

Formation of a New Cell Wall by Protoplasts of *Candida albicans*: Effect of Papulacandin B, Tunicamycin and Nikkomycin

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Incorporation of polysaccharides into the walls of regenerating protoplasts of *Candida albicans* was followed in the presence of papulacandin B, tunicamycin and nikkomycin. With the first drug, chitin was incorporated normally whereas incorporation of glucans and mannoproteins was significantly decreased. Tunicamycin decreased incorporation of all wall polymers when added at the beginning of the regeneration process but blocked only mannan and alkali-insoluble glucan incorporation when added after 5 h. Nikkomycin inhibited chitin synthesis, and the walls formed by the protoplasts were enriched in alkali-soluble glucan. Pulse-chase experiments suggested that a precursor-product relationship between the alkali-soluble and alkali-insoluble glucans existed in the wall. The results obtained with the antibiotics were confirmed and extended by cytological studies using wheat-germ agglutinin labelled with colloidal gold and concanavalin A-ferritin as specific markers of chitin and mannoproteins respectively. The results support the idea that regeneration of walls by protoplasts occurs in two steps: firstly, a chitin microfibrillar skeleton is formed, and in a later step glucan-mannoprotein complexes are added to the growing structure. The chitin skeleton probably allows the orderly spatial arrangement of the other polymers giving rise to the regenerated cell wall.

INTRODUCTION

The walls of *Candida albicans* and other fungi are complex structures both chemically and structurally (Chattaway *et al.*, 1968; Cassone *et al.*, 1973; Poulain *et al.*, 1978). Chemically they are characterized by the presence of β -glucans, chitin and mannoproteins. Glucans are the main structural component of the walls, whereas chitin is found only in small amounts (Sullivan *et al.*, 1983). The proteins are grouped in two independent families; one of them (apparent molecular mass 150 kDa or larger) comprises molecules with large polymannose chains (mannan) linked through *N*-glycosidic bonds, probably connected covalently to the glucan network (Shibata *et al.*, 1983; Frevert & Ballou, 1984; Pastor *et al.*, 1984; Zlotnik *et al.*, 1984; Elorza *et al.*, 1985). The other family found in isolated walls comprises a 30 kDa mannoprotein possessing a single *N*-linked carbohydrate chain and several low molecular mass protein species (between 15 and 80 kDa) most of which lack *N*-glycosidic linkages: they are associated with the wall structure by non-covalent bonds and their physiological significance is unknown (Pastor *et al.*, 1984; Elorza *et al.*, 1985).

The architecture of the cell walls is not well defined (Farkaš, 1985), but it has been described as being a multilayered structure (Cassone *et al.*, 1979; Hilenski *et al.*, 1986) with a fine network of microfibrils, probably chitin, in its inner part (Tronchin *et al.*, 1981; Kopecká, 1984).

Localization of the mannoproteins in the walls has been investigated by the use of specific

Abbreviations: Au-WGA, wheat-germ agglutinin complexed with colloidal gold; Con A-fe, concanavalin A-ferritin; I-glucan, alkali-insoluble glucan; S-glucan, alkali-soluble glucan.

antibodies, lectin binding, extraction with solvents, scanning and transmission electron microscopy, and enzyme digestion. The results obtained suggest that they are concentrated on the external surface of the wall and next to the plasma membrane (Shepherd *et al.*, 1985). In normal growing cells, incorporation of covalently linked mannoproteins into the wall structure seems to require concomitant formation of glucan (Murgui *et al.*, 1985).

Various antibiotics have been reported to interfere with the normal formation of fungal walls: nikkomycins are inhibitors of chitin synthesis (Gooday *et al.*, 1985; Hector & Braun, 1986); papulacandin B interferes with formation of glucans (Baguley *et al.*, 1979; Kopecká, 1984; Pérez *et al.*, 1983); and tunicamycin is an inhibitor of the *N*-glycosylation of mannoproteins (Lehle & Tanner, 1976; Tkacz & Lampen, 1975).

Formation of the wall structure has been studied in *Saccharomyces cerevisiae* (Necas & Svoboda, 1985) and other fungal protoplasts, but rather little information has been obtained as their regeneration capacity in liquid media is limited. However, protoplasts of *C. albicans* revert to normally growing cells (Miragall *et al.*, 1986; Murgui *et al.*, 1986). In the present work, we extend our previous observations on the regeneration of the cell wall by *C. albicans* protoplasts and on the effect of the inhibition of synthesis of some wall polymers on the incorporation of specific mannoproteins into the wall (Murgui *et al.*, 1986). We also report the electron microscopic localization of chitin with wheat-germ agglutinin complexed with colloidal gold and of mannoproteins with concanavalin A-ferritin.

METHODS

Organism, growth and preparation of protoplasts. *C. albicans* ATCC 26555 was used; it was maintained by subculturing every 2–3 weeks on slopes of Sabouraud dextrose agar and propagated in Lee medium (Lee *et al.*, 1975). Protoplasts were prepared as described by Elorza *et al.* (1983).

Regeneration of protoplasts and radioactive labelling. Protoplasts adjusted to OD₆₀₀ 0.22 as measured with a Perkin Elmer Coleman 250 spectrophotometer were suspended in Lee medium (20 ml) supplemented with 0.6 M-KCl in individual flasks and incubated at 28 °C. Each incubation contained 0.4 µCi [¹⁴C]glucose (0.24 Ci mmol⁻¹; 8.88 GBq mmol⁻¹) ml⁻¹ and continued for the indicated times. In some cases, papulacandin B (5 µg ml⁻¹), nikkomycin (50 µg ml⁻¹), tunicamycin (20 µg ml⁻¹) or trichodermin (2 µg ml⁻¹) were added to the regeneration medium. The stock solutions were: papulacandin, 5 mg ml⁻¹ in methanol; nikkomycin, 1 mg ml⁻¹ in water; tunicamycin, 2 mg ml⁻¹ in 50 mM-NaOH and trichodermin, 2 mg ml⁻¹ in dimethyl sulphoxide.

Preparation of walls and isolation of polysaccharide fractions. Protoplast cultures were taken at intervals and collected by centrifugation (1200 g for 10 min). The protoplasts were washed with 0.6 M-KCl, broken by shaking with glass beads in a vortex mixer (three periods of 20 s), and the walls were sedimented (1200 g for 10 min) and purified as described by Elorza *et al.* (1985). Walls were extracted with 2 M-NaOH at 100 °C for 90 min; the insoluble residue was removed by centrifugation (1200 g for 10 min) and washed with distilled water. One part of the alkali-soluble supernatant was treated with Fehling's solution (Haworth *et al.*, 1941) and the precipitate collected (mannan fraction); the other part was precipitated by adding 3 vols ethanol [mannan plus alkali-soluble glucan (S-glucan)]. The actual value of S-glucan was obtained by subtracting the latter value from that obtained for mannan. The alkali-insoluble residue was extracted with 0.5 M-acetic acid at 100 °C (90 min). The supernatant was discarded and the residue washed extensively with distilled water. Part of this fraction containing alkali-insoluble glucan (I-glucan) plus chitin was collected on glass fibre filters (Schleicher & Schuell). After drying, the filters were counted in a Beckman scintillation spectrometer (model 7500) using toluene-based scintillation fluid. The remaining fraction was mixed with Zymolyase 20T (a glucanase complex) (2 mg ml⁻¹) in 2 ml 0.1 M-sodium acetate buffer pH 5.6 and incubated for 4 h at 30 °C to remove the glucan present. The radioactivity in the insoluble residue was determined (chitin).

Analysis of the spent medium. The medium obtained after incubation of protoplasts (20 ml) was dialysed against distilled water at 4 °C (three times, 24 h each) and concentrated to 5 ml with polyethylene glycol (molecular mass 3350). The medium was then divided in two portions; one was treated with Fehling's solution (mannoproteins) and the other was precipitated by adding 3 vols ethanol (total polysaccharides).

Preparation of samples for transmission electron microscopy. Isolated walls were fixed in 0.1 M-phosphate buffer pH 7.0 containing 2.5% (v/v) glutaraldehyde (1 h). After three washings with the phosphate buffer, walls were suspended in 0.05 M-Tris/HCl pH 7.0, 0.15 M-NaCl, 1 mM-CaCl₂, 1 mM-MnCl₂, 1 mM-MgCl₂ containing concanavalin A-ferritin (Con A-fe) (0.5 mg ml⁻¹) and incubated for 30 min at room temperature. The samples were processed for conventional electron microscopy according to Spurr (1969). Thin sections of the partially regenerated walls of protoplasts incubated under different conditions and labelled with Con A-fe were floated for 1 h on wheat-germ agglutinin complexed with colloidal gold (Au-WGA) prepared according to Geoghegan &

Ackerman (1977), and deposited on copper grids. All specimens were examined in a Jeol 100s electron microscope operating at 60 kV.

Chemicals. Zymolyase 20T was purchased from Miles Laboratories. Papulacandin B was a gift from Ciba Geigy, Basel, Switzerland. Trichodermin was kindly provided by Dr A. Jiménez, Centro de Biología Molecular, Madrid, Spain. Nikkomycin, a complex of nikkomycin Z and nikkomycin X as major components, was a generous gift of Bayer AG. [U-¹⁴C]Glucose was purchased from Amersham, and tunicamycin, Con A-fe and wheat-germ agglutinin from Sigma.

RESULTS

Synthesis of cell wall polymers by protoplasts

The kinetics of incorporation of mannoproteins into the cell walls of regenerating protoplasts after incubation in the presence of [U-¹⁴C]protein hydrolysate had been determined previously (Murgui *et al.*, 1986), but it was of interest to learn how they and other polymers interacted once synthesized and before being incorporated into the walls. With this aim, protoplasts were incubated in an osmotically protected medium with [U-¹⁴C]glucose as a precursor of polysaccharides and supplemented with papulacandin B, nikkomycin or tunicamycin.

When the protoplasts were regenerated in the presence of papulacandin B, the distribution of label into various wall polysaccharides changed with respect to control cultures. Chitin, the first polymer detected on the protoplast surface (Murgui *et al.*, 1986), was incorporated normally whereas incorporation of the S- and I-glucans was significantly decreased (Table 1). Accumulation of mannoproteins on the walls was also affected, being reduced to 11% after 5 h of incubation. [We have recently found a similar result with respect to mannoprotein incorporation in *S. cerevisiae* using aculeacin A, another antibiotic that inhibits glucan formation (Valentin *et al.*, 1986).] These results indicated that the synthesis of chitin and its incorporation into the wall were independent of the presence of the other polysaccharides, and that glucan inhibition also interfered with incorporation of mannoprotein into the growing wall.

In the presence of tunicamycin, incorporation of mannoproteins into the regenerating walls was brought to a halt, suggesting that some proteins of the wall need polymannose chains for correct incorporation into the growing wall structure. Tunicamycin also decreased the formation of glucans and chitin (Table 1). This result is interesting because at the concentration used, the drug caused only 20% inhibition of total cell protein synthesis after 5 h of incubation (data not shown), suggesting that the glucan- and chitin-synthases might be mannoproteins or that their transport is indirectly blocked by the drug (see below).

Nikkomycin blocked formation of chitin by 85%; in its presence the amount of I-glucan found on the walls was lower than in the control whereas that of S-glucan was higher (Table 1). These results suggest that chitin must be present on the surface of the protoplasts before incorporation of the I-glucan can take place. In parallel with the low levels of chitin and I-glucan, mannoprotein incorporation in the presence of the drug was also significantly reduced.

Since mannoprotein incorporation into the walls was greatly decreased in the presence of the antibiotics, the possibility that mannoproteins accumulated in the growth medium was examined. Accumulation of mannoproteins in spent medium was detected in the presence of nikkomycin and papulacandin B, but not in the presence of tunicamycin (Table 2). Other ethanol-precipitable materials were also found in higher amounts in the presence of nikkomycin (Table 2) but they were not further analysed. These results indicate that nikkomycin and papulacandin B did not prevent the synthesis of mannoproteins, but blocked their incorporation into the growing wall, probably as a side effect of the inhibition of formation of the chitin and β -glucan skeletons, respectively, on the protoplast surface.

Effect of antibiotics added at different times during the regeneration process

As tunicamycin added at the beginning of the regeneration process decreased the formation of all cell-wall polymers (Table 1), and in order to obtain more information about the initial steps of wall formation, the antibiotics were added at different times during regeneration. First, the

Table 1. *Effect of papulacandin B, tunicamycin and nikkomycin on the distribution of label from [U-¹⁴C]glucose into various polysaccharide fractions of regenerating protoplasts*

Different suspensions (20 ml) of protoplasts (OD₆₀₀ 0.22) were regenerated in stabilized Lee medium containing [U-¹⁴C]glucose (0.4 µCi ml⁻¹) in the absence or presence of antibiotics added at the start of the regeneration. After 3, 5 and 7 h incubation at 28 °C, protoplasts were collected and the walls isolated and fractionated as described in Methods. The radioactivity in each fraction is expressed as total c.p.m. (mean of three experiments); values in parentheses represent percentage incorporation relative to the control.

Incubation conditions	Regeneration time (h)	Mannan	S-glucan	I-glucan	Chitin
Control	3	2100	6960	7800	25600
	5	22650	13910	31500	58600
	7	47640	24360	75050	95410
Papulacandin B (5 µg ml ⁻¹)	3	670 (31.9)	910 (13.1)	3830 (49.1)	21570 (84.3)
	5	2600 (11.5)	1930 (13.9)	9190 (29.2)	52000 (88.7)
	7	5330 (11.2)	3570 (14.7)	16540 (22.0)	110190 (116.2)
Tunicamycin (20 µg ml ⁻¹)	3	580 (27.6)	2600 (37.4)	2470 (31.7)	7960 (31.0)
	5	950 (4.2)	5190 (37.3)	7540 (23.9)	12080 (20.6)
	7	1010 (2.1)	4060 (16.7)	11770 (15.7)	14630 (15.3)
Nikkomycin (50 µg ml ⁻¹)	3	900 (42.9)	5300 (76.1)	3900 (50.0)	2050 (8.0)
	5	2140 (9.4)	17000 (122.2)	6930 (22.0)	8760 (14.9)
	7	4770 (10.0)	30730 (126.1)	16510 (22.0)	14360 (15.0)

Table 2. *Polysaccharides accumulated in the regeneration medium of C. albicans protoplasts incubated in the presence of antibiotics*

The spent medium from regenerated protoplasts, labelled with [U-¹⁴C]glucose in the absence or presence of antibiotics, was treated with Fehling's solution (mannan) or ethanol precipitated (mannan plus glucan). Radioactivity is expressed as total c.p.m. (mean of three experiments); values in parentheses represent percentage incorporation relative to the control.

Incubation conditions	Regeneration time (h)	Mannan	Glucan
Control	3	10680	5840
	5	37150	14140
	7	38650	24360
Papulacandin B (5 µg ml ⁻¹)	3	13600 (127)	5520 (95)
	5	45340 (122)	14220 (101)
	7	79160 (205)	17920 (74)
Tunicamycin (20 µg ml ⁻¹)	3	2040 (19)	7930 (136)
	5	3340 (9.0)	19260 (136)
	7	3690 (9.5)	36620 (150)
Nikkomycin (50 µg ml ⁻¹)	3	13740 (129)	11120 (190)
	5	65730 (177)	19520 (138)
	7	125810 (325)	31240 (128)

effect of inhibition of protein synthesis on wall formation was studied. Trichodermin, which at 2 µg ml⁻¹ suppressed protein synthesis by 93% (results not shown), blocked incorporation of all cell-wall polymers when added at the beginning of the incubation period. When the drug was added 3 h after the onset of regeneration, thus allowing initial protein synthesis and deposition of the chitin skeleton, mannoprotein incorporation stopped, chitin deposition was not affected, incorporation of I-glucan decreased to 70% of the control and that of S-glucan was totally inhibited (Fig. 1). After 7 h incubation, incorporation of chitin was still 80% relative to the control. These results indicated that the cell machineries for synthesizing chitin and glucans are stable systems which remain active for long periods; once synthesized, they are not affected by the drug, whereas the formation of mannoproteins requires the continuous synthesis and glycosylation of protein, as in *S. cerevisiae* (Elorza & Sentandreu, 1969).

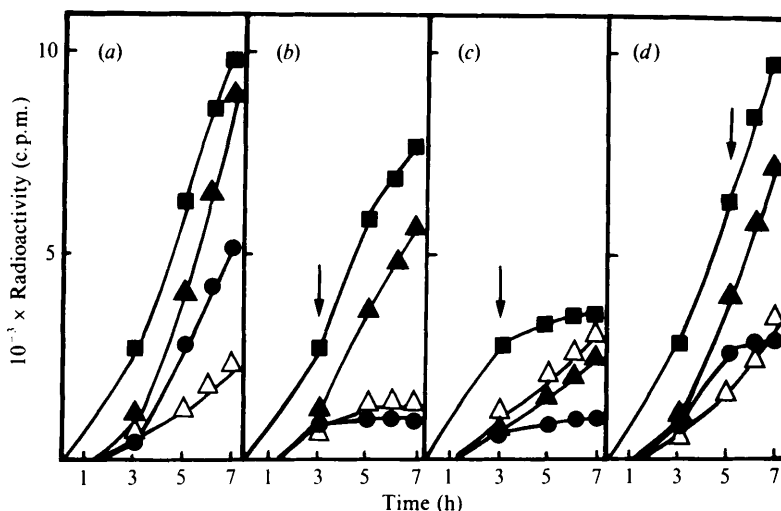


Fig. 1. Distribution of radioactivity among the polysaccharide fractions during regeneration of protoplasts. Various suspensions (20 ml) of protoplasts (OD_{600} 0.22) were regenerated in osmotically stabilized Lee medium in the presence of $0.4 \mu\text{Ci}$ $[\text{U-}^{14}\text{C}]\text{glucose ml}^{-1}$. (a) Control culture without antibiotics; (b) trichodermin or (c) nikkomycin added after 3 h of regeneration; (d) tunicamycin added after 5 h of regeneration. At the times indicated samples were taken, walls were isolated and the radioactivity in the polysaccharide fractions was determined: ■, chitin; ▲, I-glucan; △, S-glucan; ●, mannan.

Addition of nikkomycin after 3 h regeneration resulted in an almost complete blockage of new chitin incorporation as well as that of I-glucan and mannoproteins. Incorporation of S-glucan, on the other hand, was not inhibited but even stimulated (Fig. 1). These results agreed with those found when the antibiotic was added at the beginning of the regeneration process (Table 1).

Tunicamycin when added initially or 3 h after the onset of regeneration process gave similar results (Table 1). However, when tunicamycin was added after 5 h, mannoprotein synthesis was severely inhibited, and chitin was found in amounts similar to those in the control walls. After 2 h in the presence of this antibiotic, the amount of I-glucan was slightly decreased (20%) with a slight increase in the S-glucan.

Relationship between the S-glucan and the I-glucan

Some of the above results suggested a possible precursor-product relationship between S- and I-glucans in *C. albicans* similar to that described in *Schizophyllum commune* (Sonnenberg *et al.*, 1982). In order to obtain further information on this, the radioactivity incorporated into the wall glucans of regenerating protoplasts after a pulse of $[\text{U-}^{14}\text{C}]\text{glucose}$ was followed. The label found in the S-glucan after 45 min chase was only 13% of the radioactivity originally incorporated, with a parallel increase in c.p.m. in I-glucan (Table 3). Further information about the conversion of the S-glucan to I-glucan was obtained when the radioactive pulse was carried out in the presence of nikkomycin to block chitin formation. Again, the only polymer whose label was practically blocked during the chase period was that of the S-glucan (Table 3). The results obtained are consistent with the notion of a precursor-product relationship between the S- and I-glucan.

Ultrastructural studies

In order to learn how the different polymers are deposited during regeneration, ultrastructural studies were also done. Protoplasts were cultivated for 3 h and 5 h in the presence of antibiotics, and the isolated walls were labelled with Con A-fe and Au-WGA. After 3 h of regeneration, gold particles were uniformly distributed upon a material of low electron density and resembling wall

Table 3. *Distribution of radioactivity in a pulse-chase experiment among various wall polymers during the regeneration of C. albicans protoplasts in the absence (expt A) and presence (expt B) of nikkomycin*

Different suspensions (20 ml) of protoplasts were incubated in osmotically protected Lee medium. After 4.5 h (expt A) or 3 h (expt B) of regeneration, the protoplasts were supplemented with [U - ^{14}C]glucose ($0.8 \mu\text{Ci ml}^{-1}$ in A; $0.4 \mu\text{Ci ml}^{-1}$ plus $20 \mu\text{g nikkomycin ml}^{-1}$ in B), and after 30 min (A) or 2 h (B) they were transferred to prewarmed non-radioactive medium. At the times indicated samples were taken and the wall polymers extracted. Radioactivity is expressed as total c.p.m. (mean of three experiments); values in parentheses represent percentage incorporation relative to the chase at time 0.

Chase period (min)	Mannan	S-glucan	I-glucan	Chitin
Expt A				
0	10005	5000	9840	7774
45	11900 (119)	636 (13)	13190 (134)	10970 (141)
90	12700 (127)	1412 (28)	13398 (136)	11318 (146)
Expt B				
0	4000	7934	1424	5450
45	8660 (217)	5662 (71.4)	3358 (235.8)	8662 (158.9)
90	8968 (224)	4954 (62.4)	4358 (306.0)	9566 (175.5)

ghosts. The latter seemed to be the very first material deposited on the surface of regenerating protoplasts, confirming previous analytical and cytological observations that indicated it to be chitin (Elorza *et al.*, 1983; Murgui *et al.*, 1986). This initial fibrillar skeleton retained mannoproteins from the very beginning (Elorza *et al.*, 1985; Sentandreu *et al.*, 1983), as some Con A-fe receptors were also clearly seen associated within the fibrillar structures (Fig. 2).

When protoplasts were regenerated for 5 h, the appearance of the wall was clearly different. The walls showed a thick electron-transparent layer with many Con A receptors on both surfaces. The Au-WGA receptors seemed to be located preferentially in the inner part of the wall extending to the nearby transparent layer as observed in whole cells (Hilenski *et al.*, 1986). The two surfaces seemed to be enriched in glycoproteins, as Con A-fe markers were present in high amounts (Fig. 2b). When the walls were boiled in SDS only some of the Con A-fe receptors disappeared and it then seemed that the receptors located on the outer surface were more abundant than in the inner part of the wall (Fig. 2c). When the walls were treated with Zymolyase their appearance changed dramatically. The electron-transparent layer completely disappeared, as well as almost all the Con A receptors. Only a material of low electron density which could be marked with colloidal gold and had a few Con A-fe receptors was detected (Fig. 2d). These walls remained resembled the walls formed by 3 h regenerating protoplasts (Fig. 2a).

Walls obtained from protoplasts which had been incubated in the presence of tunicamycin or papulacandin B were also characteristic. The walls obtained from tunicamycin-treated protoplasts were thinner than those of control protoplasts, and almost completely lacked the electron-transparent layer, which was substituted by a material of low electron density that was randomly labelled with Au-WGA and had very few Con A-fe receptors distributed throughout the structure (Fig. 3a). These receptors probably corresponded to the very few mannoprotein molecules incorporated, as the drug suppressed glycosylation of proteins (Table 1) and the walls were similar to those found in 3 h regenerating protoplasts or after 5 h following Zymolyase treatment (Fig. 2). Treatment of these walls with SDS did not modify the basic appearance of the walls although the Con A-fe receptors almost disappeared (Fig. 3b).

When the protoplasts were incubated for 5 h in the presence of papulacandin B under conditions in which 70% of glucan synthesis was inhibited, the isolated walls showed a material of low electron density interspersed with large amounts of gold markers (Fig. 3c). Con A receptors did not disappear when these walls were treated with SDS (data not shown), suggesting that the small amounts of β -glucans incorporated in the presence of the drug (about 30% of the control walls) were also able to retain some mannoproteins.

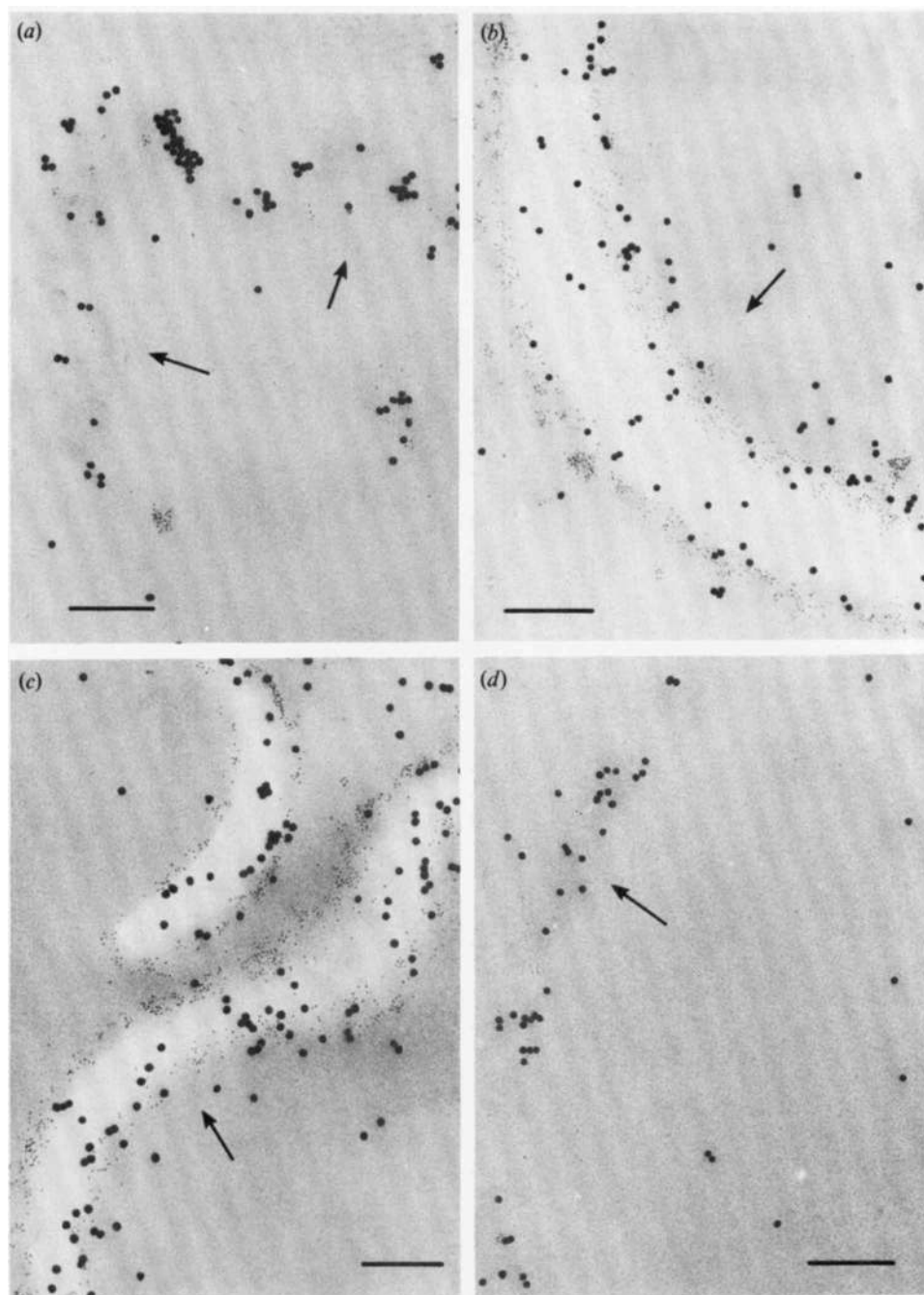


Fig. 2. Isolated walls obtained from protoplasts of *C. albicans* regenerated for 3 h (a) and 5 h (b). Walls of the latter protoplasts were also extracted with SDS (c) or Zymolyase (d). Chitin was detected with Au-WGA (large label) and mannoproteins with Con A-fe (small label). Arrows indicate the inner surface of the walls. Bars, 0.2 μ m.

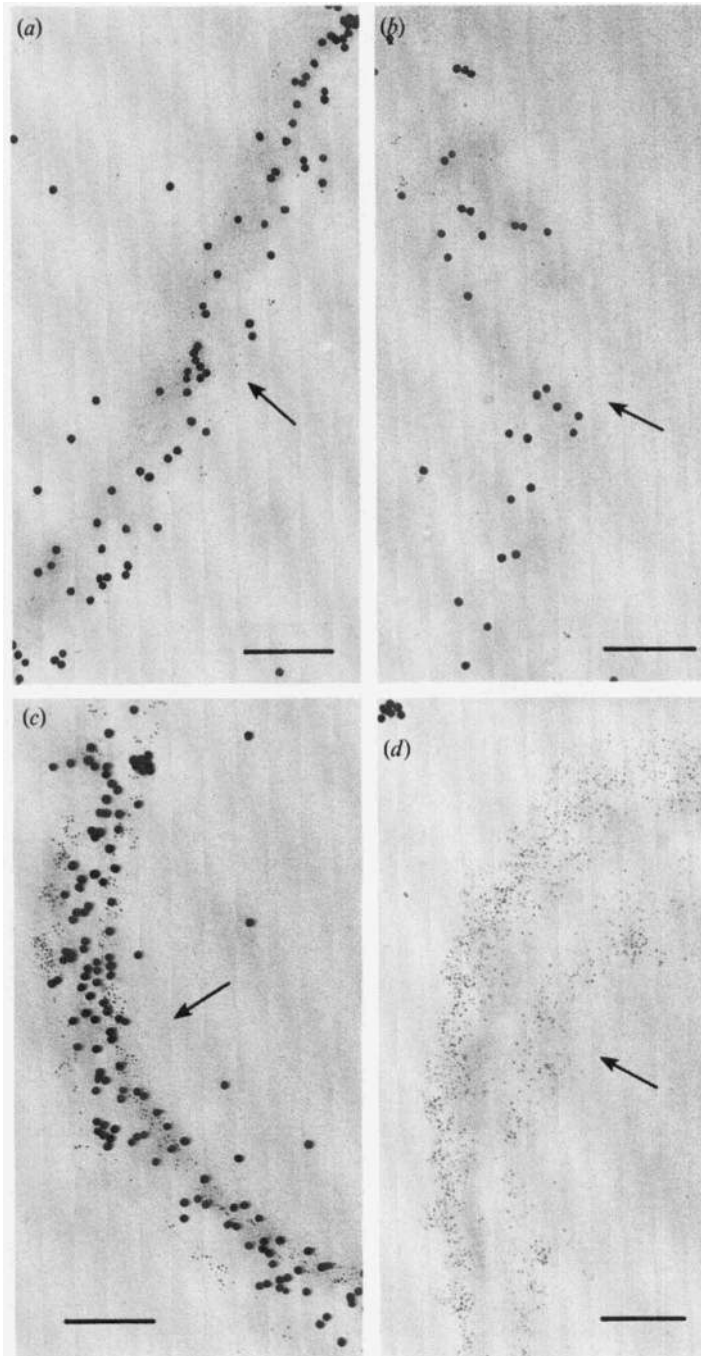


Fig. 3. Isolated walls obtained from protoplasts of *C. albicans* incubated in regeneration medium for 5 h in the presence of tunicamycin prior to (a) or after (b) extraction with SDS, or in the presence of papulacandin B (c) or nikkomycin (d). Chitin and mannoproteins were detected as in Fig. 2. Arrows indicate the inner surface of the walls. Bars, 0.2 μ m.

In the presence of nikkomycin the protoplasts formed walls which were not labelled by Au-WGA, although the Con A-fe receptors were present as well as some electron-transparent material (Fig. 3*d*), confirming that in the presence of this antibiotic chitin was not synthesized but the S-glucan (Table 1) was able to form an abnormal wall (Hilenski *et al.*, 1986).

DISCUSSION

Formation of a new wall by fungal protoplasts is initiated by deposition of a chitin skeleton which allows incorporation of other wall polymers (Elorza *et al.*, 1983; Gibson *et al.*, 1976; Murgui *et al.*, 1986). This is interesting, as chitin is a minor component of the wall of normal growing cells, suggesting that the walls formed by protoplasts are initially abnormal structures, as previously reported (Nečas & Svoboda, 1985).

In the case of *C. albicans* in the presence of nikkomycin, a potent competitive inhibitor of chitin synthase (Gooday *et al.*, 1985; Gow & Selitrennikoff, 1984; Hector & Braun, 1986), synthesis of this skeleton was severely reduced (Table 1, Fig. 3) with the formation of an abnormal wall characterized by greater amounts of S-glucan and very low concentrations of I-glucan and mannoproteins. As the S-glucan seems to be the precursor of the I-glucan, it appears that this conversion needs the previous presence of chitin in the newly formed walls. This interpretation would be in agreement with the reported existence of covalent bridges between chitin and glucan in other fungal cells (Stagg & Feather, 1973). Polyoxin D, another inhibitor of chitin synthase, blocks formation of chitin and I-glucan in *Schizophyllum commune*, but not accumulation of the S-glucan (de Vries & Wessels, 1975). We speculate that in *C. albicans*, as in *Schiz. commune* (Sietsma & Wessels, 1981; Sonnenberg *et al.*, 1982), a progressive cross-linking between S-glucan and chitin occurs during normal extension of the wall, changing the solubility characteristic of the former polymer. In the absence of chitin, the process does not occur and, consequently, the solubility characteristic of the S-glucan does not change. This view is supported by the fact that when nikkomycin was added 3 h after the initiation of regeneration (that is, when the initial chitin skeleton had already been formed) accumulation of I-glucan was again reduced whereas S-glucan was incorporated in higher amounts. When nikkomycin was present from the beginning of the regeneration process, the wall formed by the protoplasts lacked not only chitin and I-glucan but also mannoproteins, suggesting that they are also probably incorporated in a coordinated way with glucan into the growing cell wall (see below). However, they do not seem to be essential components in the process of connecting glucan subunits to the chitin network, since tunicamycin did not suppress it when added 3 h after the onset of the regeneration process.

Baguley *et al.* (1979) reported that papulacandin B inhibited glucan synthesis, and we have found that under these conditions formation of the chitin network takes place normally. However, incorporation of mannoproteins, but not their synthesis, is prevented, as they could be recovered from the regeneration medium (Table 2) (Murgui *et al.*, 1986). These results confirm that formation of the initial chitin skeleton is completely independent of the other wall polymers, and that incorporation of mannoproteins into the growing structure takes place only in a coordinated way with the formation of I-glucan.

The analytical results were confirmed and extended by the cytochemical localization of polymers in the walls formed by protoplasts in the presence of antibiotics. Chitin was marked with WGA, a lectin that binds to (1→4)- β -N-acetylglucosamine residues, complexed to colloidal gold (Horisberger & Vonlanthen, 1977; Molano *et al.*, 1980; Roberts *et al.*, 1983). Incorporation of mannoproteins was followed with ferritin-labelled concanavalin A, a lectin that binds to α -linked mannose and glucose residues. This lectin does not react with the other polysaccharides present in *C. albicans* cell walls (Tronchin *et al.*, 1979). Initially, protoplasts formed a net of chitin (Fig. 2). A thick layer of β -glucan, marked neither by Au-WGA nor by Con A-fe, was then deposited; this layer disappeared with Zymolyase digestion and did not appear in the presence of papulacandin B. The glucan layer was covered with mannose-containing compounds which were probably mannoproteins, possibly covalently bonded to the wall structure as they were not removed by SDS, and could only be eliminated by Zymolyase digestion (Fig. 2).

Fractionation of the wall material formed in the presence of nikkomycin demonstrated that S-glucan was probably the main polymer present (Table 1). Mannoproteins were not incorporated (Table 1), but accumulated in the medium (Table 2). In these circumstances the morphogenetic capacity of the protoplasts was reduced and they did not revert to normal growing cells.

The results reported in this and other papers suggest that building of the walls by protoplasts occurs in two steps: (a) formation of the chitin microfibrillar skeleton, and (b) incorporation of glucans and mannoproteins which give rise to a defined mould responsible for the specific wall and cell morphologies (Elorza *et al.*, 1985). This hypothesis is also supported by the fact that an initial deposition of chitin occurs not only during protoplast regeneration but also during normal apical growth (Cabib, 1981; Wessels *et al.*, 1983) and in the formation of the cross-septum during cell division (Cabib, 1981), suggesting that this polymer is principally responsible for the wall ontogeny. Glucans and mannoproteins are then deposited, being the main morphogenetic components of the walls.

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