Arch Microbiol (1995) 164 : 186–193

ORIGINAL PAPER

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Involvement of transglutaminase in the formation of covalent cross-links in the cell wall of *Candida albicans*

Received: 30 January 1995 / Accepted: 11 April 1995

Abstract Activity of the enzyme glutaminyl-peptide-γ-glutamylyl-transferase (EC 2.3.2.13; transglutaminase), which forms the interpeptidic cross-link N^{ε}-(γ-glutamic)-lysine, was demonstrated in cell-free extracts obtained from both the yeast like and mycelial forms of *Candida albicans*. Higher levels of enzymatic activity were observed in the cell wall fraction, whereas the cytosol contained only trace amounts of activity. Cystamine, a highly specific inhibitor of the enzyme, was used to analyze a possible role of transglutaminase in the organization of the cell wall structure of the fungus. Cystamine delayed protoplast regeneration and inhibited the yeast-to-mycelium transition and the incorporation of proteins into the cell wall. The incorporation of covalently bound high-molecular-weight proteins into the wall was sensitive to cystamine. Proteic epitopes recognized by two monoclonal antibodies, one of which is specific for the mycelial walls of the fungus, were also sensitive to cystamine. These data suggest that transglutaminase may be involved in the formation of covalent bonds between different cell wall proteins during the final assembly of the mature cell wall.

Key words Transglutaminase · Cell wall · *Candida albicans* · Wall epitopes · Covalent bonds · Protein interactions · Protoplasts · Dimorphism

Abbreviation MAb · Monoclonal antibody

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Introduction

The cell walls of *Candida albicans* and other fungi are complex structures composed mainly of β-glucans and mannoproteins, together with minor amounts of chitin. The β-glucans show different degrees of solubility in alkali and/or acid, depending upon the amounts of β-1,3 and β-1,6 linkages and branching points. Wall mannoproteins are responsible for this as well as for structural functions of the major immunological differences between yeast and mycelial walls of *C. albicans*. Chitin is an important component of the cell walls because the insolubility of the other components depends on their interaction with it.

The idea that covalent links help to maintain the organization of the fungal cell walls has been discussed for some time (for revision of wall structure see: Wessels 1986; Ruiz-Herrera 1992; Sentandreu et al 1994). Accumulated evidence shows that a defined population of high-molecular-weight proteins is covalently linked to the structural polysaccharides of the cell wall in diverse fungi (Elorza et al. 1985; Herrero et al. 1987; Van Rinsum et al. 1991), and the study of these materials released from *S. cerevisiae* walls has permitted the identification of a novel type of carbohydrate side chain (Montijn et al. 1994). The existence of a covalent association between glucan and chitin has also been substantiated (Sietsma and Wessels 1979, 1981; Sonnenberg et al. 1982; Mol and Wessels 1987; Surarit et al. 1988; Hartland et al. 1994). Recently, using two monoclonal antibodies (MAb), one specific for mycelial cells (MAb 4C12; Elorza et al. 1989; Marcilla et al. 1991) and the other that immunoreacts with a mannoprotein found in both yeast and mycelial cells (MAb 1B12; Marcilla et al. 1993), the processes of incorporation of two defined proteins into the cell wall of *C. albicans* were studied. In both cases, the MAbs recognize *O*-glycosylated proteins in the protoplast supernatant fluids and immunoreact with high-molecular-weight mannoproteins having *O*- and *N*-linked sugars found in material released from cell walls. These results suggest that both *O*- and *N*-glycosylated mannoproteins are secreted as individual species, but are

released from the walls as part of supramolecular complexes with covalent bonds (Elorza et al. 1989; Marcilla et al. 1993).

In animal systems, an enzymatic activity that catalyzes the formation of covalent bonds between protein chains through the interaction of glutamine and lysine residues has been described. This enzyme (EC 2.3.2.13, glutaminyl-peptide-γ-glutamylyl transferase, or glutamylyl transferase, or transglutaminase) catalyzes a reaction involving the carboxamide group of a peptide-bound glutamine residue and the ε-amino group of a peptide-bound lysine residue. Formation of this interprotein linkage has been described in multiple animal cells, in lower eukaryotes including *Paramecium aurelia* and *Physarum polycephalum*, and in the prokaryote *Escherichia coli* (reviewed by Folk 1980; Loewy 1984). Based on our results and the wide distribution of the enzyme on the phylogenetic scale, we tested whether this enzymatic activity is involved in covalent bonding between different proteins of the fungal cell walls. Our results suggest that this enzyme is indeed present in *C. albicans*, where it plays an important role in cell wall organization.

Materials and methods

Strains and culture conditions

The strain used in this study, *Candida albicans* ATCC 26555, was maintained by periodic transfer on slants of Sabouraud agar (Difco, Detroit, Mich., USA). A loopful of cells was transferred into modified Lee's medium (Lee et al. 1975; Elorza et al. 1985). Cells were incubated with shaking at 28°C for 24 h, recovered by centrifugation, washed twice with sterile distilled water, and kept at 4°C for not less than 48 h. Adequate volumes were used to inoculate fresh medium and were incubated at 28°C to obtain yeast populations, or at 37°C to obtain mycelial cells, as described by Elorza et al. (1985).

Formation and regeneration of protoplasts

Cells grown in Lee-glucose medium for 24 h as described above were harvested by centrifugation and washed once with water. The number of cells was calculated by the turbidity of the cell suspension at 600 nm; this value was compared with a calibration curve. Cells were centrifuged (3000 × g, 10 min) and resuspended at a density of 10 μg (dry wt./ml) in a 10 mM sodium phosphate buffer (pH 8.0) containing 0.5 mg/ml pronase, 10 mM EDTA, and 0.1 M mercaptoethanol. The suspension was shaken at 30°C for 30 min and centrifuged. Cells were washed once with 0.6 M KCl, resuspended in twice the original volume of 0.6 M KCl containing 0.375 mg/ml Zymolyase 20T (Seikagaku Corporation, Tokyo, Japan), and shaken at 30°C for 30–45 min to obtain protoplasts as determined by their osmotic fragility. Protoplasts were centrifuged at low speed (1200 × g, 10 min) and carefully washed four times with 0.6 M KCl. Protoplasts were regenerated in Lee-glucose medium made hypertonic with 0.6 M KCl.

Staining with monoclonal antibodies

Cells were fixed with formaldehyde and aliquots were air-dried on glass slides, covered with an adequate dilution of monoclonal antibody (MAb), washed with phosphate-buffered saline, treated with goat anti-mouse IgG bound to fluorescein, washed, and examined by fluorescence microscopy (Casanova et al. 1989).

Measurement of amino acid incorporation into protein in whole cells

Aliquots of cultures that had been grown in the presence of radioactive amino acids were mixed with an equal volume of ice-cold 10% TCA and left on ice for not less than 1 h. Samples were heated in a boiling-water bath, filtered through glass fiber filters (2.5 cm in diameter), and washed twice with 5% TCA and twice with ethanol. Filters were dried, placed into scintillation vials containing toluene-based scintillation fluid, and their radioactivity was measured in a scintillation counter.

Isolation of cell walls and measurement of covalently and non-covalently bound proteins

Cells or protoplasts grown in the presence of [^{14}C]protein hydrolysate with or without 50 mM cystamine were harvested by centrifugation. They were washed twice with 50 mM phosphate buffer (pH 7.4). The pellet was mixed with approximately 4 g of glass beads (425–600 microns) per gram of cells and broken by agitating on a Vortex mixer for periods of 30 s with intermediate cooling periods of 1 min in an ice bath. Breakage was assessed by phase-contrast microscopy and the extracts were recovered by resuspension in the above buffer. Extracts were centrifuged at 1200 × g, and the sedimented walls and supernatant fluid were saved. Walls were washed twice with 50 mM phosphate buffer (pH 7.4), twice with 2 M NaCl, and twice with distilled water. Non-covalently bound proteins were extracted by heating for 10 min with 2% SDS in a boiling-water bath. Cell walls and solubilized material were separated by centrifugation at 3000 × g. Cell walls were washed twice with water, twice with ethanol, and twice with water. Wall glucan-bound proteins were released by incubation with Zymolyase 20T (1 mg/ml in water containing 1 mM phenylmethylsulfonyl fluoride) for 3 h at 30°C and separated by centrifugation as above. Walls were washed three times and treated with 0.5 mg/ml chitinase (Sigma, St. Louis, Mo., USA) in 10 mM phosphate buffer (pH 7.0) for 3 h at 30°C. Released proteins were separated from the final insoluble residue by centrifugation (3000 × g, 10 min). Aliquots were taken at each step and dried on glass-fiber filters to measure their radioactivity. Proteins extracted by SDS were precipitated at 4°C with 75% ethanol, centrifuged, and washed three times with ethanol.

SDS-PAGE, autoradiography and immunodetection

Samples were mixed with two volumes of solubilizing mixture (40% glycerol, 8% SDS, 20% mercaptoethanol), heated for 10 min in a boiling-water bath, and then subjected to electrophoresis in 10% polyacrylamyde gels using the discontinuous system of Laemmli (1970). Gels were stained with Coomassie blue. After being photographed, gels were immersed in Amplify solution (New England Nuclear, Beverly, Mass., USA), dried, and autoradiographed exposing to Kodak X-Omat S film at –70°C for various periods. Alternatively, wall proteins were electrophoretically transferred from the gel to nitrocellulose sheets and immunodetected by the method of Towbin et al. (1979), as modified by Burnette (1981) using monoclonal antibodies (MAb) 1B12 or 4Cl2. MAb 1B12 recognizes a wall protein epitope present in both the yeast and the mycelial cells of *C. albicans* (Marcilla et al. 1993); MAb 4C12 recognizes a wall protein epitope specific for the mycelial form of the fungus (Casanova et al. 1989; Elorza et al. 1989).

Quantitative determination of cell wall epitopes by ELISA

Cell wall epitopes were quantitatively determined using the method of Voller et al. (1980). The number of units was calculated as the inverse of the epitope dilution that produced 50% of the saturating antigen-antibody reaction.

Table 1 The distribution of tranglutaminase activity in cell-free extract fractions of *Candida albicans* mycelial cells. Transglutaminase total activity was determined by measuring the amount of [^{14}C]putrescine incorporated into TCA-precipitable material in presence or absence of 50 mM cystamine under the conditions described in Material and methods. The specific activity and relative distribution (%) of the transglutaminase were calculated from the total activity

Fraction	Specific activity [dpm h^{-1} (mg protein)$^{-1}$]	Total activity (dpm)	Relative activity (%)	Total activity (plus cystamine) (dpm)
Cell walls	4347	50312	58	200
Mixed membrane	1862	32119	37	122
Cytosol	147	3675	4	126

Preparation of cell-free extracts and determination of transglutaminase activity

Cell-free extracts were obtained by breaking cells with glass beads in a Braun cell homogenizer in 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 1 µg pepstatin A per ml. The extracts were centrifuged at 2000 × g to remove cell walls, and the supernatant fraction was centrifuged at 40 000 × g in a Beckman JA20 rotor to separate the membrane fraction (pellet) from the cytosol (supernatant). Transglutaminase reaction mixtures were in a final volume of 1 ml of the above described buffer, 2 mM $CaCl_2$, 2.5 µCi carrier-free [^{14}C] putrescine, and enzymatic fraction. Following 2–6 h of incubation at 30°C with occasional shaking, the reaction was stopped by addition of 2 ml 10% TCA. After at least 2 h on ice, samples were centrifuged and the pellet was resuspended in 5 ml 10% TCA, heated in a boiling-water bath for 10 min, and centrifuged. Sediments were washed twice with 5% TCA and twice with ethanol. They were then resuspended in 1.5 ml Solvable (NEN-Dupont, Boston, Mass., USA) and incubated overnight at 37°C to dissolve the protein. Ten milliliters of NEE-989 scintillation fluid (NEN-Dupont) was added and radioactivity was measured with a liquid scintillation counter.

Results and discussion

Determination of transglutaminase activity in cell-free extracts

In animal systems, where the role of the secondary covalent bonding among different proteins is well established (Folk 1980; Loewy 1984), enzyme activity has been measured by the incorporation of radioactive putrescine as a di-amino acid analogue into TCA-precipitable material (Simon and Green 1985; Piacentini et al. 1988; Coussons et al. 1991; Klein et al. 1992). We used a similar protocol to determine the presence of this enzymatic activity in *C. albicans*, except that a more severe washing procedure was employed to minimize nonspecific adsorption. Initial experiments were performed using a cell-free extract as the enzymatic source and demonstrated incorporation of a significant amount of [^{14}C]putrescine by the extracts in a time-dependent fashion. As shown in Table 1, most of the enzymatic activity was present in the cell wall, whereas only minimal activity was detected in the cytosol. In all cases, putrescine incorporation was inhibited by cystamine, a specific active site-directed transglutaminase inhibitor (Lorand and Conrad 1984; Barsigian et al. 1988). A similar enzyme distribution was demonstrated for the yeast form of the fungus (not shown). Mixing cell wall and membrane fractions had no inhibitory or stimulatory effects, but mixing cytosol with either membranes or walls had a synergistic effect that increased activity by 30–40% (Table 2) and suggests that the cytosol contains acceptor material for the transferase reaction. Activity was linearly dependent on time for 6 h and on the amount of enzymatic extract up to 0.5 mg of wall protein (data not shown).

Table 2 Synergistic effect of cytosol and particulate fractions in transglutaminase activity. The protocol was the same described for Table 1, but mixtures contained half the volume of each individual fraction. The theoretical incorporation was calculated by adding half of the total purtescine incorporation values of the respective individual fractions

Fractions	Putrescine incorporated (dpm)	Theoretical incorporation (dpm)	Stimulation (%)
Cell wall	20,002	––	––
Mixed membranes	12,854	––	––
Cytosol	1,471	––	––
Wall + membranes	16,440	16,428	0
Wall + cytosol	15,646	10,736	46
Membranes + cytosol	8,221	6,662	28

Effect of transglutaminase inhibition of protoplast regeneration and yeast-to-mycelium transition

To determine whether protein cross-linking through transglutaminase was an important reaction in the formation of a tighter net in the cell wall of *C. albicans*, we made use of cystamine, which has been described as a specific inhibitor that blocks transglutaminase reaction (Simon and Green 1985; Martinez et al. 1989). The addition of cystamine to protoplasts of the fungus delayed their regeneration for several hours. Quantitative data from several experiments demonstrated that under normal conditions, after 8 h of incubation, the number of regenerating protoplasts sensitive to osmotic shock was less than 20% of the original population, whereas in the presence of 50 mM cystamine, more than 50% of regenerated protoplasts remained sensitive to the osmotic shock. Interestingly, cystamine delayed yeast-to-mycelium transition of the fungus without severely affecting growth as measured by incorporation of radioactive amino acids into protein (Fig. 1). The effect of cystamine on cell morphology was also observed in solid media. Using the conditions described in Materials and methods, colonies of *C. albicans* with a

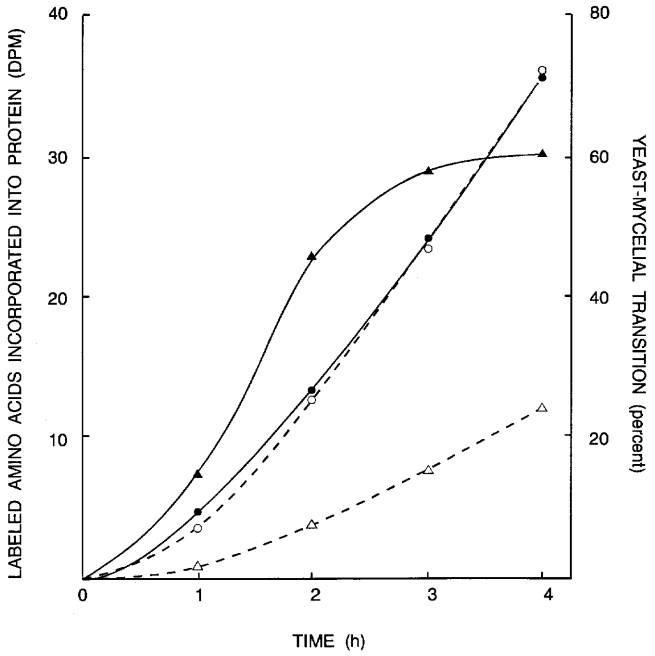


Fig. 1 The effect of cystamine on growth and dimorphism of *Candida albicans*. Starved cells maintained at 4°C were inoculated into Lee's medium containing [^{14}C]protein hydrolysate and casamino acids in the absence and presence of 50 mM cystamine. Cells were incubated at 37°C, and samples were withdrawn at intervals to measure incorporation of radiolabeled amino acids into TCA-insoluble material (*open and closed circles*), and yeast-mycelial conversion (*open and closed triangles*). *Open* symbols indicate samples with 50 mM cystamine; *closed* symbols indicate controls

rough, twisted surface formed. These colonies were made of mixtures of mycelial, pseudomycelial, and yeastlike cells. When cystamine was added to solid medium, growth was delayed, colonies appeared smooth, and only budding yeasts were observed.

Effect of cystamine on protein incorporation into cell walls

Because of the important role of the cell wall in morphogenetic phenomena, we turned our attention to the effect of cystamine on the incorporation of proteins into the wall. Cells were grown in Lee's medium containing [^{14}C] protein hydrolysate at either 28°C or 37°C to obtain the yeast or mycelial forms of the fungus, respectively, and [^{14}C]amino acid incorporation into cell walls was measured in the presence or absence of 50 mM cystamine (Table 3).

Non-covalently bound cell-wall proteins were extracted with SDS. Covalently bound cell-wall proteins resistant to this treatment were separated into two classes: proteins released by Zymolyase treatment, and those that remained associated with the wall after this treatment and were solubilized only by chitinase. An insoluble residue remained after the three treatments were carried out sequentially. Incorporation of covalently bound proteins into the wall was drastically reduced by cystamine; the incorporation of proteins from mycelial walls solubilized by chitinase was inhibited by more than 90% (Table 3). Since protein synthesis was not inhibited by cystamine, the remaining radiolabelled wall proteins were released into the incubation medium (M. Iranzo et al., unpublished results).

Effect of transglutaminase inhibition on the synthesis and incorporation of specific high-molecular-weight proteins

Material solubilized from the wall by either SDS or by Zymolyase treatment was subjected to SDS-PAGE, followed by autoradiography. The results obtained with Zymolyase-released material (Fig. 2) demonstrated that cystamine noticeably inhibited the incorporation of high-molecular-weight proteins (Fig. 2) into the mycelial cell wall (compare lanes 2 and 3 and lanes 6 and 7). Interestingly, a high-molecular-weight material that accumulated at the top of the stacking gel completely disappeared in the presence of cystamine (compare lanes 6 and 7). This type of high-molecular-weight material is usually a result of transglutaminase activity (Barsingan et al. 1988; Martinez et al. 1989). No differences were observed in the absence or presence of cystamine in the relative amounts of SDS-extractable proteins incorporated into walls, (Fig. 2; compare lanes 4 and 5 and lanes 8 and 9).

Further alterations induced by cystamine in the cell wall of *C. albicans* were noticed when mycelial cells were

Table 3 Inhibition of protein incorporation into the cell wall in *C. albicans* by cystamine (50 mm). *C. albicans* was grown as mycelial or yeast cells for 5 h in medium containing a [^{14}C]amino acid mixture in the presence or absence of 50 mM cystamine. Cell walls were obtained and sequentially treated with SDS, Zymolyase, and chitinase

Fraction	Mycelium protein			Yeast protein		
	Control (dpm)[a]	Cystamine (dpm)[a]	Inhibition (%)	Control (dpm)[a]	Cystamine (dpm)[a]	Inhibition (%)
Cell walls	258.5	116.2	55	145.8	115.1	21
Soluble in SDS	109.4	69.0	37	69.1	42.8	38
Zymolyase extract	5.2	25.9	53	41.0	35.3	14
Chitinase extract	27.3	2.5	91	13.7	10.4	24
Insoluble residue	22.1	1.0	95	4.9	2.3	53

[a] In thousands

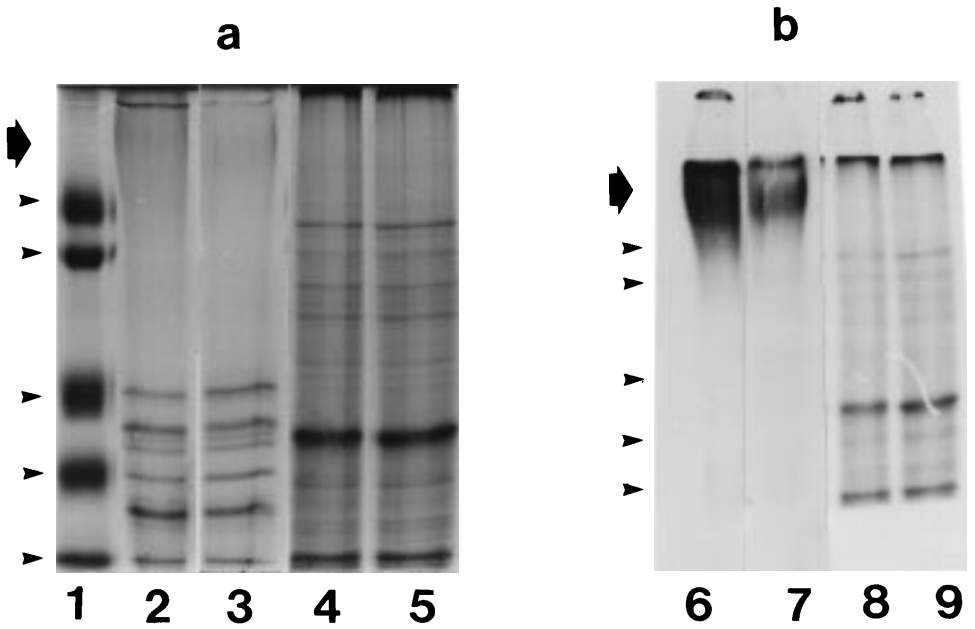


Fig. 2a, b The inhibition of high-molecular-weight protein incorporation into the cell wall by cystamine. Cell walls isolated from control mycelial cells or cells grown in the presence of 50 mM cystamine were sequentially extracted with SDS and Zymolyase. Equal amounts of released material (by radioactivity) were subjected to SDS-PAGE and the gels were stained with **a** Coomassie blue and **b** subjected to auto-radiography. *Lane 1* molecular weight standards; *lanes 2 and 6*, control Zymolyase-released proteins; *lanes 3 and 7*, cystamine Zymolyase-released proteins; *lanes 4 and 8*, control SDS-extracted proteins; *lanes 5 and 9*, cystamine SDS-extracted proteins. *Small arrows* indicate molecular weight standards (in descending order: 118, 84, 64, 48 and 36 kDa). *Large arrows* indicate high-molecular-weight material extracted by Zymolyase

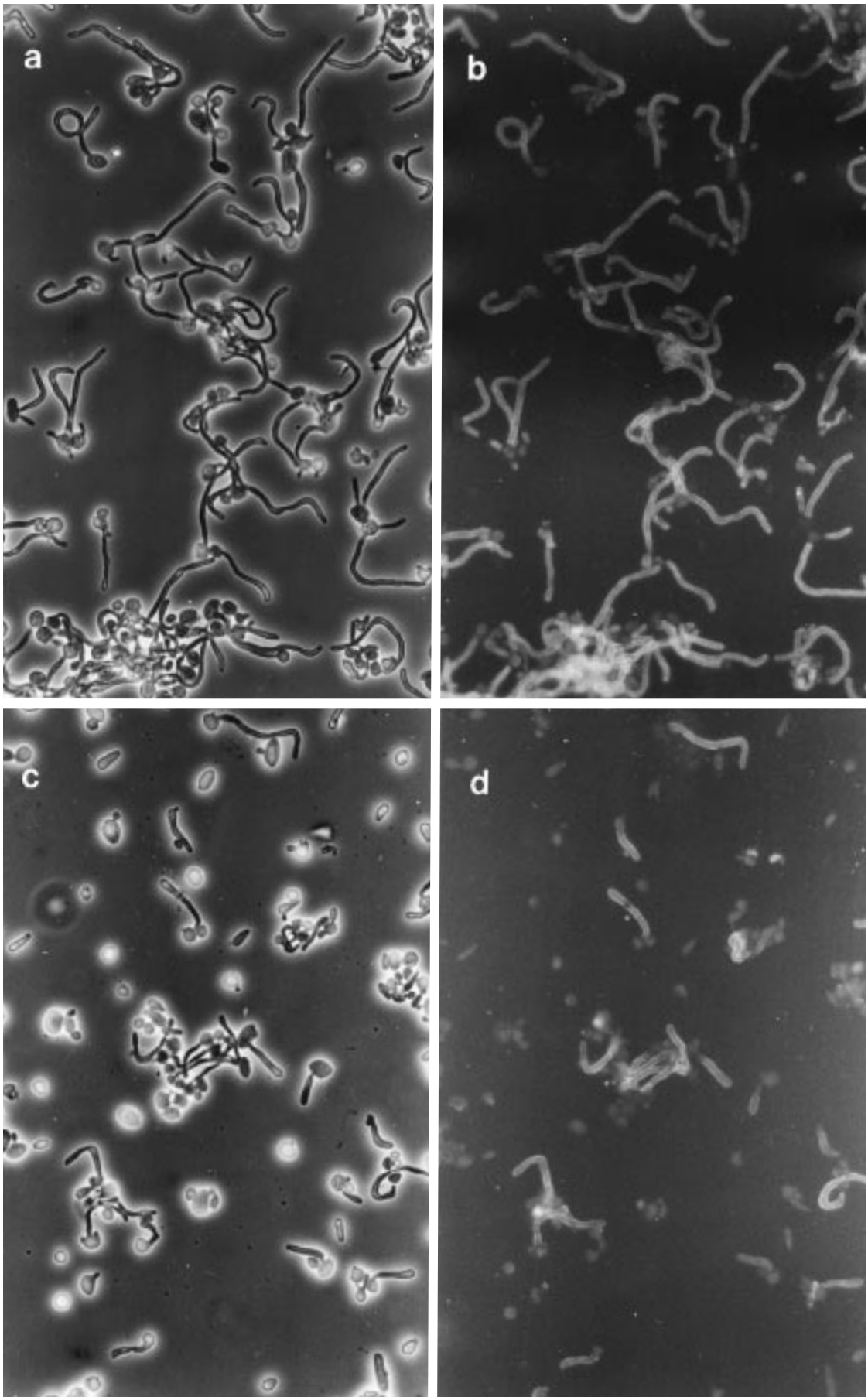
stained with the monoclonal antibody (MAb) 4C12. This antibody recognizes specifically a proteinaceous epitope present in the cell walls of the mycelium of the fungus (Casanova et al. 1989). Figure 3 shows the appearance of control cells observed under phase contrast (Fig. 3a) and cells stained by indirect immunofluorescence with MAb-4C12 (Fig. 3b). The appearance of cells grown in the presence of cystamine is shown in Fig. 3c (phase contrast) and Fig. 3d (indirect immunofluorescence). In contrast to the long hyphal cells of the control, cells grown in the presence of cystamine appear as dwarf mycelia, pseudomycelia, and budding yeasts.

More detailed studies were made by subjecting the material solubilized by SDS or released by Zymolyase to SDS-PAGE. In these studies we detected specific epitopes by immunoreaction with polyclonal or monoclonal antibodies. To load the same amount of protein per lane, cells were radioactively labeled with [^{14}C] protein hydrolysate. As observed previously by autoradiography and Coomassie blue staining, the patterns of SDS-extracted proteins detected with polyclonal antibodies directed against mycelial (Fig. 4a) or yeast (Fig. 4b) walls failed to reveal any differences between control and cystamine-treated cells (Fig. 4; compare lanes 4 and 5 and lanes 9 and 10). On the other hand, the incorporation of polydisperse high-molecular-weight wall proteins solubilized by Zymolyase and recognized by either set of antibodies was inhibited by cystamine (Fig. 4a). The most noticeable difference occurred in those proteins of very high-molecular-weight that remained in the stacking gel (Fig. 4, large arrows).

Similar results were obtained when monoclonal antibodies were used as probes. The incorporated amount of non-covalently bound MAb-1B12 epitope extracted by SDS was not reduced in mycelial cells grown in the presence of cystamine (Fig. 5a; compare lanes 4 and 5). On the other hand, incorporation of covalently bound epitopes present in mycelial cell walls was severely reduced by the cystamine (Fig. 5a; compare lanes 2 and 3). The incorporation of both covalent and non-covalent MAb-4C12 epitope into the walls was inhibited by growth in the presence of cystamine (Fig. 5b; lanes 7 and 8 and lanes 9 and 10, respectively).

Quantitative data of epitope release and incorporation into the cell walls were obtained during protoplast regeneration. Protoplasts were inoculated into hypertonic media in the absence or presence of cystamine and regenerated for 4 h at 37°C. Regenerating protoplasts were recovered, and nascent walls were isolated and extracted with SDS, followed by Zymolyase. The amounts of protein epitopes in the medium and in wall material released by SDS and Zymolyase were analyzed by ELISA using MAb 1B12 and 4C12. Epitope was extracted from the nascent walls by Zymolyase, but not by SDS. Results showed that cyst-

Fig. 3a–d The effect of cystamine on cell morphology and content of MAb 4C12 epitope. Cells grown in the absence or presence of 50 mM cystamine were stained with MAb 4C12 by indirect immunofluorescence and observed under the microscope. **a**, **c** Phase contrast; **b**, **d** immunofluorescence; **a**, **b** control cells; **c**, **d** cystamine-treated cells. Notice the low proportion of mycelial cells in **c** compared to the control (a) ▶


a
b
c
d

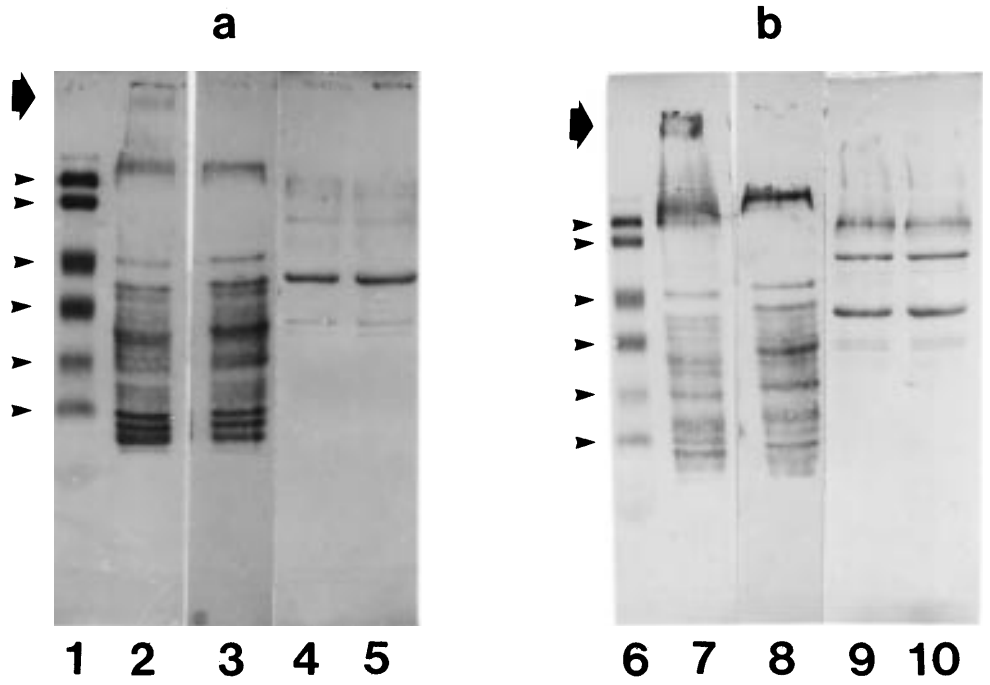


Fig. 4a, b Cystamine inhibition of incorporation of epitopes recognized by polyclonal antibodies. Equal amounts of SDS- or Zymolyase-released material from the walls of control and Zymolyase-treated mycelial cells were subjected to SDS-PAGE and immunoblotting. Gels were stained with polyclonal antibodies directed against **a** mycelial or **b** yeast cell walls of *Candida albicans*. *Lanes 1 and 6* protein standards; *lanes 2 and 7* control Zymolyase-released material; *lanes 3 and 8* cystamine-treated Zymolyase-released material; *lanes 4 and 9* control SDS-extracted material; *lanes 5 and 10* cystamine-treated SDS-extracted material. Notice the difference in high-molecular-weight material (especially that which barely entered the stacking gel, *large arrows*) between control and cystamine-treated cells. *Arrows* indicate protein molecular mass standards (in descending order: 205, 116, 97, 66, 45, and 29 kDa)

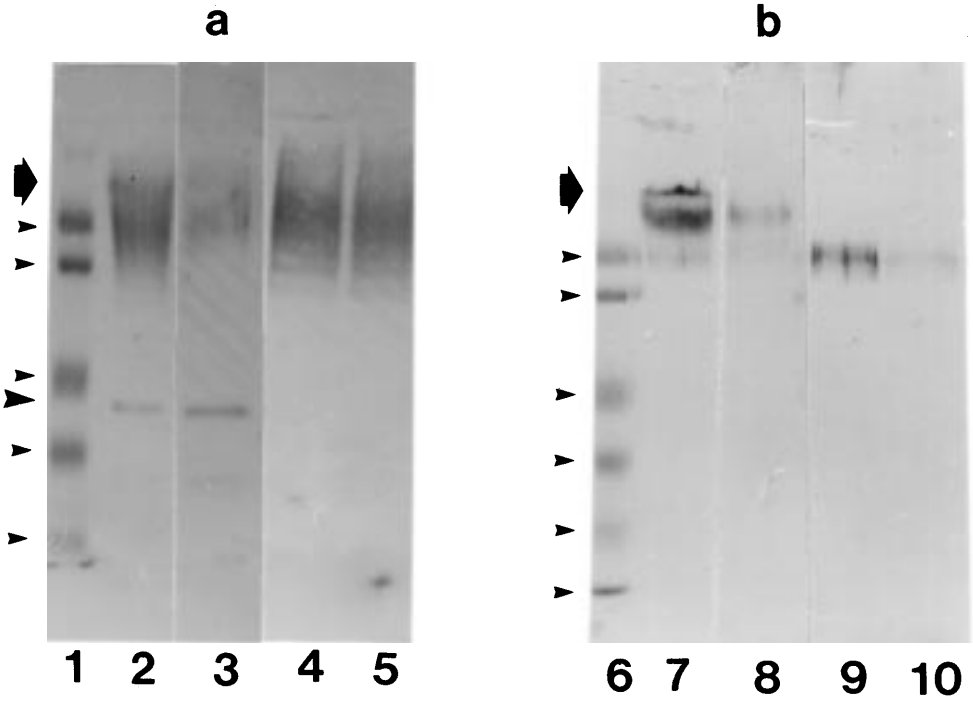


Fig. 5a, b The effect of cystamine on the incorporation of epitopes recognized by monoclonal antibodies. Material prepared from mycelial cells and separated by SDS-PAGE was blotted and stained with **a** MAb1B12 or **b** MAb4C12. *Lanes 1 and 6* protein standards; *lanes 2 and 7* control Zymolyase-released material; *lanes 3 and 8* cystamine Zymolyase-released material; *lanes 4 and 9* control SDS-extracted material; *lanes 5 and 10* cystamine SDS-extracted material. *Arrows* indicate protein molecular mass standard weight (in descending order: 205, 116, 97, 66, 45, and 29 kDa)

amine did not inhibit the release of either epitope into the regeneration media (Table 4); however, their incorporation into the nascent wall was almost completely inhibited, suggesting that the corresponding antigens become associated into the wall by means of establishing interprotein covalent bonds. From the data obtained in the present work, these covalent bonding reactions must be catalyzed at least in part by transglutaminase, suggesting that this activity is an important element in the structural organization of the cell wall of *C. albicans* and other fungi and

Table 4 Effect of cystamine on the secretion and wall incorporation of MAb epitopes by regenerating protoplasts. Protoplasts were regenerated in hypertonic media containing no cystamine or 50 mM cystamine for 4 h at 37°C. Cells and culture media were separated by centrifugation. Cell walls were obtained and extracted sequentially with SDS and Zymolyase. ELISA determinations were performed using the indicated fractions (in thousands)

Fraction	Epitope units MAb-1B12			Epitope units MAb-4C12		
	Control	Cystamine	Inhibition (%)	Control	Cystamine	Inhibition (%)
Medium	44.8	44.8	0	89.6	89.6	0
Zymolyase	2.6	0	100	10.2	0	100

probably establishes cross-links among structural glycoproteins.

Acknowledgments This work was partially supported by grants from CONACYT (Mexico), the Dirección General de Investigación Científica y Técnica (PB 93–0051, Spain), and the Fondo de Investigaciones Sanitarias (FIS 95/1602, Spain).

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