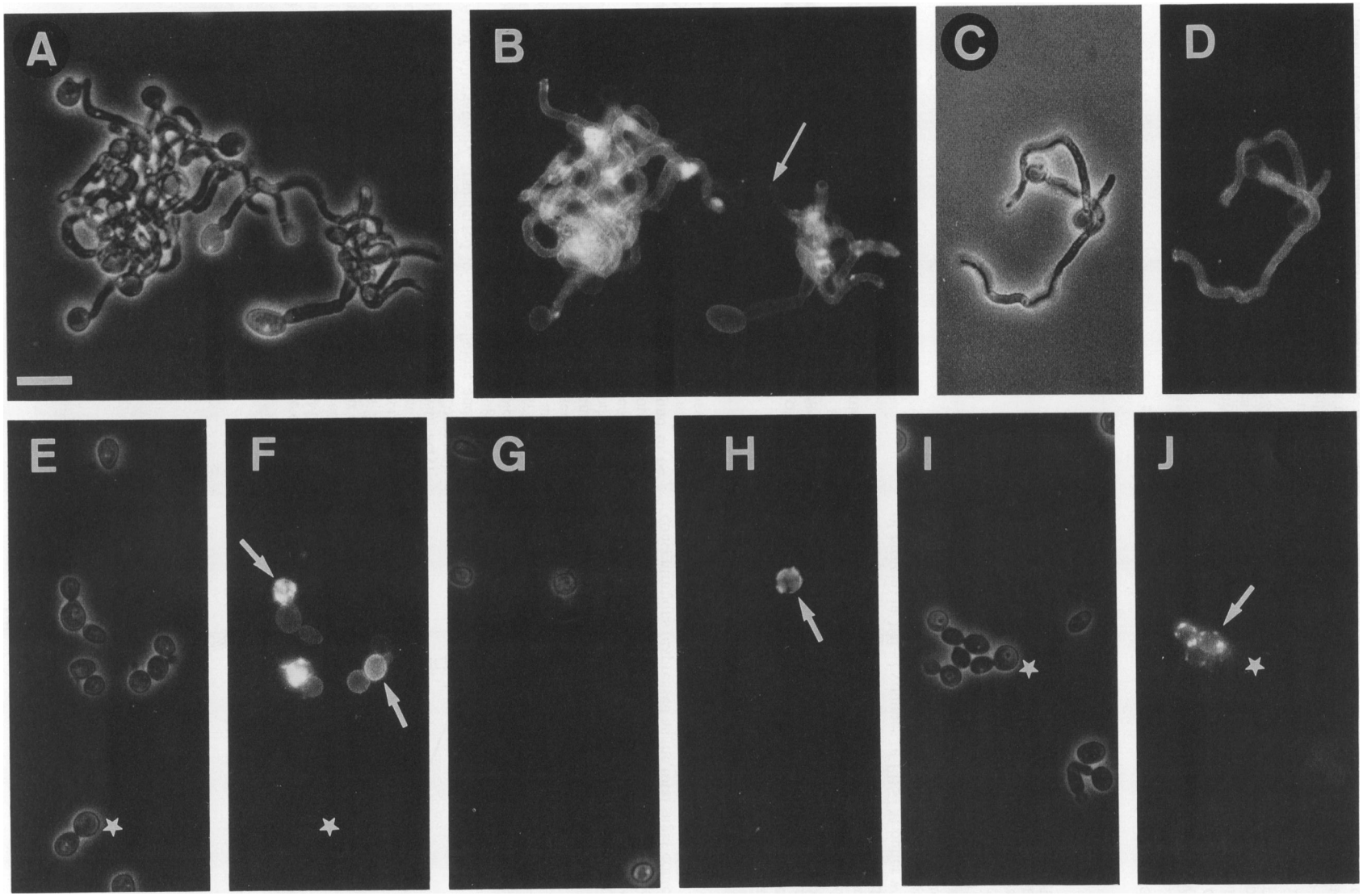

FIG. 1. Binding of fibrinogen to cells of *C. albicans* incubated at 37°C to induce germ tube formation (A to D) or at 28°C (budding growth forms) (E to J), as seen by an indirect immunofluorescence assay. Phase-contrast (A, C, E, G, and I) and fluorescence (B, D, F, H, and J) microscopy was used. In panel B, the arrow points to a weakly fluorescent hyphal element. In panels E to J, stars indicate yeast cells showing no fluorescence, whereas arrows point to blastoconidia exhibiting different fluorescent (homogeneous or patched) patterns. Bar, 10 μm.

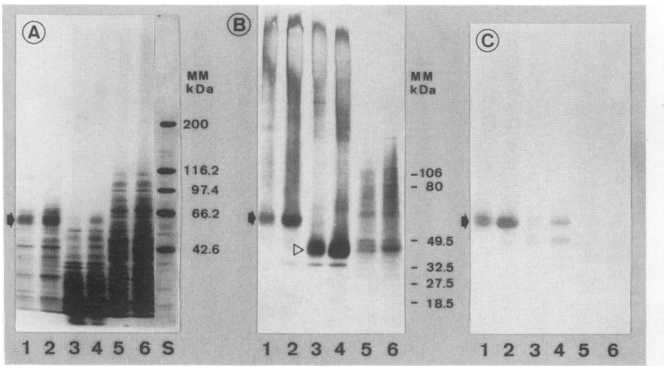

FIG. 2. SDS-PAGE and Western blotting analysis of the candidal receptors for fibrinogen. Wall components released by βME treatment (βME extract) (lanes 1 and 2; samples applied to each well contained ca. 200 μg of material expressed as the total sugar content) and Zymolyase 20T digestion (Zymolyase extract) (lanes 3 and 4; 600-μg samples here, also expressed as the total sugar content) of blastoconidia (lanes 1 and 3) and germinated blastoconidia (lanes 2 and 4), respectively, were analyzed by electrophoresis with acrylamide (5 to 15%) slab gradient gels and then stained with Coomassie blue (A) or, alternatively, transferred to nitrocellulose and stained with ConA-peroxidase (B) or immunoblotted with fibrinogen–anti-fibrinogen antibody (C) (see Materials and Methods). Samples (40 μg of protein) of blastoconidia (lane 5) and blastoconidia with germ tubes (lane 6) in the form of protoplast homogenates were also analyzed. Solid arrows point to a 58-kDa mannoprotein species present in the βME extracts from both growth forms that exhibited an intense reactivity following detection with an anti-fibrinogen antibody subsequent to incubation of the nitrocellulose sheet in purified human fibrinogen. The open arrowhead points to a broad mannoprotein band (average apparent molecular mass, 40 kDa) that was found to be slightly reactive toward fibrinogen (C). Lane S, mixture of molecular weight standards run in parallel and stained with Coomassie blue; MM, molecular masses of standard proteins.

cosylation on the binding of fibrinogen. The effects of different deglycosylation treatments on the mp58 species solubilized with βME from germinated blastoconidia allowed further characterizations of its glycoprotein nature and of the

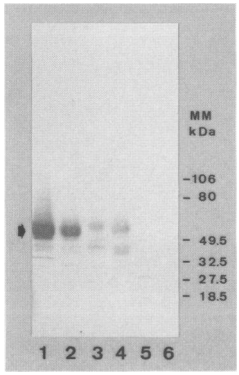

FIG. 3. Western blot of βME (lanes 1 and 2) and Zymolyase 20T (lanes 3 and 4) extracts and protoplast homogenates (lanes 5 and 6) reacted with a specific polyclonal rabbit antiserum raised against the 58-kDa fibrinogen-binding mannoprotein (PAb anti-mp58; 1:1,000 dilution) and detected with goat anti-rabbit polyclonal antiserum coupled with peroxidase. The amounts of sample applied to each well are described in legend to Fig. 2. The arrow points toward a 58-kDa band that was the major antigen recognized by the PAb anti-mp58. MM, molecular masses of standard proteins.

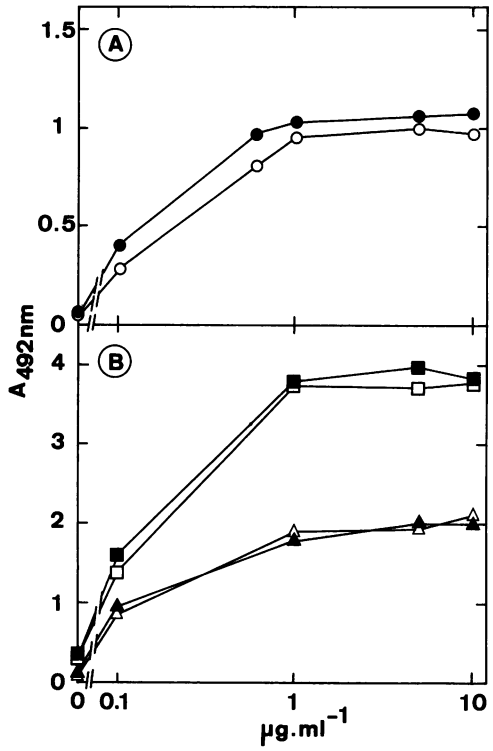

FIG. 4. Effect of PAb anti-mp58 (purified IgG fraction) on the binding of fibrinogen. (A) Non-competition ELISA. Duplicate series of wells of standard titration plastic plates were coated with various concentrations (expressed as the total sugar content) of the material released by βME from germinated blastoconidia. Fixed amounts of purified IgG fraction (100 μg of protein per well) from the antiserum anti-mp58 in 100-μl volumes of PBSTM buffer (○) were added to one of the duplicate series of wells, whereas a similar volume of buffer containing no antibody (control) (●) was added to each well of the other duplicate series. Binding of fibrinogen was then determined with an anti-fibrinogen antibody subsequent to incubation in purified human fibrinogen. (B) Competition ELISA. Duplicate series of wells were coated with βME-released material from germinated blastoconidia as described for panel A. Fixed amounts of a mixture of the purified IgG fraction (10 μg of protein) and fibrinogen (10 μg of protein) in 100-μl volumes of PBSTM buffer were added to each well (□, △), whereas the same amount (10 μg) of purified IgG fraction (■) or fibrinogen (▲) alone was added to the wells of the reference series. After incubation, 100 μl of a peroxidase-conjugated goat anti-rabbit IgG polyvalent serum dilution per well was added to a duplicate series of wells (□, ■), while a similar volume of a rabbit anti-fibrinogen antiserum coupled with peroxidase was added to each well of the other duplicate series (△, ▲). Values shown in both panels are representative of triplicate determinations with no more than 5% (A) or 10% (B) variation.

role played by the polypeptide and carbohydrate moieties of this molecule in its ability to bind fibrinogen. Treatment with endo H, which cleaves high-molecular-weight, N-linked mannose polysaccharides of *C. albicans* cell wall-related mannoproteins (16), resulted in the conversion of the native mp58 molecule (Fig. 5A, lane 1, solid arrow) to a 47-kDa species (Fig. 5A, lane 2, open arrowhead). This band, which was still able to bind ConA (Fig. 5A), thus indicating that it contained carbohydrate, reacted with PAb anti-mp58 (Fig. 5B) and retained the ability to bind fibrinogen (Fig. 5C). β-elimination, which removes O-linked oligosaccharides (16), did not significantly modify the electrophoretic mobility

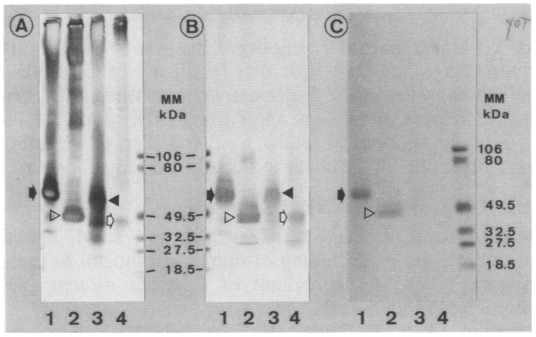

FIG. 5. Effects of different deglycosylation treatments on the 58-kDa fibrinogen-binding mannoprotein. Material released from germinated blastoconidia by treatment with βME was subjected to different treatments to remove N- and/or O-glycosidically bound carbohydrate moieties (endo H digestion [lanes 2]; β-elimination treatment [lanes 3]; combined endo H digestion–β-elimination treatment [lanes 4]; native material is shown in lanes 1). Samples of the different materials were subjected to SDS-PAGE in acrylamide (5 to 15%) slab gradient gels (amounts of sample loaded in each well [expressed as the total protein] were 5 μg in panel A, 15 μg in panel B, and 30 μg in panel C), transferred to nitrocellulose paper and stained with ConA-peroxidase (A), immunoblotted with PAb anti-mp58 (B), and assayed for fibrinogen binding as described previously (see Materials and Methods and the legend to Fig. 2). Relevant bands are indicated by solid and open arrows and arrowheads (see the text for further explanations). The electrophoretic mobilities of prestained molecular weight standards run in parallel are shown in all panels. MM, molecular masses of standard proteins.

of mp58, as its molecular mass was reduced approximately by 1.5 kDa (Fig. 5A, lane 3). Interestingly, the O-deglycosylated mp58 species was unable to interact with fibrinogen (Fig. 5C, lane 3). Although other possible chemical and/or structural alterations of the mp58 molecule caused by the β-elimination treatment could be responsible for the observed inability to bind fibrinogen, this seems not to be the case, as the O-deglycosylated molecule exhibited an unaltered reactivity against the lectin (Fig. 5A, lane 3, solid arrowhead) and the antiserum anti-mp58 (Fig. 5B). Finally, endo H digestion followed by β-elimination treatment also caused the abolition of fibrinogen binding (Fig. 5C, lane 4) along with an almost complete lack of reactivity against ConA (Fig. 5A, lane 4, open arrow), although the reactivity of the N- and O-deglycosylated molecule with PAb anti-mp58 was basically unaffected (Fig. 5B, lane 4, open arrow).

DISCUSSION

This study adds to observations from other authors on fibrinogen binding to *C. albicans* cells (1, 35) in that we have characterized a 58-kDa mannoprotein (mp58) which is a genuine cell wall component (8) and which may represent a specific, receptorlike molecule for human fibrinogen, since other mammalian proteins, such as laminin, fibronectin, and type IV collagen, did not bind to mp58 species under our experimental conditions. Although attachment of fibrinogen to yeast cells in tests using other *C. albicans* strains has not been reported (1, 35), this molecule was found to be present in both growth forms of the fungus. In this context, the results presented here are in line with those of reports indicating that not only hyphae but also *C. albicans* yeast cells possess cell wall-bound components (some of them mannoproteins) that may be related to the interaction of the latter with host ligands (23, 42) and adhesion to epithelial cells (18) and that may modulate cell surface hydrophobicity (29). However, only a low number of yeast cells examined were able to bind fibrinogen, while most of the filamentous forms do, as revealed by the IIF assay used. Alternative explanations for the distinct in vitro fibrinogen-binding behavior exhibited by yeast and mycelial elements include the following theories: (i) the mp58 species is quantitatively less abundant in the yeast cell wall (as suggested by ELISA titration with PAb anti-mp58), (ii) irregular deposition of the mp58 species takes place in the yeast cell wall, (iii) expression of the receptor for fibrinogen occurred in only a minor percentage of nongerminating cells, or (iv) the receptor molecule is located deep within the wall structure in the greater part of yeast cells whose walls are thicker than those of mycelial cells. It is also possible that the results presented here may reflect a particular characteristic and/or physiological response of *C. albicans* ATCC 26555 to the in vitro growth conditions used. However, this seems not to be the case, as both pseudohyphal and yeast forms present in infected human tissues showed strong reactivities with the antiserum anti-mp58. A similar observation (i.e., in vitro and in vivo expression) has been also reported for the candidal C3d receptor (23).

The polyclonal antiserum against the 58-kDa mannoprotein appeared to be specific for the fibrinogen-binding species present in βME and Zymolyase extracts, as evidenced by the detection of the same pattern of bands on Western blots incubated with either fibrinogen of PAb anti-mp58. On the other hand, PAb anti-mp58 recognized an immunodeterminant(s) which seemed to be associated mostly with the protein moiety of the mp58 molecule and which was not shared by other unrelated molecules present in the cell wall structure. The following evidence supports these contentions. First, a significant reduction of the antiserum reactivity was not observed following the removal of the N- and O-linked sugars. Second, multiple bands from both extracts, instead of a single major band, would be expected to exhibit reactivity against PAb anti-mp58 if distinct wall components share common epitopes. The slight reactivities with different bands (78, 70, 45, 43, and 38 kDa) observed for the βME extracts from blastoconidia are of doubtful significance at the dilution of antiserum used. Besides, some of these minor bands may also represent degradation products of mp58. The presence of fibrinogen-binding bands from 60 to 40 kDa in the Zymolyase extracts that also cross-react with the antiserum deserves some comment. The mp58 species appeared to be associated mostly with the outermost layers of the cell wall where receptors for other host ligands (e.g., laminin) have been also found (2), as βME treatment apparently extracted most of the mp58 present in the cell wall (under our experimental conditions, βME has been found to solubilize ca. 15% of the protein content of the wall only [29]). Although the suggested preferential location of mp58 is consistent with its suspected role as a receptor for fibrinogen, it is possible that degradation of the wall β-glucan network by Zymolyase released minor amounts of the mp58 species that might remain deep within the wall structure after βME treatment of cells. The particular solubilizing effect exerted by the β-glucanase complex, along with the contaminating proteolytic activity present in the commercial Zymolyase preparation (17, 19), may account for both the lack of reactivity toward ConA and the slight differences we noticed in the molecular mass of the major fibrinogen-binding band present in both βME and Zymolyase extracts. The same reasons may apply for the appearance of a low-molecular-mass (40-kDa) fibrinogen-binding species which was de-

tected exclusively in Zymolyase extracts and which shares at least one common epitope with the 58-kDa mannoprotein.

Some experimental evidence which indirectly suggests that carbohydrates may play an active role in the interaction between candidal receptors and host ligands has been reported (see reference 3 for a review on that topic). The results presented here are somewhat in line with this contention, as we have found that O-linked oligosaccharides (presumably no more than 10 mannose residues) present in the mp58 species could be involved in the interaction of the latter with fibrinogen. The lack of inhibition in fibrinogen binding with purified anti-mp58 IgG in either a noncompetitive or a competitive assay suggests that the fibrinogen-binding site on mp58 is distinct from that of PAb anti-mp58 binding and represents indirect evidence of the role that O-linked sugars may play in the interaction of mp58 with fibrinogen (as already stated, PAb anti-mp58 seems to recognize an immunodeterminant[s] present mostly in the polypeptide moiety of the mp58 species). Nevertheless, the possibility that the fibrinogen site on mp58 is not immunogenic (thus, anti-mp58 IgG did not compete for those sites) cannot be dismissed out of hand. In any case, as mannose and methyl-α-D-mannoside did not block binding of fibrinogen to mp58 in ELISA and Western blot assay (data not shown), the precise chemical nature and structural conformation of sugar residues in the O-linked sugar domain of the mp58 species as well as the role (if any) that carbohydrates may play in the binding of fibrinogen by mp58 remain to be determined.

The relationship of the mp58 fibrinogen-binding species with other candidal receptors for human proteins is unknown. *C. albicans* cell wall proteins and glycoproteins within a range of molecular masses from 68 to 60 kDa have been identified as surface receptors for laminin (2) and C3d (4, 28, 40). Proteins with molecular masses of 60, 62, and 68 kDa were found to have multiple biological activities, as they exhibited affinities for laminin, fibrinogen, and C3d (2, 43). Binding of laminin and fibrinogen to germ tubes may imply the same candidal receptors, since the latter greatly reduced the binding of laminin to *C. albicans* (2), and evidence indicating the existence of a candidal CR3-like protein that recognizes the RGD sequences that are common to many mammalian cell proteins, such as iC3b, fibrinogen, fibronectin, and laminin, exists (3). Finally, it has to be mentioned here that the mp58 species characterized in this work was previously found to have hydrophobic characteristics (29).

In conclusion, the observations above mentioned, including the results reported in this article, suggest the existence of a particular family of cell wall proteins and mannoproteins within a concrete range of molecular masses (from 55 to 70 kDa) that are the modulators of several cell surface properties that can be survival or virulence factors (e.g., antigenicity, cell surface hydrophobicity, adherence to inert surfaces, and interactions with host ligands) for *C. albicans* cells. Some of these wall components could be common to both growth morphologies (e.g., the mp58 fibrinogen-binding species described here), whereas others appear to be form specific. However, further studies are required to establish (i) the existence of multiple biological activities for some of these molecules, as suggested by different authors (2, 3, 43), (ii) the molecular mechanisms that promote the interaction of candidal receptors with animal cell or plasma ligands, and (iii) the specific presence of receptorlike molecules associated with each *C. albicans* growth form. This information is essential to understanding the mechanisms by which the fungus is able to colonize the host.

ACKNOWLEDGMENTS

This work was partially supported by grants to R.S. from the Dirección General de Investigación Científica y Técnica (PB90-0424) and the Commission of the European Communities (EEC Group on *Candida albicans* Cell Biology and Pathogenicity and Cl1*.0631.M). J.L.L.-R. is a predoctoral fellow from the Plan de Formación del Personal Investigador, Ministerio de Educación y Ciencia.

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