Characterization of Cell Wall Proteins from Yeast and Mycelial Cells of *Candida albicans* by Labelling with Biotin: Comparison with Other Techniques

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Candida albicans ATCC 26555 blastoconidia and blastoconidia bearing germ tubes were metabolically labelled by incubating the cells with ^{14}C-labelled protein hydrolysate and were subsequently tagged with biotin. Double-labelled (radioactive and biotinylated) cell wall proteins and glycoproteins were extracted from intact cells of both growth forms by treatment with 2-mercaptoethanol (βME) and with β-glucanases (Zymolyase) after treatment with βME. The βME- and Zymolyase-extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotted (immunoblotted) to nitrocellulose paper. Polyacrylamide gels were stained with Coomassie blue and processed for fluorography. Western blot analysis was performed either with peroxidase conjugated-concanavalin A (ConA) or Extravidin. Blotted proteins were also reacted with polyclonal antibodies and monoclonal antibodies against mannoprotein components from mycelial cell walls of the ATCC 26555 strain. Labelling with biotin allowed identification of a complex array of cell wall protein and glycoprotein components within a very wide molecular mass range (from 650 to 13 kDa). These appeared to be genuine cell wall components. Biotinylated high-molecular-mass glycoproteins that were not stained with Coomassie blue or that appeared as poorly resolved polydisperse bands by indirect ConA-peroxidase staining of Western blots were detected as sharply defined bands following reaction with the Extravidin-peroxidase conjugate. Biotinylated molecules retained unaltered reactivities against ConA, polyclonal antibodies, and monoclonal antibodies.

The major components of the cell wall of the opportunistic dimorphic fungal pathogen *C. albicans* are polymers of mannose (mannan) covalently associated with proteins (mannoproteins), polymers of glucose (glucans), and chitin (40). Mannoproteins appear to be involved in morphogenesis and pathogenicity-related aspects (i.e., adhesion to inert materials and animal tissues and presentation of antigens affecting the host immune system) in this fungus (4, 9–11, 13, 18, 19, 26, 32, 39). Hence, information about the chemical nature, distribution, and interactions of mannoproteins is important for a precise characterization of the mechanisms involved in virulence and in modulation of the host immune response and for an understanding of how the cell wall of *C. albicans* is built.

Polyacrylamide gel electrophoresis alone or in association with Western blot techniques has been used to analyze protein and glycoprotein constituents solubilized from intact cells and from isolated cell walls by different chemical and/or enzymatic treatments (6–8, 10, 12, 15, 17, 26, 28, 34, 35, 41). In general, these studies evidenced both a complex array of protein-containing components and quantitative and qualitative differences in the protein composition of yeast and mycelial cell walls. Some of the solubilized cell wall components have been characterized as high-molecular-weight mannoproteins (HMWM) that appear to be important in *Candida* morphogenesis and immunity (8, 9, 16, 17, 19, 35, 41). Visualization of radioactive polypeptides in polyacrylamide gels by fluorography was the most suitable procedure for the detection of HMWM (8, 15, 17), as other methods which utilize reagents that detect proteins (i.e., Coomassie

blue and silver nitrate) or carbohydrates (i.e., silver nitrate and lectins, such as Concanavalin A, coupled to peroxidase) either did not stain these macromolecular species or produced diffuse staining patterns because of the large carbohydrate content and polydisperse character of *C. albicans* HMWM (15). In the medium- to low-molecular-weight range, over 40 polypeptide bands have been obtained from isolated cell walls (12, 17), although it is unknown whether all these protein moieties are bona fide cell wall constituents.

Expression, chemical characteristics and behavior observed in vitro, and biological properties of *C. albicans* cell wall-bound proteins and glycoproteins appear to be dependent on multiple environmental factors. These include growth conditions (3), organism-related factors (such as growth state, morphology of the cells, strain and serotype, and phenotypic switching) (19, 29), and the nature of the biological specimens (intact cells or isolated walls) that are subjected to analysis (6). This makes it difficult to compare results reported by different authors regarding characterization of cell wall proteins and glycoproteins in *C. albicans*.

In the present study, we have attempted to delineate further the protein composition of the cell wall of *C. albicans* by using the avidin-biotin complex technique, which in recent years has become a very useful tool in a wide variety of bioanalytical applications (44). The results reported here indicate that labelling of living cells with biotin and subsequent detection with avidin of biotinylated moieties chemically and enzymatically released from intact cells and transferred to nitrocellulose is a simple and suitable procedure for the characterization of autochthonous cell wall proteins and glycoproteins within a wide molecular mass range from both growth forms of *C. albicans*.

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MATERIALS AND METHODS

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

A loopful of cells from a slant culture grown for 24 to 36 h was inoculated in an Erlenmeyer flask (500 ml) containing 150 ml of minimal liquid synthetic medium (24) and incubated at 28°C for 14 to 16 h in a gyratory incubator (200 rpm) to reach an A_{650} (measured with a Perkin-Elmer Coleman 250 spectrophotometer) of 0.6 U, which corresponds to the exponential growth phase. Cells (blastoconidia) were recovered by centrifugation at 3,000 × g for 10 min, washed twice with sterile glass-distilled water, and resuspended at a concentration of about 1 mg (dry weight) of cells per ml in sterile glass-distilled water. The cell suspension was incubated at 28°C for 3 h with shaking and then stored at 4°C for 48 to 96 h to produce starvation.

Development of yeast or mycelial forms. Starved blastoconidia were used to obtain exponential-phase cultures by inoculating them into fresh liquid synthetic medium to a final density of 0.2 mg (dry weight) per ml (as calculated from a standard curve relating dry weight to optical density at 600 nm) and incubated with shaking at 28°C to grow yeast cells or at 37°C to develop mycelial cells. The detailed protocol followed to obtain blastoconidia (yeast forms) or germinated blastoconidia (also defined here as blastoconidia bearing germ tubes or mycelia) has been described elsewhere (8, 26).

Labelling with radioactive precursors. For radioactive metabolic labelling of peptides, starved blastoconidia were inoculated into Lee medium supplemented with 0.3 μCi of ¹⁴C-labelled protein hydrolysate (specific activity, 56 mCi [2.072 MBq] per milliatom of carbon) per ml and incubated with shaking for 6 h at 28 or 37°C. After the pulse, the cells were collected, washed three times with 0.05 M phosphate (pH 7.4), and subjected to the enzymatic and chemical treatments described below.

Surface labelling of cells with biotin. Fungal cells (blastoconidia and germinated blastoconidia) were collected by centrifugation, washed once with 100 mM phosphate buffer (pH 8), and resuspended in a volume of the same buffer equivalent to 1/10th of the volume of culture medium in which the cells were grown, containing 1 mg of NHS-biotin (Sigma) per ml (NHS-biotin was previously dissolved in dimethyl sulfoxide). After incubation for 1 h at 22°C in a shaking water bath, the cells were recovered and washed four times with 50 mM phosphate buffer (pH 6) and then once with 10 mM phosphate buffer (PB), pH 7.4. Biotinylated cells were chemically and enzymatically extracted (see below).

Preparation of cell wall extracts. Intact cells were treated with 2-mercaptoethanol (βME) and with β-glucanases after treatment with βME to release the entire population of cell wall proteins and glycoprotein components, basically by following the procedure described previously (6). This procedure has been used to minimize the possibility of an incomplete solubilization, the loss of certain cell wall constituents, or other masking effects that may lead to erroneous or incomplete findings.

Metabolically ¹⁴C-labelled and biotinylated blastoconidia and blastoconidia bearing germ tubes were harvested, washed as described above, resuspended in PB containing 1% (vol/vol) βME (using a volume of buffer equivalent to 1/10th of the original volume of culture medium in which they were grown), and incubated for 30 min at 37°C with

shaking. 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). The βME-extracted cells were subsequently treated with a (1→3)-β-glucanase complex (Zymolyase 20T) to obtain protoplasts by the following procedure. Cells were washed twice with 0.6 M KCl in distilled water, resuspended in 100 ml of 0.6 M KCl containing 0.5 mg of Zymolyase 20T per ml, and incubated at 28°C with gentle agitation. Under these conditions, protoplast formation (which was checked microscopically) usually occurred within 60 to 90 min. Protoplasts were sedimented at 3,000 × g for 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, spheroplasts were washed twice with 0.6 M KCl plus 3 mM PMSF (phenylmethylsulfonylfluoride), and resuspended in 10 ml of PB containing 1 mM CaCl₂, 1 mM MgCl₂, 3 mM PMSF, and 200 mM N-octylglucoside. Mild detergent agents such as octylglucoside or octylthioglucoside have been used to solubilize surface adhesion receptors from animal cells (36). In this work, N-octylglucoside was used to solubilize any existing transmembranal plasma membrane proteins that may have had domains within the cell wall structure. Once resuspended in the solution described above, protoplasts were lysed by being stirred in a Vortex mixer. The homogenate thus 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 a small volume of PB and were also stored at −30°C. The experimental protocol used to generate the different extracts described above is summarized in Fig. 1. The different samples (the βME and Zymolyase extracts and the lysate) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then blotted to nitrocellulose paper as described below. Prior to the performance of SDS-PAGE, radioactivity in the extracts was determined in duplicate 10-μl samples adsorbed to glass fiber filters (no. 8; Schleicher & Schuell). Filters were air dried at 70 to 90°C and placed in toluene-based scintillation fluid for dried samples. Measurements were made in a Beckman LS-7500 liquid scintillation counter. The total sugar and protein contents in the different samples were determined by the methods of Dubois et al. (14) and Lowry et al. (27), respectively, with glucose and bovine serum albumin as standards.

SDS-PAGE and Western blotting techniques. SDS-PAGE was performed basically as described by Laemmli (23), using slab gradient gels (5 to 15%; ratio of acrylamide to bisacrylamide, 30:0.2) with 3.5 to 4% polyacrylamide stacking gels. Samples were prepared for electrophoresis by the procedure reported previously (8). After electrophoresis, the gels either were stained for protein with Coomassie blue or were treated with Amplify (Amersham), dried, and exposed for adequate periods of time on Kodak X-Omat film at −70°C for fluorographic detection of radioactively labelled polypeptides.

Electrophoretic transfer (Western blotting) of proteins from polyacrylamide gels to nitrocellulose paper (Bio-Rad) was carried out as described by Burnette (2), except that the transfer buffer used contained 0.025 M Tris–0.192 M glycine (pH 8.3)–20% (vol/vol) methanol, and the electrophoretic transfer was carried out at 6 to 8 V/cm for 15 to 17 h at 4°C in a Trans-Blot cell (Bio-Rad) with an LKB 2197 power supply. Indirect concanavalin A (ConA)-mediated peroxidase staining of mannoproteins in nitrocellulose blots was
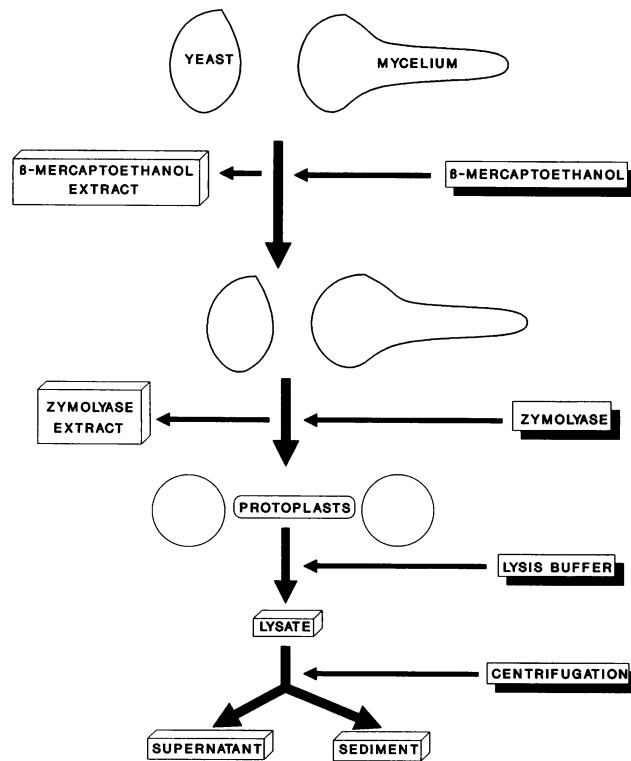
FIG. 1. Schematic diagram summarizing the experimental procedure used to solubilize protein and glycoprotein components from the cell walls of both growth forms of *C. albicans*.

TABLE 1. Biotinylated proteins and glycoproteins solubilized with βME from blastoconidia and germinated blastoconidia

Band no.[a]	Apparent molecular mass (kDa)[b]	Presence in cell wall of[c]:		Reactivity with ConA[d]
		Blastoconidia	Germinated blastoconidia	
1	>650	y	y	+
2	220	y	n	+
3	170	y	y*	+
4	140	y	y*	+
5	124	y	n	+
6	100	y	n	+
7	78	y	y	−
8	72	y	y	+
9	58	y	y	+
10	43	y	n	−
11	33	y	y	−
12	550	y*	y	+
13	400	y*	y	+
14	80	n	y	−
15	42	n	y	−
16	36	n	y	−
17	30	n	y	−
18	28	y*	y	−
19	23	y*	y	−
20	16	y*	y	−
21	13	n	y	−

[a] See Fig. 2.

[b] Calculated by linear regression. In the case of broad bands (i.e., bands 12 and 13), apparent molecular masses are given as average values.

[c] Common components strictly indicate molecular species that exhibited the same electrophoretic mobility. Some of the common components have a weaker biotin label (*) than their counterparts. y, present; n, not present.

[d] As detected by Western blotting (see Fig. 5).

conducted by the method of Hawkes (20) with the modifications of Millette and Scott (31).

Biotinylated proteins transferred to nitrocellulose paper were detected as follows. The nitrocellulose membranes were blocked with 3% bovine serum albumin (BSA) in 10 mM Tris hydrochloride buffer (pH 7.4) containing 0.9% NaCl (TBS) for 1 h at 37°C. After being washed once with TBS containing 0.05% Tween 20 (TBST), the nitrocellulose sheets were incubated with Extravidin-peroxidase conjugate (Sigma) at a 1:3,000 dilution in TBST plus 1% BSA. After incubation for 1 h at room temperature with agitation, blots were washed four times with TBST, and reactive bands were developed with hydrogen peroxide and with 4-chloro-1-naphtol as the chromogenic reagent.

Immunochemical detection of proteins on blots was performed by following the protocol described in the Bio-Rad Immun-Blot (GAR-HRP) assay kit, which is based on the procedures of Burnette (2) and Towbin et al. (42). A polyclonal rabbit serum enriched in antibodies against the overall mannoprotein composition of purified mycelial walls and a mouse monoclonal antibody (MAb 4C12) raised against a mannoprotein band with an average apparent molecular weight of 260 kDa specific for the cell wall of the mycelial form of *C. albicans* ATCC 26555 were prepared as previously reported (8) and used as antibody probes. Antibodies were used at a final concentration of 1:500 (for polyclonal antibodies), or 1:3,000 (for monoclonal antibodies, in 0.01 M Tris hydrochloride buffer, pH 7.4, containing 3% (wt/vol) BSA as a blocking agent, 0.9% NaCl, and 0.05% Tween 20. Diluted (1:2,000) peroxidase-labelled goat anti-rabbit or goat anti-mouse immunoglobulin G (Bio-Rad) was used as an indicator antibody. Colored reactive bands were developed as described above.

Chemicals. Gel electrophoresis and blotting reagents were obtained from Bio-Rad. Zymolyase 20T was obtained from Miles Laboratories. ^{14}C-labelled protein hydrolysate was obtained from Amersham International. Prestained molecular mass markers were obtained from Bio-Rad and Sigma Chemical Co. Unless otherwise indicated, all other chemicals used were purchased from Sigma Chemical Co.

RESULTS

Proteins identified by reaction with Extravidin-peroxidase. Biotinylated protein and glycoprotein components were extracted from the cell walls of both intact blastoconidia and germinated blastoconidia by βME treatment. When separated by SDS-PAGE under reducing conditions on 5 to 15% acrylamide slab gradient gels and analyzed by Western blotting with Extravidin-peroxidase conjugate, βME extracts from blastoconidia (Table 1 and Fig. 2, lane 1) and germinated blastoconidia (Table 1 and Fig. 2, lane 2) were seen to contain about 21 major polypeptide chains with molecular masses ranging from >650 to 13 kDa. Although several bands appeared to be common to both populations of βME-released species, possible form-specific constituents were detected. Thus, high- and medium-molecular-mass (from 220 to 43 kDa) species were found to be yeast form-specific moieties (Fig. 2, bands 2, 5, 6, and 10, and Table 1), whereas mycelium-specific components were observed preferentially in the medium- to low-molecular-mass range (from 82 to 13 kDa) (Fig. 2, bands 14, 15, 16, 17, and 21, and Table 1). Some common bands appeared to be
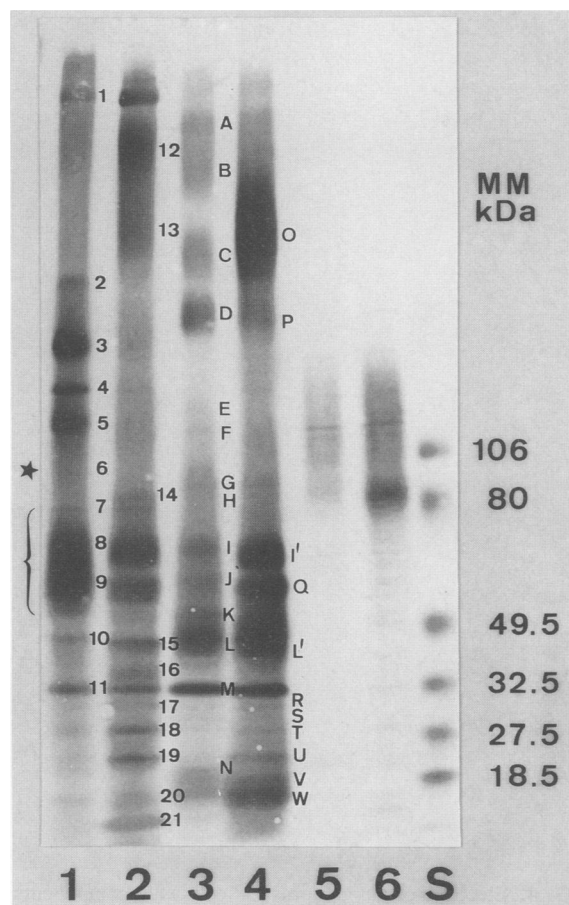
FIG. 2. Electrophoretic patterns of biotinylated polypeptides released from the cell surface of blastoconidia (lanes 1 and 3) or germinated blastoconidia (lanes 2 and 4) by βME treatment (lanes 1 and 2 [containing 100 μg of material expressed as total sugar content per well]) and Zymolyase digestion (lanes 3 and 4 [containing 300 μg of material, also expressed as total sugar content per well]). Samples of protoplast homogenates from blastoconidia and germinated blastoconidia were run in lanes 5 and 6, respectively (containing 20 μg of total protein per well). Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with the Extravidin-peroxidase conjugate. Lane S shows a mixture of prestained molecular weight standards run in parallel. Molecular masses (in kilodaltons) are indicated. Letters and numbers are used to identify the most relevant bands (see text).

TABLE 2. Biotinylated proteins and glycoproteins solubilized with Zymolyase from blastoconidia and germinated blastoconidia

Band designation[a]	Apparent molecular mass (kDa)[b]	Presence in the cell wall of[c]:		Reactivity with ConA[d]
		Blastoconidia	Germinated blastoconidia	
A	650	y	y*	+
B	500	y	?	+
C	340	y	?	+
D	200	y	?	+
E	140	y	?	−
F	125	y*	y	+
G	90	y	y	?
H	84	y	y	+
I	68	y	y	?
I'	66–67	n	y	+
J	58	y	n	+
K	52	y	n	+
L	45	y	y	+
L'	43–44	n	y	+
M	33	y	y	+
N	18	y	n	−
O	260	n	y	+
P	180	n	y	+
Q	56	n	y	+
R	30	n	y	−
S	29	n	y	−
T	28	n	y	−
U	22	n	y	+
V	18	y*	y	+
W	16	n	y	−

[a] See Fig. 2.
[b] See Table 1, footnote b.
[c] See Table 1, footnote c. ? is used to indicate that it was not possible to establish clearly the presence of a particular band in both patterns.
[d] See Table 1, footnote d. ? is used to indicate an unclear response of a particular band towards ConA.

quantitatively more important (as suggested by the intensity of the biotin-avidin complexes developed on Western blots) in the βME extracts from blastoconidia (bands 3 and 4) or germinated blastoconidia (Fig. 2, bands 12, 13, 18, 19, and 20; compare lane 1 with lane 2), yet a distinct accessibility of biotin to target molecules during in vivo labelling of cells may account for the quantitative differences observed in vitro.

Biotinylated proteins solubilized with Zymolyase after treatment with βME from both yeast (Fig. 2, lane 3) and mycelial forms (Fig. 2, lane 4) also represented a complex array of high- to low-molecular-mass bands (from 650 to 13 kDa). In addition to species common to both populations of Zymolyase-released molecules, form-specific constituents were also detected (Fig. 2, lanes 3 and 4, and Table 2). Low-molecular-mass species (from 32 to 15 kDa) appeared

to be present preferentially in the extracts from germinated blastoconidia (compare lane 4 with lane 3 in Fig. 2), although the possibility that these species represent cleavage products of large cell wall components because of the contaminating proteolytic activity present in the commercial Zymolyase preparation (16, 19) cannot be dismissed. Patterns of βME- (Fig. 2, lanes 1 and 2) and Zymolyase-released (Fig. 2, lanes 3 and 4) materials were markedly different (especially in the high-molecular-mass range, i.e., >120 kDa). This represents additional evidence in support of the notion that the *C. albicans* cell wall contains form-specific protein components.

Western blot analysis of protoplast homogenates (see Materials and Methods) with Extravidin-peroxidase conjugate (Fig. 1) revealed only two major bands, of 80 and 115 kDa, along with two or three minor components in the case of the sample corresponding to germinated blastoconidia (Fig. 2, lane 6), whereas the 80-kDa species was not detected in homogenates from yeast spheroplasts (Fig. 2, lane 5). These biotinylated moieties may represent transmembranal plasma membrane proteins with domains within the cell wall structure (see below).

Proteins identified by fluorography. Double-labelled (biotinylated and ¹⁴C-labelled) molecules (see Materials and Methods) characterized on Western blots as described above were analyzed in parallel by fluorography.

A comparison of patterns of biotinylated and radiolabelled proteins revealed that all components detected with the
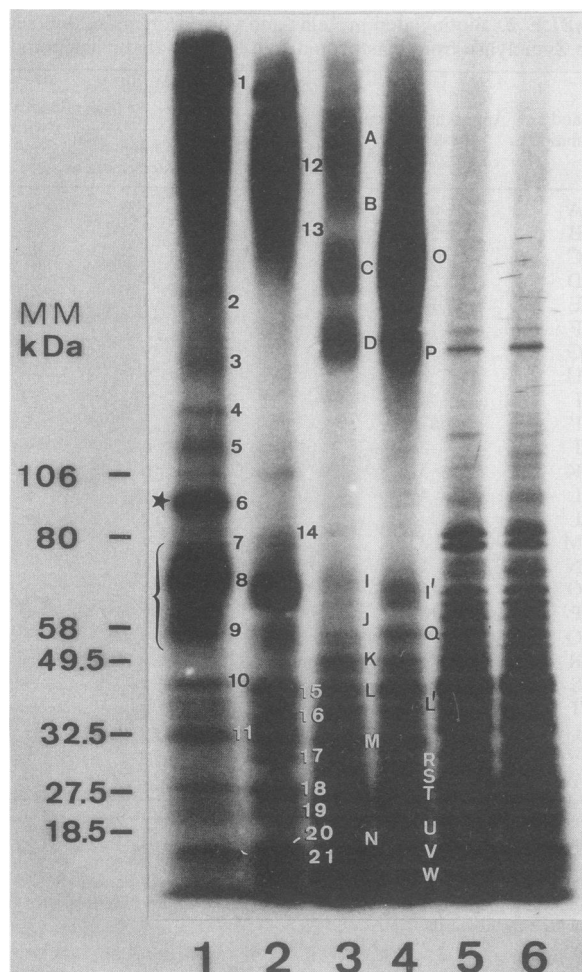
FIG. 3. Fluorography (10,000 cpm per well) of ^{14}C-labelled proteins present in the materials released by βME (lanes 1 and 2) and Zymolyase (lanes 3 and 4) from the cell surface of blastoconidia (lanes 1 and 3) or blastoconidia bearing germ tubes (lanes 2 and 4). Samples of protoplast homogenates from blastoconidia and germinated blastoconidia were run in lanes 5 and 6, respectively. Molecular masses (in kDa) of standard proteins are given on the left. Letters and numbers are used to identify relevant bands (see text).
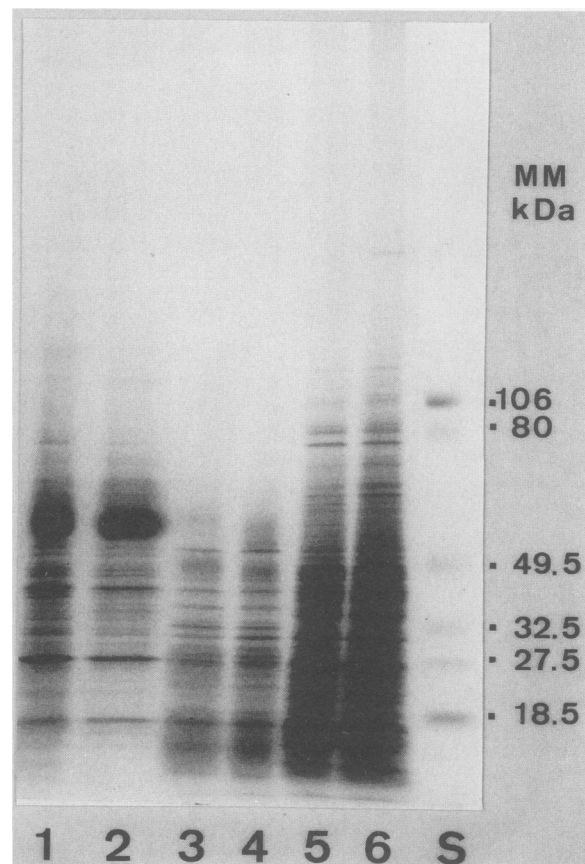

FIG. 4. Electrophoretic patterns of cell wall components released by βME treatment (βME extract) (lanes 1 and 2 [containing 200 μg of material expressed as total sugar content per well]) and Zymolyase digestion (Zymolyase extract) (lanes 3 and 4 [containing 600 μg of material, also expressed as total sugar content per well]) of blastoconidia (lanes 1 and 3) and germinated blastoconidia (lanes 2 and 4). After electrophoresis, polypeptides were revealed by Coomassie blue staining. Samples (40 μg of protein) of blastoconidia (lane 5) and blastoconidia bearing germ tubes (lane 6) protoplast homogenates (lysate; see Materials and Methods and Fig. 1) were also analyzed. Lane S shows a mixture of prestained molecular weight standards run in parallel. Molecular masses (MM) are indicated in kilodaltons.

Extravidin-peroxidase conjugate on Western blots (Fig. 2) were also observed in the fluorograms (Fig. 3), yet resolution of individual bands (those reactive with NHS-biotin), especially in the high-molecular-mass range, appears to be better in Western blots (Fig. 2). It has to be stressed that the X-ray films were overexposed so as to detect weakly radioactive labelled bands. Several weakly biotinylated bands (i.e., Fig. 2, band 6 [star]), however, exhibited a strong radioactive label (Fig. 3). Such bands may reflect the existence of (i) minor cell wall components with a fast metabolic turnover, (ii) proteins with low affinity for biotin, (iii) proteins that are located deep within the cell wall structure or are masked by other cell wall components (both possibilities would hamper accessibility of biotin to target molecules), or (iv) cytosolic proteins, as it has been suggested that treatment of intact C. albicans cells with reducing agents such as dithiothreitol or βME may release some intracellular macromolecular components (4).

Fluorographic analysis of protoplast homogenates re-

vealed a very complex array of polypeptide species, with molecular masses ranging from 180 to 13 kDa (Fig. 3, lanes 5 and 6), yet polydisperse high-molecular-mass species (Fig. 2 and 3, lanes 1 to 4, bands 1, 2, A, B, C, D, and O) were not detected in these samples (Fig. 3, lanes 5 and 6). Protoplast homogenates were found to have an identical qualitative and quantitative polypeptide composition (Fig. 3 and 4, lanes 5 and 6) regardless of the growth form of C. albicans considered. Most of these polypeptides may be cytosolic (intracellular) components, as they did not exhibit any detectable biotin label (Fig. 2, lanes 5 and 6), yet some of the bands observed by fluorography of the cell homogenates (Fig. 3) or by Coomassie blue staining (Fig. 4) could represent transmembranal plasma membrane proteins with cell wall-related domains, which are only detectable in this work by the biotin-avidin technique.

Proteins identified by reaction with Coomassie blue, ConA, and polyclonal and monoclonal antibodies. Coomassie blue staining allowed detection of the medium- to low-molecular-
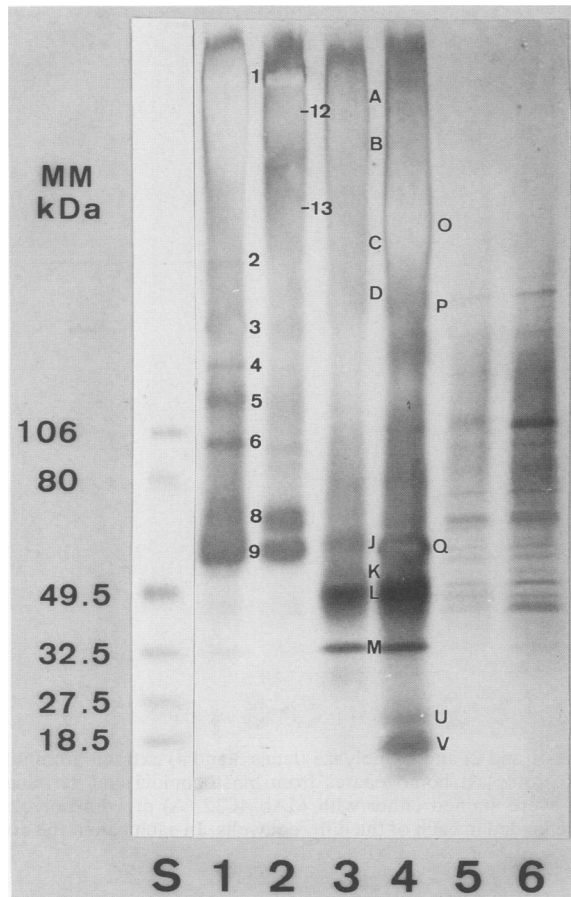
FIG. 5. Characterization of glycoproteins released by βME (lanes 1 and 2 [containing 100 μg of material expressed as total sugar content per well]) and Zymolyase digestion (lanes 3 and 4 [containing 300 μg of material, also expressed as total sugar content per well]) from the cell surface of blastoconidia (lanes 1 and 3) or germinated blastoconidia (lanes 2 and 4). Samples of protoplast homogenates from blastoconidia and germinated blastoconidia were run in lanes 5 and 6, respectively (containing 20 μg of total protein per well). Glycoproteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with peroxidase-conjugated ConA. Lane S shows a mixture of prestained molecular weight standards run in parallel. Molecular masses (in kDa) are indicated. Letters and numbers are used to identify the most relevant bands (see text).

mass proteins present in the different samples (Fig. 4), although the high-molecular-mass species (>120 kDa) present in the extracts were not sensitive to the dye. Indirect ConA-peroxidase staining of nitrocellulose blots revealed the mannoprotein nature of the high-molecular-weight bands (high-molecular-weight-mannoproteins; HMWM [8]), yet detection with the lectin led to the appearance of poorly stained, poorly resolved patterns (Fig. 5, lanes 1 to 4) because of the high carbohydrate content in these molecules and the polydisperse nature of their mannan moieties (15). It must be stressed here that the almost negligible reactivity of HMWM species with ConA on nitrocellulose blots (i.e., bands 12, 13, A, B, C, D, O, and P in Fig. 5) occurs when the extracts subjected to electrophoresis and subsequent blotting contain large amounts of these mannoprotein moieties. In this case, the bulk of the blotted HMWM species (>160 to 180 kDa) does not exhibited detectable reactivity against the

lectin, and only a weak peripheral colored reaction is usually observed. Some of the medium-molecular-mass mannoprotein species (i.e., band Q in Fig. 5) also exhibited this peripheral staining pattern with the ConA-peroxidase technique. Nevertheless, experimental evidence (obtained by using very small amounts of sample in SDS-PAGE and in the lectin-blotting analysis) have revealed the mannoprotein nature of the high-molecular-mass species solubilized by treatment with βME and Zymolyase digestion. In this context, bands A, B, C, D, O, and P (Fig. 3 and 5 and Table 2) correspond to previously identified yeast- and germ tube-specific HMWM antigens (8, 17, 19, 29). On the other hand, the high carbohydrate content of HMWM species may account for the absence of reactivity of HMWM following Coomassie blue staining (Fig. 4). Most of the medium- and low-molecular-mass species (<70 kDa) showed no reactivity with the lectin (Fig. 5, lanes 1 to 4), suggesting that these species contain no mannose polymers.

The reactivities of proteins on blots with polyclonal and monoclonal antibodies against different cell wall mannoprotein antigens of *C. albicans* ATCC 26555 (8) were not apparently affected by the in vivo biotinylation process to which fungal cells were subjected. The monoclonal antibody (MAb 4C12) tested in this work was raised against a mycelium-specific cell wall mannoprotein band with an apparent molecular mass of 260 kDa (8) present in Zymolyase extracts (Fig. 6A, lane 4). It recognizes an antigenic determinant present in the polypeptide moiety of a mycelium-specific cell wall HMWM species whose apparent molecular mass may vary depending on the extraction procedure employed (8, 16, 28). Accordingly, MAb 4C12 reacted exclusively with high-molecular-mass polydisperse antigens present in the βME-released material from germinated blastoconidia (Fig. 6A, lane 2). The electrophoretic mobility of the proteinaceous species containing the immunodeterminant recognized by the monoclonal antibody is very low in this case, resulting in an apparent molecular mass of >650 kDa (Fig. 6A, lane 2). The polyclonal antibody used as a probe to detect antigens on blots was enriched in antibodies against the overall mannoprotein composition of purified mycelial cell walls (8). The pattern of reactive bands detected in the material released with Zymolyase from intact germinated blastoconidia (Fig. 6B, lane 4) was found to be basically similar to that previously reported for Zymolyase digests of isolated cell walls from mycelial cells (8, 29). Some HMWM released with βME from germinated blastoconidia (Fig. 6B, lane 2) along with mannoprotein species solubilized from yeast cells with βME (Fig. 6B, lane 1) and with Zymolyase (Fig. 6B, lane 3) also exhibited reactivity with the mPAb preparation. It has to be stressed that the polyclonal antibody, which was prepared by repeated absorption of an antiserum raised against mycelial cell walls with heat-killed blastoconidia, still contained antibodies against yeast-specific cell wall components (8). This may account for the cross-reactivity of mPAb with antigens present in the βME and Zymolyase extracts obtained from blastoconidia. In any case, immunodetection with MAb 4C12 and mPAb represents additional evidence in support of the notion that proteins and glycoproteins released by βME and Zymolyase from intact cells of both morphologic phases of *C. albicans* are genuine cell wall-related components, since no reactive bands were observed by Western blot analysis of protoplast homogenates using both antiserum preparations as probes (Fig. 6A and B, lanes 5 and 6).
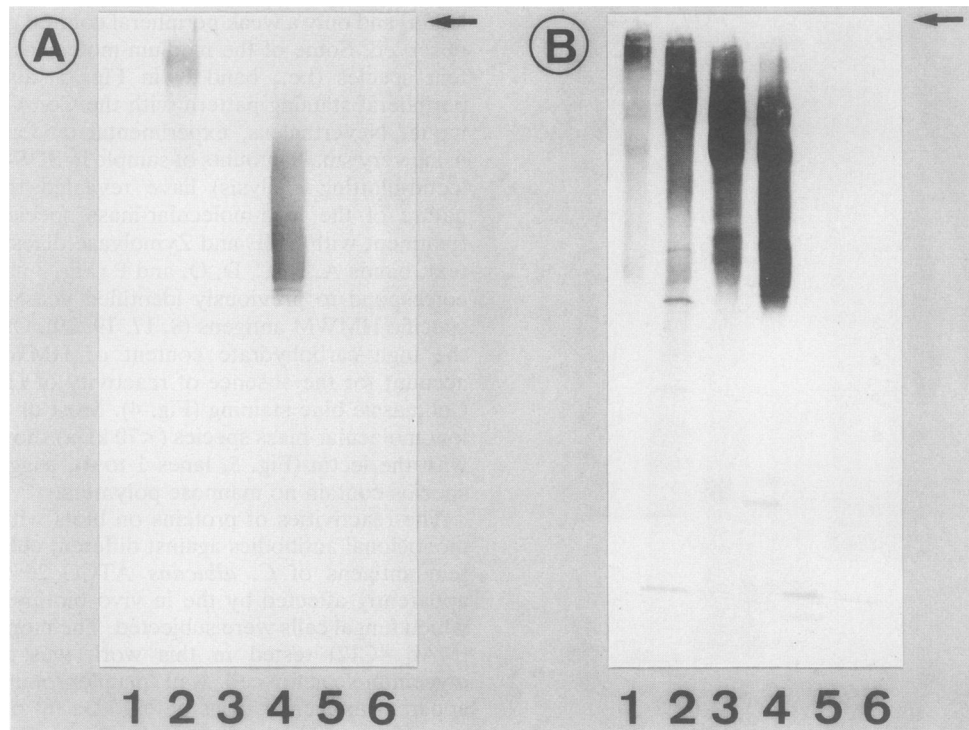
FIG. 6. Western blots of 5 to 15% slab gradient gels loaded with βME (lanes 1 and 2) and Zymolyase (lanes 3 and 4) extracts from intact blastoconidia (lanes 1 and 3) or mycelial cells (lanes 2 and 4). Samples of protoplast homogenates from blastoconidia and germinated blastoconidia were run in lanes 5 and 6, respectively. Nitrocellulose sheets were stained either with MAb 4C12 (A) or with polyclonal antibodies (B). See the legend to Fig. 2 for information on the amount of sample loaded in each of the different wells. In each panel, the arrow indicates the upper edge (origin) of the resolving gel.

DISCUSSION

The results presented in this study show that labelling of intact, living *C. albicans* cells with biotin, followed by treatment of whole cells with βME and subsequent digestion with Zymolyase, allows characterization of autochthonous cell-surface (cell wall)-related proteins and glycoproteins following SDS-PAGE and Western blot analyses of the βME and Zymolyase extracts. A complex array of proteins was labelled in both growth forms of the fungus, supporting the notion that the cell wall of *C. albicans* contains numerous protein constituents, and, as already reported in work from several authors using other techniques, labelling with biotin also demonstrated qualitative and quantitative differences in the cell wall proteins of yeast-form cells and germinated blastoconidia (6–8, 10, 12, 15, 17, 26, 28, 34, 35, 41).

It is possible that structural changes occurring in the cell wall structure of *C. albicans* during germination or other factors (i.e., expression of new form-specific gene products with low affinities for biotin) may alter or modulate the accessibility and/or the reactivity to the labelling reagent. Either possibility may account for the qualitative and quantitative differences observed in the electrophoretic patterns. In any case, several similarities that can be established between the cell wall protein and glycoprotein species detected in this report and cell wall components described in work by other authors support the contention that the biotin conjugation technique is a suitable, high-resolution, reproducible technique for the labeling of *C. albicans* cell wall proteins.

Thus, in the medium- to low-molecular-mass range (from 78 to 33 kDa), a cluster of bands common to both growth forms (bands 7, 8, and 9) was detected in the βME extracts (Fig. 2 and 3, lanes 1 and 2 [bracket]). Some of these species were found to be glycoproteins (Fig. 5, lanes 1 and 2). Several cell wall-bound proteins and glycoproteins within a molecular mass range from 68 to 60 kDa may act as candidal surface receptors for laminin (1) and complement (C3d) (5, 25, 38) and appear to play an important role in the adherence of *C. albicans* cells to plastic and to epithelial cells (18, 43). On the other hand, band 9 (Fig. 2, 3, and 5, and Table 1) has been previously identified as a major hydrophobic cell wall mannoprotein component (26).

Also in the medium- to low-molecular-mass range is a 33- to 34-kDa mannoprotein released from both growth forms by Zymolase treatment of intact cells (Fig. 2 and 5, lanes 3 and 4; band M). A mannoprotein with a similar molecular mass, but whose physiological role has not yet been established, was detected as a major component in Zymolyase digests of isolated, purified cell wall preparations obtained from different fungal species, including *C. albicans* (17, 19, 21, 22, 33, 37). On the other hand, the broad band (from 49 to 40 kDa) detected in the enzymic digests of germinated blastoconidia (Fig. 2, lane 4) seems to be formed by several overlapping mannoprotein species (Fig. 5, lane 4) that may include the 47- and 43-kDa germ tube-specific cell wall mannoprotein antigens of *C. albicans* that have been identified previously (10). Besides, some of the components with apparent molecular masses of <260 kDa expressed preferentially in myce-lial-phase extracts and recognized by the polyclonal antise-rum (mPAb [8]) used in this work (Fig. 6) could correspond to some of the antigenic species detected by other authors (35, 41).

Finally, Zymolyase extracts of blastoconidia also contained four major HMWM species (Fig. 2, lane 3, bands A, B, C, and D), whereas the enzymic digests obtained from germinated blastoconidia were found to contain only two major HMWM components (Fig. 2, lane 4, bands O and P). These bands may correspond to previously identified form-specific mannoprotein antigens released by Zymolyase from purified preparations of yeast and mycelial cell walls (8, 17) that may play an important role in the regulation of cell wall morphogenesis (9, 16, 19, 28–30, 39). On the other hand, among the HMWM species released with βME, band 3 (Fig. 2), which appears to be a major mannoprotein (Fig. 5) component in yeast cell walls, has an apparent molecular mass (170 kDa) similar to that of the recently characterized 165-kDa glycoprotein that may be related to an adhesin function specific for *C. albicans* yeast cells, along with other glycoprotein species with lower apparent molecular masses (18).

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