DISTRIBUTION OF CHITIN IN THE YEAST CELL WALL

An Ultrastructural and Chemical Study

JESUS MOLANO, BLAIR BOWERS, and ENRICO CABIB

From the National Institute of Arthritis, Metabolism and Digestive Diseases, and the National Heart, Lung and Blood Institute, the National Institutes of Health, Bethesda, Maryland 20205. Dr. Molano's present address is the Departamento de Laboratorio, Ciudad Sanitaria La Paz, Madrid-34, Spain.

ABSTRACT

The distribution of chitin in *Saccharomyces cerevisiae* primary septa and cell walls was studied with three methods: electron microscopy of colloidal gold particles coated either with wheat germ agglutinin or with one of two different chitinases, fluorescence microscopy with fluorescein isothiocyanate derivatives of the same markers, and enzymatic treatments of [^{14}C]glucosamine-labeled cells. The septa were uniformly and heavily labeled with the gold-attached markers, an indication that chitin was evenly distributed throughout. To study the localization of chitin in lateral walls, alkali-extracted cell ghosts were used. Observations by electron and fluorescence microscopy suggest that lectin-binding material is uniformly distributed over the whole cell ghost wall. This material also appears to be chitin, on the basis of the analysis of the products obtained after treatment of ^{14}C-labeled cell ghosts with lytic enzymes. The chitin of lateral walls can be specifically removed by treatment with β-(1 $\rightarrow$ 6)-glucanase containing a slight amount of chitinase. During this incubation, ~7% of the total radioactivity is solubilized, about the same amount liberated when lateral walls of cell ghosts are completely digested with snail glucanase to yield primary septa. It is concluded that the remaining chitin, i.e., >90% of the total, is in the septa. The facilitation of chitin removal from the cell wall by β-(1 $\rightarrow$ 6)-glucanase indicates a strong association between chitin and β-(1 $\rightarrow$ 6)-glucan. Covalent linkages between the two polysaccharides were not detected but cannot be excluded.

Previous studies of our laboratory (4, 7) and of others (1) have suggested that most or all of the chitin contained in *Saccharomyces* cell walls is localized in the bud scars. Morphological observations (3, 5) and studies on the effect of a chitin synthesis inhibitor on cell division (3) support the view that chitin forms the primary septum between mother cell and bud and subsequently remains in the parental bud scar. Our extensive investigations about the regulation of septum morphogenesis (7) were based on this specific localization of the polysaccharide.

Formation of the primary septum in budding yeasts seems to take place in two steps (5). The first, at early budding, is the appearance of a chitin ring around the "neck" between mother and daughter cells; the second, before cell division, is a centripetal growth of material to form a disk-

shaped cross-wall between the two cells. As our most purified preparations of septa contained, in addition to chitin, ~15% of anthrone reacting material,[1] it was conceivable that glucan, rather than chitin, might be the principal substance that participates in the second step, giving rise to the central portion of the septal disk. Furthermore, it was recently reported that chitin may not be exclusively confined to septa. Horisberger and Vonlanthen (15), by using gold-linked wheat germ agglutinin (WGA-Au) as a marker for cell wall chitin in electron microscopy sections, found that the gold particles adhered not only to the bud scar region but also in some measure to lateral walls. It was, therefore, important to ascertain the nature of the wheat germ agglutinin (WGA)-binding material in lateral walls and to determine quantitatively its amount.

In the investigation of this problem, we used three complementary methods: (*a*) visualization of chitin distribution by electron microscopy of colloidal gold-attached WGA or chitinase; (*b*) localization of fluorescein isothiocyanate-WGA (FITC-WGA) and FITC-chitinase by fluorescence microscopy; (*c*) specific labeling of chitin with [^{14}C]glucosamine and effect of different treatments on the release of label.

MATERIALS AND METHODS

Materials

WGA and FITC-WGA were obtained from Miles Laboratories (Elkhart, Ind.). FITC-celite was from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Polyethylene glycol, mol wt 20,000, was purchased from Polysciences Inc. (Warrington, Pa.), chloroauric acid from ICN K & K Laboratories Inc. (Plainview, N. Y.), and Sephadex G-25 and G-100 from Pharmacia Inc. (Piscataway, N. J.). Pustulan (β-(1 → 6)-glucan) was obtained from Calbiochem and laminarin (β-(1 → 3)-glucan) from ICN K & K Laboratories Inc. [1-^{14}C]glucosamine (50 mCi/mmol) was purchased from New England Nuclear (Boston, Mass.). Chitin oligosaccharides for use as chromatography standards or inhibitors of WGA or chitinase binding were obtained as described (19). *N*-acetylglucosaminitol was obtained by reduction of *N*-acetylglucosamine with sodium borohydride.

Methods

YEAST GROWTH: *Saccharomyces cerevisiae* X2180 (ATCC 26109) was grown in YEPD medium (6) and harvested in the late logarithmic phase of growth.

To obtain incorporation of [^{14}C]glucosamine into the cell wall, galactose had to be substituted for glucose in the medium. *S. cerevisiae* X2180 grows very slowly in galactose, because of a mutation in the gal-2 (permease) gene (R. K. Mortimer, personal communication). Nevertheless, we found that rapid growth occurs after prolonged incubation in a galactose-containing medium. It is not known whether this is caused by long-term adaptation or by selection of a mutant. For this purpose, *S. cerevisiae* X2180 was inoculated in galactose minimal medium (0.7% Difco yeast nitrogen base [Difco Laboratories, Detroit, Mich.], 2% galactose) and incubated at 30°C. After 4–5 d some growth was visible. Growth ceased at a relatively low turbidity, but became much faster and more abundant upon reinoculation into fresh medium. The yeast was maintained on slants containing YEP Gal medium (2% galactose, 2% peptone, 1% yeast extract and 2% agar) and transferred for growth to the same medium, but lacking the agar.

PREPARATION OF CELL GHOSTS AND SEPTA: Cell ghosts were obtained from cells by cyclical treatments with alkali and acetic acid as previously reported (1, 4). Septa were prepared by digestion of the cell ghosts with purified snail glucanase (4). The sugar composition of cell ghosts has already been reported (4). In septa, *N*-acetylglucosamine was determined colorimetrically after complete enzymatic hydrolysis of chitin (4), and glucan was measured with anthrone. Glucan represented ~16% and chitin 84% of the total polysaccharide. These are relative values. Absolute values are not available, because dry weights were not measured on account of the very small amount of material.

PREPARATION OF ENZYMES: *Streptomyces griseus* chitinase was purified from culture filtrates by adsorption on chitin, as already described (18). Wheat germ chitinase was purified to homogeneity as reported (19). Snail glucanase was purified by adsorption on yeast glucan (4). The preparation contained both β-(1 → 3)- and β-(1 → 6)-glucanase activity, as measured with laminarin and pustulan, respectively, as substrates. It also contained some chitinase activity, which was eliminated by adsorption on chitin, as follows: To 7.75 ml of glucanase, 13 mg of regenerated chitin (18) was added. After 10 min at 10°C, the suspension was centrifuged for 10 min at 27,000 *g*. The treatment was repeated a second time.

Endo β-(1 → 6)-glucanase was obtained (11) from culture filtrates of *Bacillus circulans* grown on yeast cell walls as carbon source and purified up to and including the Sephadex G-100 step. The column fractions containing the activity were concentrated in an Amicon pressure cell (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) with a UM-10 filter and dialyzed overnight against 10 mM sodium succinate, pH 5.

This preparation contained a small amount of chitinase, most of which could be eliminated by passage through a chitin column. In a typical preparation, β-(1 → 6)-glucanase containing 53 U of activity was applied to a 0.9 × 44 cm chitin (18) column, previously equilibrated with 20 mM potassium phosphate, pH 6.3, containing 0.1 mg/ml of bovine serum albumin. Elution was performed with the same buffer and fractions of 1.8 ml were collected. The fractions containing β-(1 → 6)-glucanase activity were pooled and sodium azide was added as a preservative to a final concentration of 0.02%. The recovery of β-(1 → 6)-glucanase activity was almost quantitative, whereas 95% of the contaminating chitinase was lost.

ENZYMATIC ASSAYS AND UNITS: β-(1 → 6)- and β-(1 → 3)-glucanase were assayed as described by Fleet and Phaff (11), with minor modifications, and chitinase as already reported (18). In all cases, 1 U of enzyme is defined as that amount that gives rise to the liberation of 1 μmol of product (calculated as monosaccharide) per min at 30°C.

TREATMENT OF UNLABELED CELL GHOSTS WITH ENZYMES: For chitinase treatment, the mixture contained 1 ml of cell ghosts (2 mg [dry weight]/ml), 25 μl 1 M potassium phos-

[1] E. Cabib. Unpublished observations.

phate, pH 6.3, and 60 μl (0.36 U) of *Streptomyces* chitinase. Incubation was for 6 h with shaking at 30°C. The treated ghosts were recovered by centrifugation and washed with distilled water.

For β-(1 → 6)-glucanase treatment, the reaction mixture consisted of 0.5 ml of cell ghosts (for intact ghosts, 2 mg [dry weight]/ml; for chitinase-treated ghosts, an amount derived from 2 mg/ml of intact ghosts), 0.5 ml of 50 mM sodium succinate, pH 5, and 0.25 ml (0.8 U) of β-(1 → 6)-glucanase. Sodium azide was added to a final concentration of 0.02%. The mixture was incubated in a bath shaker at 30°C for 48 h. After the supernatant fluid was separated by centrifugation, two additional 48-h incubations were carried out, by resuspending the ghosts each time in an identical mixture containing fresh enzyme. Finally, the ghosts were centrifuged off and washed with distilled water.

PREPARATION OF ^{14}C-LABELED GHOSTS: An inoculum (5 ml) of galactose-adapted *S. cerevisiae* X2180 in the logarithmic phase of growth was added to 80 ml of YEP Gal medium (see above), containing 0.4 mCi of [1-^{14}C]glucosamine. After overnight incubation at 30°C, the yeast was harvested at a concentration of ~8 × 10^7 cells/ml. The cells (0.5 g, wet weight) were washed several times with water and used for ghost preparation by alkali and acetic acid treatments (see above). The final preparation contained a total of 34 μmol of anthrone-reacting material (as glucose) and 1.08 μmol of chitin (as *N*-acetylglucosamine). The sp act of the chitin was 2.7 × 10^7 cpm/μmol.

It appears that the cell walls of the galactose-grown cells were much more resistant to attack by alkali and acetic acid than those of glucose-grown cells, as the glucan:chitin ratio was sevenfold higher in ghosts from galactose-grown cells. Nevertheless, the appearance in the fluorescence microscope with FITC-WGA, before and after different enzymatic treatment, was the same for both types of ghosts.

Treatment of ^{14}C-labeled cell ghosts with *Streptomyces* chitinase, β-(1 → 6)-glucanase, or snail glucanase was carried out as for the unlabeled preparations (see above). After incubation, the remaining insoluble material was centrifuged off, and the supernatant fluid was saved for further analysis as described below.

STREPTOMYCES CHITINASE DIGEST: Supernatant fluid, obtained after chitinase treatment of radioactive ghosts containing 29,500 cpm, was desalted by passage through an Amberlite MB-3 (mixed bed; acetate and hydrogen forms) 0.4 × 3 cm column (Rohm and Haas Co., Philadelphia, Pa.). After evaporation under reduced pressure, the solution was applied to Whatman 1 paper and the chromatogram was developed with isoamyl alcohol:pyridine:water 1:1:0.8.

β-(1 → 6)-GLUCANASE DIGEST: ^{14}C-labeled ghosts, containing 2.07 × 10^6 cpm, were subjected to two successive 48-h treatments with β-(1 → 6)-glucanase, as described above, and the supernatant fluids were pooled (total radioactivity, 3.07 × 10^5 cpm).

For total hydrolysis, a portion of the digest (95,000 cpm) was concentrated under reduced pressure to a final volume of 100 μl. An equal volume of 12 N HCl was added, the tube was sealed in the flame, and the mixture was placed in a boiling water bath for 3 h. After evaporation to dryness, addition of 0.5 ml of water and new evaporation, the residue was redissolved in 0.2 ml of water and adjusted to slightly alkaline pH with NaOH.

The aminosugars liberated in the acid hydrolysis were reacetylated as follows: To the hydrolysate, 0.05 ml of freshly prepared 12.5% acetic anhydride in water and 0.03 ml of 2 M Na_2CO_3 were added. The mixture was kept at room temperature for 10 min and subsequently transferred to a boiling water bath for 5 min. The N-acetylated material was desalted by passage through a mixed bed resin and subjected to paper chromatography as described above for the chitinase digest.

Other portions of the β-(1 → 6)-glucanase digest were either directly deionized or first N-acetylated (see above) and then deionized, before paper chromatography.

For further analysis of disaccharide and trisaccharide, the corresponding peaks were eluted from the paper chromatogram with water, and the eluates were concentrated under reduced pressure. Chitinase treatment was performed on this material under the same conditions as for cell ghosts, except that incubation was for 3 h at 30°C. The digest was again deionized and used for paper chromatography.

For borohydride reduction, to 50 μl of disaccharide (~19,000 cpm), 2 μl of 0.5 M NaOH and 50 μl of 0.2 M sodium borohydride in 0.01 M NaOH were added. After overnight incubation at room temperature, 100 μl of 12 N HCl was added, followed by 0.25 μmol each of *N*-acetylglucosamine and *N*-acetylglucosaminitol as carriers. Hydrolysis, reacetylation, and deionization were carried out as described above for direct hydrolysis of the β-(1 → 6)-glucanase digest. The MB-3 column eluates were evaporated, applied to Whatman No. 1 paper, and subjected to electrophoresis (20 V/cm, 2.5 h) with 2% sodium molybdate as electrolyte.

ANALYTICAL: Total hexose was determined with anthrone (22). Radioactivity was measured in a Beckman 8100 scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.), using Aquasol (New England Nuclear, Boston, Mass.) as scintillation cocktail.

PREPARATION OF FITC CONJUGATES: The method used was a modification of Rinderknecht's procedure (20). For wheat germ chitinase, to 0.5 ml of the enzyme containing 16 mg/ml protein, 30 μl of 1 M sodium bicarbonate and 10 mg of FITC-celite were added. After 5-min incubation at room temperature with stirring, the suspension was brought to 2 ml with 0.05 M sodium bicarbonate and centrifuged for 5 min at 800 *g* in the cold room. The supernatant fluid was applied to a Sephadex G-25 (medium) column, 2 × 20 cm, previously equilibrated with 50 mM sodium bicarbonate. The same buffer was used for elution. Fractions (1.5 ml) were collected and the absorbance at 280 and 495 nm was measured. Fractions corresponding to the peak at both wavelengths were pooled and stored in the refrigerator after adding sodium azide to a final concentration of 0.02%.

For the *Streptomyces* chitinase-FITC conjugate, 0.5 ml of the enzyme (3 mg/ml) was adjusted to pH 8.5 by addition of 1 M Na_2CO_3, followed by 10 mg of FITC-celite. After 30 min at room temperature with stirring, the mixture was diluted to 1 ml with 20 mM potassium phosphate, pH 6.3, and centrifuged for 5 min at 800 *g*. The supernatant fluid was subjected to Sephadex filtration as for the wheat germ chitinase adduct, except that the size of the column was 1.5 × 11 cm and the equilibrating and eluting buffer was 20 mM potassium phosphate, pH 6.3.

STAINING OF CELL GHOSTS WITH FITC CONJUGATES: To 10 μl of intact, chitinase-treated or β-(1 → 6)-glucanase-treated cell ghosts (1 mg/ml anthrone-reacting material, as glucose) were added 10 μl of FITC-WGA (2 mg/ml) and 10 μl of 50 mM sodium bicarbonate, or 10 μl of 50 mM sodium phosphate at pH 7.3. After 5 min at room temperature, samples were mounted on slides for fluorescence microscopy. Alternatively, suspensions were diluted to 0.2 ml with bicarbonate or phosphate buffer and centrifuged. The pellets were resuspended in buffer and used for microscopy.

The same technique was used for FITC derivatives of wheat germ or *Streptomyces* chitinase, except that the concentrations of the corresponding solutions were 0.4 and 0.3 mg/ml, respectively.

FLUORESCENCE MICROSCOPY: Samples were observed with a Leitz Dialux microscope (E. Leitz, Inc., Rockleigh, N. J.)

equipped with an incident light Ploemopak 2.3 illuminator. An edge filter K480, a red suppression filter BG38, and a neutral filter N4 were used. The filter system for excitation contained an excitation filter BP 390–490, a beam splitting mirror RKP510, and a suppression filter LP515. Photographs were taken with Kodak Tri-X pan film, with an exposure time of 20–40 s.

PREPARATION OF COLLOIDAL GOLD-LABELED PROTEINS: Colloidal gold was prepared as described by Geoghegan and Ackerman (12), with the same concentration of chloroauric acid (0.1 g/liter). The absorbance of the colloidal gold solution at 580 nm and pH 8.3 varied between 0.25 and 0.33. The size of the gold particles was 13.5 ± 2.4 nm. For each protein, the amount and pH required for maximal stabilization of the colloid were determined as suggested by the same authors.

WGA was dialyzed overnight against 2.5 mM HCl. To 0.16 ml of the dialyzed solution, containing 0.2 mg protein, 10 ml of the colloidal gold solution, previously adjusted to pH 8.7 with 0.2 M K_2CO_3 (12), was added, while stirring. After 2 min, 0.5 ml of 1% polyethylene glycol (mol wt 20,000) was added and, 5 min later, the colloidal solution was centrifuged in the cold for 1 h at 35,000 *g*. The pellet was resuspended in 10 ml 50 mM Tris, pH 8.3, containing 0.15 M NaCl and 0.05% polyethylene glycol. After centrifugation as above, the pellet was resuspended in 1 ml of the same buffer, and sodium azide was added to a final concentration of 0.02%. A control was prepared in the same way, but omitting WGA.

For staining, each suspension of intact or enzyme-treated cell ghosts contained 2 mg of anthrone-reacting material (as glucose) per ml. For septa, an amount originated in the same quantity of cell ghosts, as measured with anthrone, was used. To 10 μl of suspension of the material to be stained, 200 μl of 0.2% bovine serum albumin was added and the suspension was centrifuged for 5 min in a clinical centrifuge in the cold. Omission of this washing caused high blanks in the subsequent labeling step. The pellet from the centrifugation was suspended in 10 μl of the original Tris/NaCl/polyethylene glycol mixture, followed by addition of 5 μl of 1% bovine serum albumin and 20 μl of WGA-Au (or control gold particles). In some cases a fivefold dilution of WGA-Au was also used. After 3 min at room temperature, the suspensions were centrifuged as above and the pellets were washed twice, first with 0.2 ml and then with 0.5 ml of 50 mM Tris, pH 8.3, containing 0.15 M NaCl and 0.05% polyethylene glycol. The final pellets were resuspended in 20 μl of distilled water and used for electron microscopy.

The colloidal gold complexes of wheat germ and *Streptomyces* chitinase were prepared in a similar way, with the following changes:

Wheat germ chitinase (17 ml of a 0.3 mg/ml solution) at pH 8.5, was concentrated to 2 ml in an Amicon stirred cell with a PM-10 filter. The concentrate was diluted to 10 ml with water and concentrated again in the same way. This operation was repeated seven additional times. The amount of the final solution used for 10 ml of colloidal gold was only 0.2 ml, because larger amounts led to coagulation of the colloid. The final pellet after centrifugation was resuspended in 0.5 ml of buffer.

Streptomyces chitinase, 3 mg/ml, was dialyzed overnight against water. Of the dialyzate, 0.1 ml was used for each 10 ml of colloidal gold solution.

Staining with the two chitinase-gold preparations was carried out as for WGA-Au, except that the wheat germ chitinase-gold complex was diluted 10-fold before use to avoid excessive labeling. The bovine serum albumin-gold complex was prepared as directed (12), at pH 6, where its stability is maximal.

ELECTRON MICROSCOPY: Specimen support grids were made by picking up a water-cast parlodion film on 400-mesh copper grids. The parlodion film was stabilized by a thin layer of evaporated carbon and glow-discharged just before use. One drop of washed sample suspension was applied to the grid. After 30–60 s the grid was rinsed with three drops of distilled water or, in the case of the synthetic chitin, with three drops of 1% uranyl acetate. The pure chitin samples were not visible unless negatively stained. The grids were air-dried and examined at 100 kV in a Siemens Elmiskop 101.

RESULTS

Electron Microscopy of Isolated Septa

Binding of WGA-Au to yeast chitin was demonstrated with polysaccharide obtained in vitro (9)

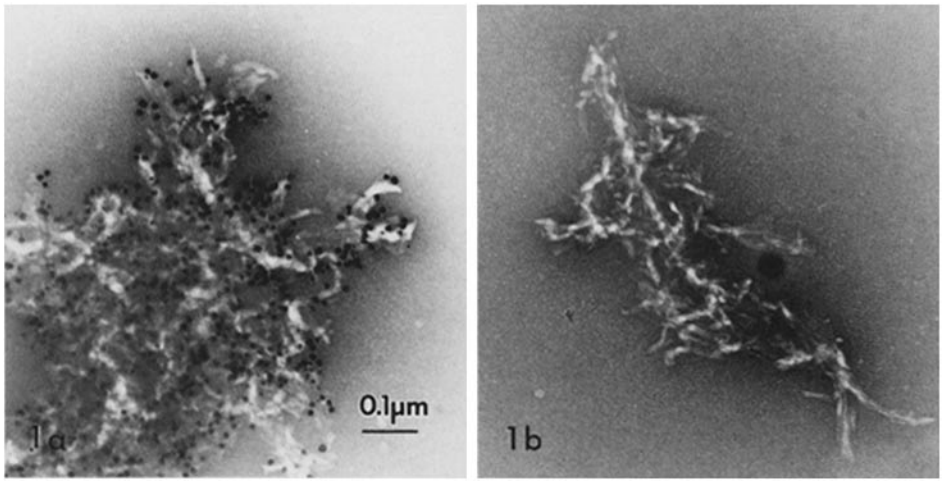

FIGURE 1 Labeling of enzymatically synthesized chitin with WGA-Au. (*a*) WGA-Au used for staining, (*b*) control with colloidal gold only. × 72,400.

by incubation of UDP-*N*-acetylglucosamine with solubilized chitin synthetase (Fig. 1 *a*). Gold particles lacking lectin did not attach to chitin (Fig. 1 *b*).

For the preparation of primary septa, yeast cells were first converted (4) into "extracted cell ghosts" (from here on called "cell ghosts" for brevity), by cyclical treatments with alkali and acetic acid. The ghosts are thin cell envelopes that have lost all of the wall mannan-peptide and most of the glucan. The remaining material is chitin and glucan (4). The latter is degraded with glucanase, thus setting free the primary septa (4). When WGA-Au was applied to the septal disks, a heavy and fairly uniform labeling over the whole surface of the disks was observed (Fig. 2 *a* and *b*). To observe the distribution of the gold particles over the material, it was necessary to use a concentration of WGA-Au below saturation (Fig. 2 *b*). There was no indication of specific areas devoid of particles. As with isolated chitin, gold particles without WGA did not bind to the septa (Fig. 2 *c*). The attachment of the WGA-Au particles was greatly diminished, although not abolished, by addition of chitin oligosaccharides before that of WGA-Au (Fig. 2 *d*).

Gold particles attached to purified *Streptomyces* (18) or wheat germ (19) chitinase[2] also bound to the septa (Fig. 2 *e*, and *f*), although the labeling was lighter than with WGA.

Treatment of septa with *Streptomyces* chitinase led to their virtually complete dissolution.

Electron Microscopy of Cell Ghosts

In intact yeast cells, chitin is covered by the other polysaccharides that form the cell wall and it is only accessible to small molecules, such as primulin and brighteners (13, 21). When yeast is observed under ultraviolet light in the presence of these compounds, fluorescence is detected only in the bud scar region and at the connection between mother cell and bud, where the bud scar will be formed during cell division (5, 13, 21). To uncover as much chitin as possible, cells were converted into cell ghosts as described in the previous section. Despite the loss of all of the mannan and most of the glucan of the cell wall, this preparation retains almost all of the chitin, which is very resistant to these treatments (4).

When cell ghosts were treated with WGA-Au, the labeling was surprisingly weak in the bud scar area (Fig. 3 *a* and *b*, compared with Fig. 2 *a* and *b*). At the same time, grains with about the same density distribution were observed on the remainder of the ghost surface. Therefore, it appears that despite extensive digestion of the cell wall, most of the chitin in the bud scar region was still shielded from the gold grains.

The amount of WGA-Au attached to the surface of the cell ghosts was relatively small, but the binding appears to be specific. Gold particles with attached bovine serum albumin did not bind (Fig. 3 *c*) and the labeling with WGA-Au was decreased by addition of chitin oligosaccharides (Fig. 3 *d*). Treatment of ghosts with chitinase abolished WGA-Au binding, except over the bud scar areas (Fig. 3 *e*).

Fluorescence Microscopy of Cell Ghosts

To complement the studies done by electron microscopy, observations of cell ghosts were made under the fluorescence microscope, with the use of FITC derivatives of both WGA and chitinases as markers. Because the fluorescein derivatives are much smaller than the gold particles, they penetrate easily the ghost cell walls and indicate the location of chitin even when it is deeply embedded in the walls.

With FITC-WGA, fluorescence was observed not only at the bud scar sites (cell poles), where most of chitin was expected to be, but also around the whole contour of the cell ghosts (Fig. 4 *a*).

After treatment with chitinase for 6 h, the fluorescence was restricted to the bud scar region (Fig. 4 *b*). Repeated additions of chitinase, followed by overnight incubation, gradually led to a decrease of the residual fluorescence until, after the third treatment, the ghosts were hardly visible (not shown).

After several incubations of cell ghosts with β-(1 $\rightarrow$ 6)-glucanase the same apparent effect was observed as with a single chitinase digestion, i.e., elimination of fluorescence from the lateral walls (Fig. 4 *c* and *d*). If the β-(1 $\rightarrow$ 6)-glucanase incubation was performed on the chitinase-treated ghosts of Fig. 4 *b*, the remaining fluorescence at the cell poles disappeared completely (Fig. 4 *e* and *f*).

The loss in fluorescence of lateral walls observed

[2] To avoid misunderstandings, it should be stressed here that *Streptomyces* chitinase was used, in different experiments, either as a label for chitin, when attached to gold particles or to FITC, or as an enzyme, to digest chitin or chitin oligosaccharides.

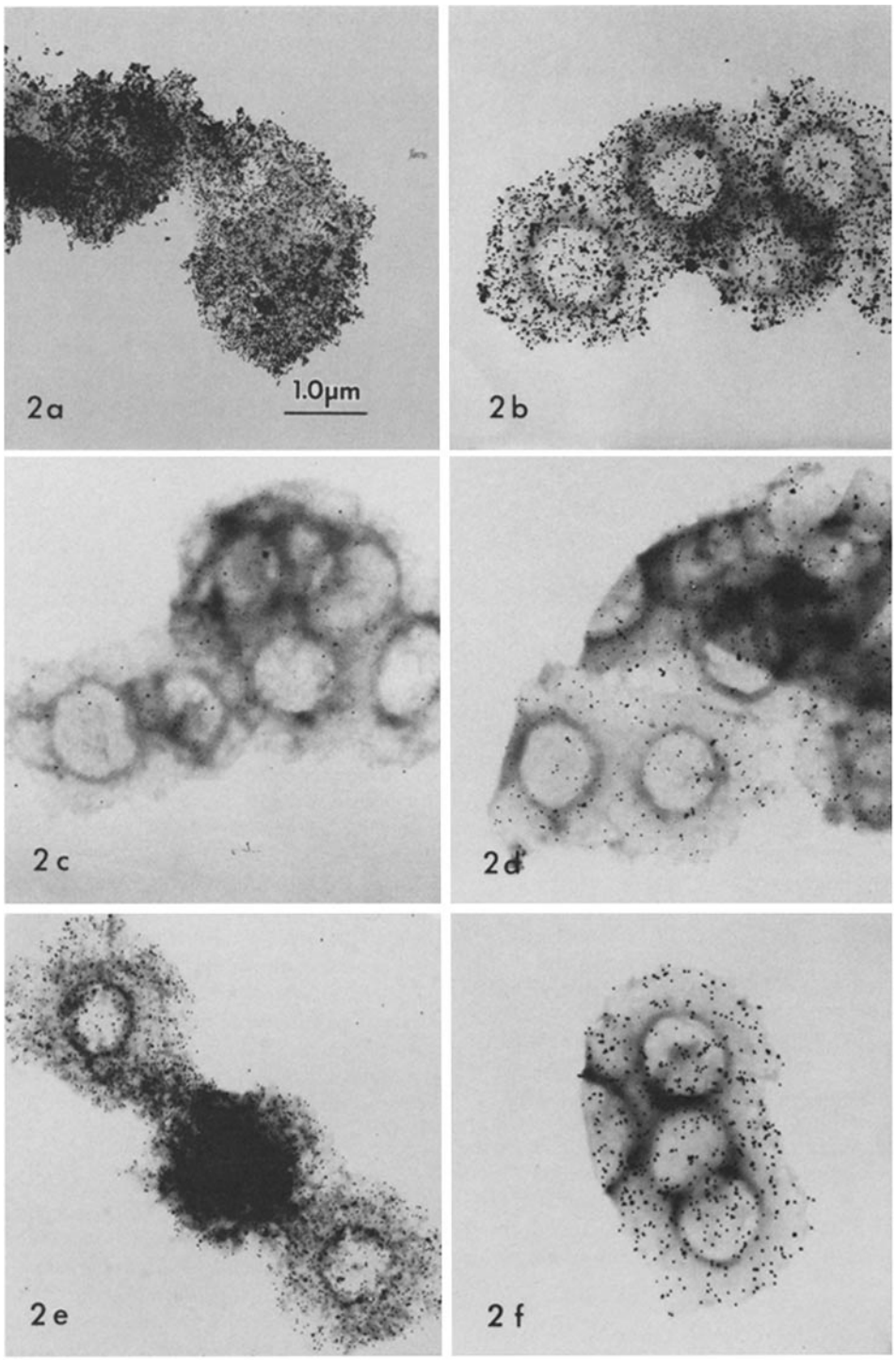

FIGURE 2 Labeling of primary septa with colloidal gold-marker particles. (*a*) WGA-Au as prepared, (*b*) WGA-Au at fivefold dilution, (*c*) control with colloidal gold only, (*d*) same experiment as *b*, but the septa were mixed with *N*-acetylchitopentaose at 1 mM final concentration, before adding WGA-Au, (*e*) labeling with *Streptomyces* chitinase-gold, (*f*) labeling with wheat germ chitinase-gold. × 11,900.

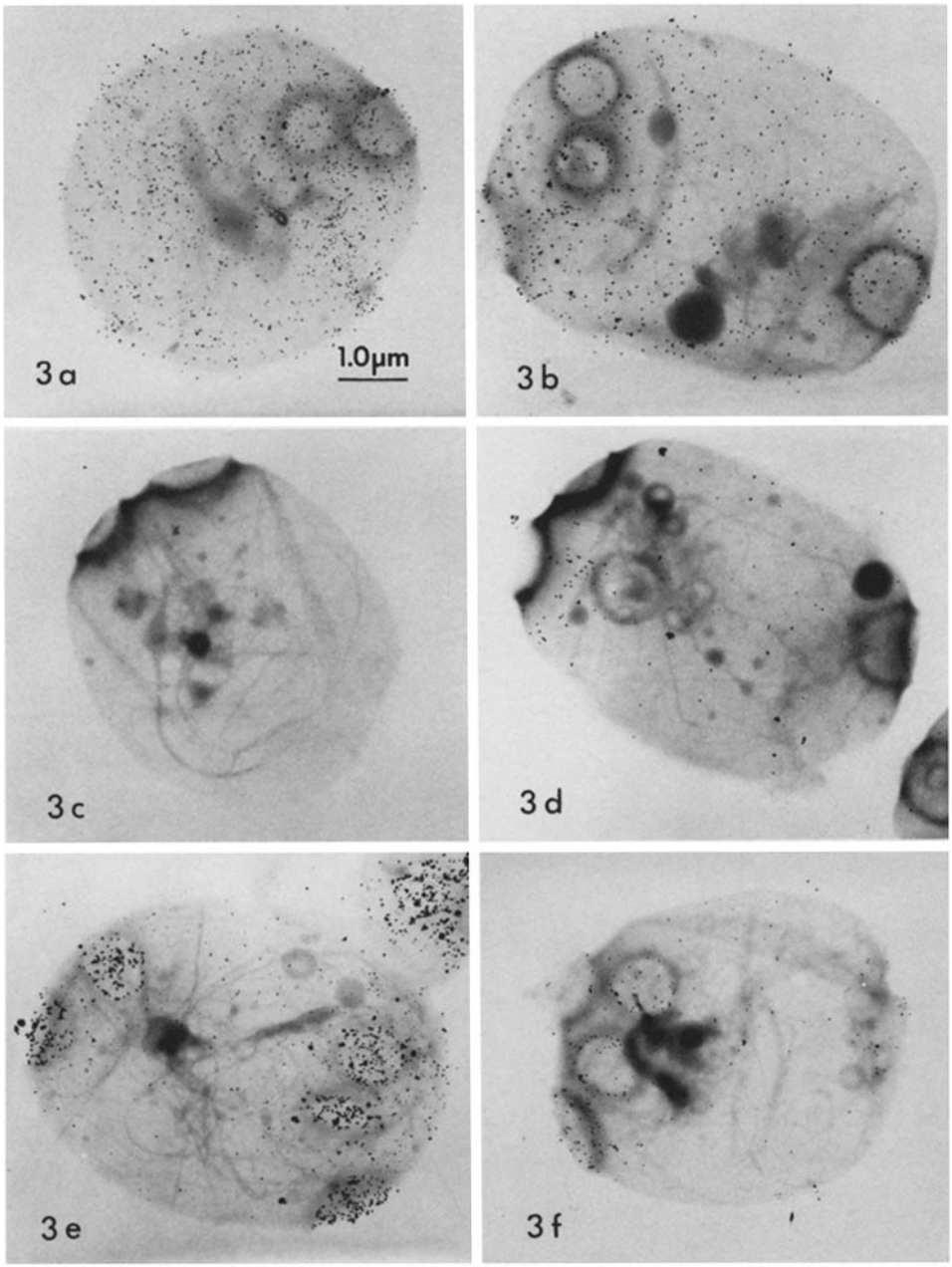

FIGURE 3 Labeling of cell ghosts with colloidal gold-marker particles. (*a*) WGA-Au, (*b*) WGA-Au at 1:5 dilution, (*c*) gold particles coated with bovine serum albumin, (*d*) same as *b*, but the ghosts were mixed with *N*-acetylchitopentaose at 1 mM final concentration before adding WGA-Au, (*e*) WGA-Au, (concentration as in *a*) on chitinase-treated ghosts, (*f*) WGA-Au, (concentration as in *b*) on β-(1 → 6)-glucanase-treated ghosts. × 9,100.

after β-(1 → 6)-glucanase treatment was paralleled by a decrease in the density of WGA-Au particles attached to the wall (Fig. 3*f*).

In contrast with the observations made with FITC-WGA, the fluorescein derivatives of wheat germ or *Streptomyces* chitinase yielded fluorescence only at the poles of the cell ghosts, i.e., in correspondence with the bud scars position (Fig.

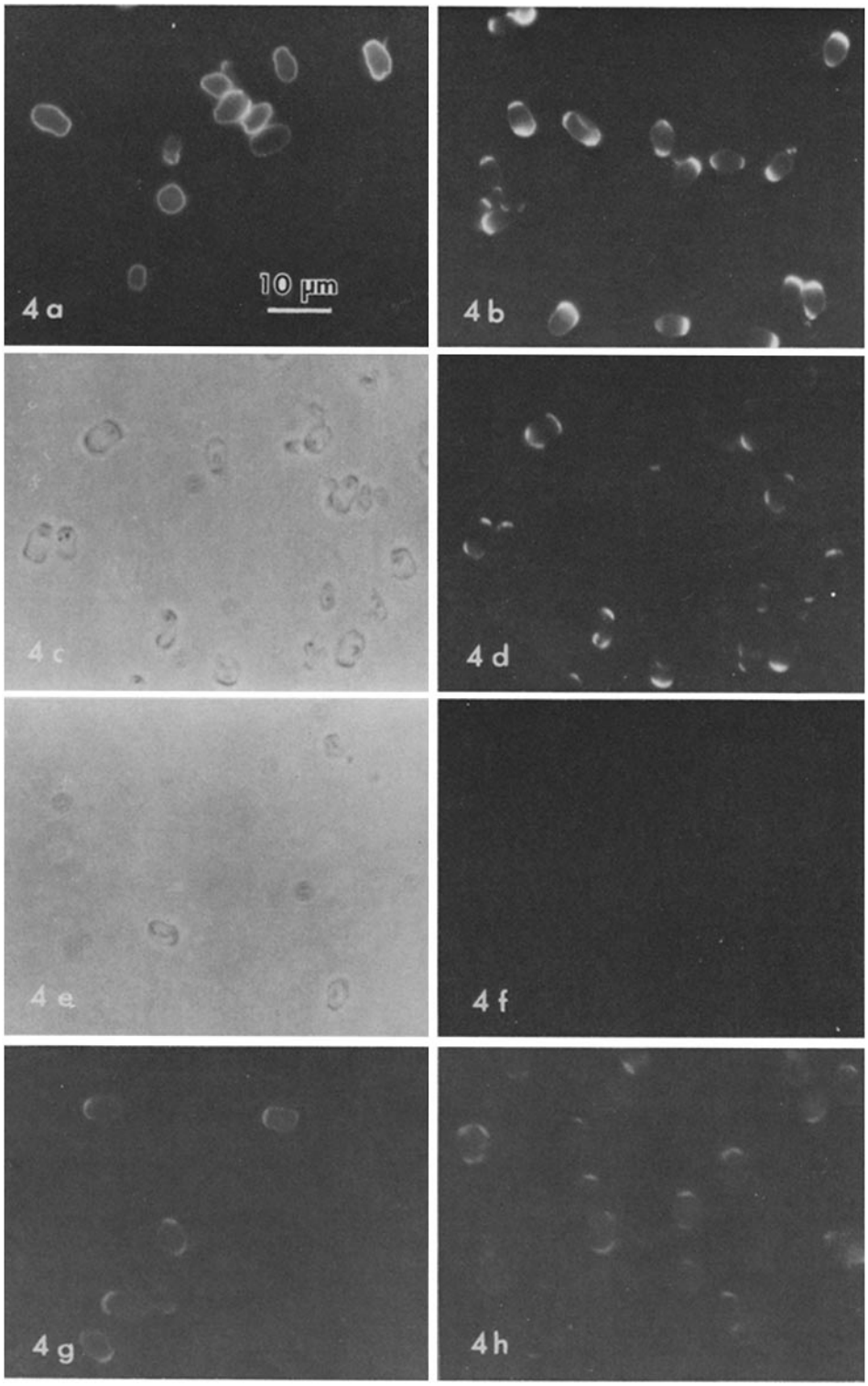

FIGURE 4 Fluorescence labeling of cell ghosts with FITC derivatives. (*a*) Intact ghosts with FITC-WGA; (*b*) chitinase-treated ghosts with FITC-WGA; (*c* and *d*) β-(1 → 6)-glucanase-treated ghosts with FITC-WGA: *c*, phase-contrast image, *d*, fluorescence image; (*e* and *f*) ghosts treated in succession with chitinase and β-(1 → 6)-glucanse, with FITC-WGA: *e*, phase-contrast image, *f*, fluorescence image; (*g*) intact ghosts with FITC-*Streptomyces* chitinase; (*h*) intact ghosts with FITC-wheat germ chitinase. × 940.

4 *g* and *h*). The result was especially clearcut with the *Streptomyces* enzyme. In these cases, previous treatment of the ghosts with *Streptomyces* chitinase led to total loss of fluorescence (not shown).

Studies with [^{14}C]Glucosamine-labeled Ghosts

To correlate the release of hexosamine-containing compounds from the cell ghosts with the morphological observations it was desirable to label the walls with radioactive glucosamine. It was not possible to do this with glucose-grown cells, because glucose competed with glucosamine for transport into the cells (V. Farkas and E. Cabib, unpublished observations). When galactose was substituted for glucose in the growth medium, however, glucosamine was taken up. At the low concentrations of aminosugar utilized, the glucose and mannose of cell wall polysaccharides were not labeled. About 45% of the total radioactivity in intact cells was recovered after conversion into cell ghosts. Besides mechanical losses, the loss in radioactivity may be ascribed to extraction of glucosamine-containing metabolites and to solubilization of mannan proteins, which contain a diacetylchitobiose bridge between polysaccharide and peptide moiety. Practically all the radioactivity remaining in the cell ghosts (97–98%, see Table I) could be solubilized with chitinase. Of this material, 85% was recovered after passage through a mixed bed exchange resin, an indication that the labeled substances were essentially uncharged (~90% recovery is routinely obtained with neutral sugars in columns of this type). The main product in the column effluent, as determined by paper chromatography, was diacetylchitobiose (Fig. 5 *A*), as expected from the specificity of *Streptomyces* chitinase (16). The small amount of free *N*-acetylglucosamine found (Fig. 5 *A*) is probably because of a small contamination of the chitinase with β-*N*-acetylglucosaminidase.

To determine the total radioactivity contained in lateral walls, the cell ghosts were treated with glucanase purified from snail intestinal juice. This preparation, which contains both β-(1 → 3) and (1 → 6)-glucanases, dissolved completely the walls, leaving only the chitin primary septa (see above). The amount of radioactivity liberated by this treatment was ~8% of the total (Table I).

By repeated digestions with β-(1 → 6)-glucanase a similar amount, ~7% of the total radioactivity, was solubilized (Table I). This figure was calculated after correcting for possible hydrolysis of bud scar chitin by the traces of chitinase present in the β-(1 → 6)-glucanase preparation. ^{14}C-labeled septa, in amount equivalent to that of the cell ghosts utilized, were used as substrate for enzyme; the liberated radioactivity was subtracted from that solubilized from intact cell ghosts (Table I). By measurements with anthrone it was found that ~25% of the cell ghosts glucan was also solubilized during incubation with β-(1 → 6)-glucanase.

The similarity between the amounts of radioactivity liberated by snail glucanase or β-(1 → 6)-glucanase suggests that β-(1 → 6)-glucanase is able to liberate all of the label associated with lateral walls of the ghosts.

Because β-(1 → 6)-glucanase incubation eliminated both the fluorescence along the lateral wall of intact ghosts and that remaining at the bud scar sites of chitinase-treated ghosts, it seemed possible that chitin and β-(1 → 6)-glucan might be cova-

TABLE I

*Solubilization of Radioactivity of [^{14}C]Glucosamine-labeled Cell Ghosts by Different Enzymatic Treatments**

Exp	Material used	Treatment	Solubilized radioactivity
			%
1	Cell ghosts	*Streptomyces* chitinase, 18 h	97, 98‡
2	Cell ghosts	Snail glucanase, 4 h§	7.9
3	Cell ghosts	β-(1 → 6)-glucanase, 48 h	7.8
4	Cell ghosts	β-(1 → 6)-glucanase, 2 × 48 h	10.5
5	Cell ghosts	β-(1 → 6)-glucanase, 3 × 48 h	12.0
6	Septa	β-(1 → 6)-glucanase, 48 h	2.3
7	Septa	β-(1 → 6)-glucanase, 2 × 48 h	4.0
8	Septa	β-(1 → 6)-glucanase, 3 × 48 h	4.8
		5 minus 8	7.2

* For conditions of different treatments, see Methods.

‡ Results of two different experiments.

§ All ghosts converted into septa, as observed by phase microscopy.

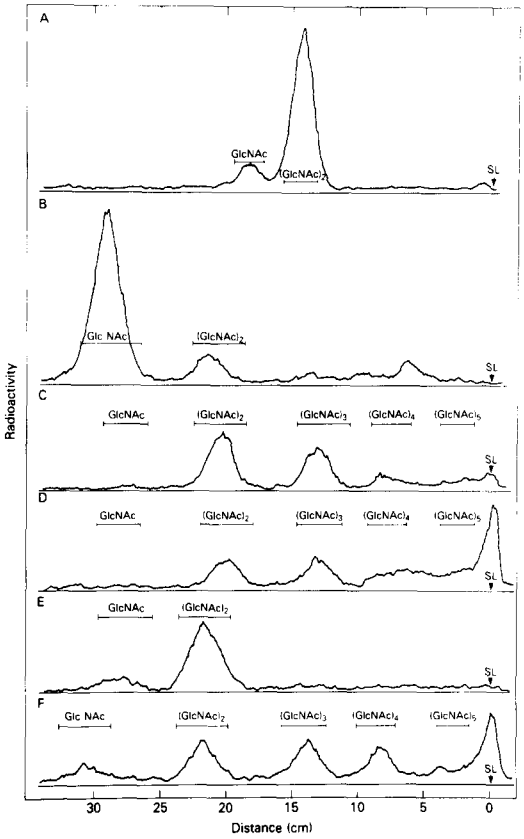

FIGURE 5 Paper chromatography of ^{14}C-labeled sugars. (*A*) Product of cell ghost incubation with *Streptomyces* chitinase, (*B*) β-(1 → 6)-glucanase digest of cell ghosts after hydrolysis with 6 N HCl and reacetylation, (*C*) β-(1 → 6)-glucanase digest after deionization, (*D*) β-(1 → 6)-glucanase digest, after *N*-acetylation and deionization, (*E*) *Streptomyces* chitinase product of the material in *D*, (*F*) snail glucanase digest of cell ghosts, after N-acetylation and deionization. GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, etc. indicate the position of *N*-acetylglucosamine and of the di-, tri- and higher oligosaccharides of the chitin series, used as standards. *SL*, starting line.

lently linked to each other. If that were the case, one would expect to find in the β-(1 → 6)-glucanase digest some oligosaccharide containing both glucose and *N*-acetylglucosamine. A search for such compounds was therefore carried out.

In the first place, practically all the radioactivity solubilized by β-(1 → 6)-glucanase corresponded to glucosamine compounds, as shown by the fact that hydrolysis with 6 N hydrochloric acid, followed by N-acetylation, desalting, and paper chromatography, resulted in a main peak of labeled *N*-acetylglucosamine (Fig. 5 *B*). A minor peak, whose position approximately coincides with that of diacetylchitobiose was also present (Fig. 5 *B*).

When the soluble products of β-(1 → 6)-glucanase digestion were applied to a mixed-bed exchange resin, only 36% of the radioactivity emerged with the effluent. Upon paper chromatography, two main radioactive peaks were observed, with the same R_f as diacetylchitobiose and triacetylchitotriose (Fig. 5 *C*). Some radioactive material with lower mobility was also detected.

Small trials with both anionic and cationic exchange resin columns indicated that most of the material retained in the mixed bed column was positively charged, perhaps as a result of deacetylation during the alkali treatments used in the preparation of cell ghosts. Indeed, after the β-(1 → 6)-glucanase digest was subjected to N-acetylation, the amount of radioactivity not retained by the mixed bed column increased to 64% of the total. Upon paper chromatography, this material yielded, in addition to the peaks observed in Fig. 5 *C*, others that moved more slowly (Fig. 5 *D*). Incubation of the N-acetylated and deionized glucanase digest with *Streptomyces* chitinase converted all the radioactive material into a substance with the same R_f as diacetylchitobiose (plus a small amount of *N*-acetylglucosamine, see Fig. 5 *E*). Thus, it appears that all the radioactive peaks of Fig. 5 *D* are chitin oligosaccharides. To verify the absence of glucose in the reducing terminals of the oligosaccharides, the substances moving as di- and trisaccharides were separately eluted from chromatograms and analyzed in greater detail.

Incubation of the putative disaccharide with *Streptomyces* chitinase followed by chromatography resulted in no visible change, as expected from the specificity of the enzyme (not shown). On the other hand, the trisaccharide yielded products moving as diacetylchitobiose and *N*-acetylglucosamine (Fig. 6), in addition to some remaining initial material. The three peaks of Fig. 6 *B* were separately eluted and counted. Of a total of 6,700 cpm recovered, 1,740 were in the *N*-acetylglucosamine area, 3,700 in the disaccharide, and 1,260 in the trisaccharide area. The ratio di- to monosaccharide was 2.1:1, close to the 2:1 value expected if the trisaccharide was uniformly labeled. This result practically excludes the possibility that an (unlabeled) glucose was at the reducing end of the trisaccharide. This was further confirmed by reducing both di- and trisaccharides with sodium borohydride. After hydrolysis with 6 N HCl and

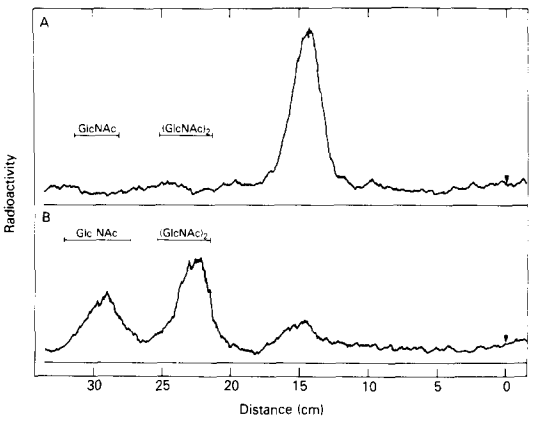

FIGURE 6 Effect of *Streptomyces* chitinase on trisaccharide. (*A*) Trisaccharide, as eluted from a chromatogram similar to that of Fig. 5 *C*, and rechromatographed; (*B*) same as *A*, after treatment with *Streptomyces* chitinase. Abbreviations as in Fig. 5. Arrows, starting line.

reacetylation, the mixture was subjected to paper electrophoresis with 2% molybdate at pH 5 as electrolyte. In both cases, peaks corresponding to *N*-acetylglucosamine and *N*-acetylglucosaminitol were detected (not shown).

The material released by snail glucanase was also analyzed. Its behavior on mixed bed exchange resin columns before and after N-acetylation was very similar to that of the β-(1 → 6)-glucanase digest. The same can be said of the paper chromatography profile of the N-acetylated and deionized material (Fig. 5 *F*). As with the β-(1 → 6)-glucanase digest, after treatment of this mixture with *Streptomyces* chitinase, practically all the radioactivity moved on paper as diacetylchitobiose, except for a small *N*-acetylglucosamine peak (not shown, but similar to Fig. 5 *E*).

Role of Contaminating Chitinase in the Action of the β-(1 → 6)-Glucanase Preparation

Preparations of β-(1 → 6)-glucanase, after purification through the Sephadex G-100 chromatography step (see Methods), were still contaminated with chitinase. Most of the chitinase (~95%) could be eliminated by passage through a chitin column, as described under Methods. Specific activities with respect to protein cannot be given, because albumin was added before the last step. On a relative basis, an amount of Sephadex G-100 eluate containing 1 U of β-(1 → 6)-glucanase activity (1 μmol of glucose equivalent liberated per min at 30°C) decomposed 39 nmol of chitin (as *N*-acetylglucosamine) in 16 h in the chitinase assay (18). After the chitin column step, the amount of chitinase product was reduced to 1.6 nmol under the same conditions. When incubated with cell ghosts, this preparation yielded small oligosaccharides of the chitin series, as mentioned in the previous section (Fig. 5 *C* and *D*). Therefore, it seemed that even the traces of chitinase present in the preparation might have an important role. To verify this point, cell ghosts were treated with three different preparations of glucanase: a Sephadex G-100 eluate, a preparation that had been further purified by passage through a chitin column, and another that had undergone a second chitin treatment. The effectiveness in eliminating FITC-WGA fluorescence from the lateral walls of the ghosts decreased from the first to the third preparation, i.e., as the chitinase contamination diminished. On the other hand, small amounts of chitinase in the absence of β-(1 → 6)-glucanase were ineffective. When ^{14}C-labeled cell ghosts were subjected to repeated incubations with an amount of *Streptomyces* chitinase that liberated about the same amount of radioactivity as the β-(1 → 6)-glucanase preparation, very little diminution of the fluorescence was observed. It is concluded that both β-(1 → 6)-glucanase and traces of chitinase (or very high amounts of chitinase alone, Fig. 4 *b*) are required to eliminate the WGA-binding material from the lateral cell walls.

DISCUSSION

The three methods used in this study complement each other. Thus, the use of gold-attached ligands allows examination of surface chitin distribution because the large gold granules cannot cross the ghost cell wall network. On the other hand, the smaller FITC derivatives can penetrate further and yield information about chitin presence in inner layers. Finally, the radioactive labeling of chitin affords the possibility of quantitative and chemical studies. The combination of the three approaches allows certain conclusions about the localization of chitin in the yeast cell wall.

In the first place, the evidence strongly supports the notion that chitin is distributed throughout the primary septum. The septa were completely covered with gold particles when either gold-bound WGA, wheat germ chitinase, or *Streptomyces* chitinase were used as probes. The small amount of glucan still present in the isolated septa is probably interspersed in between the chitin chains. These

results essentially coincide with those of Horisberger and Vonlanthen (15) obtained with yeast cell sections. In contrast, previous observations from the same laboratory on isolated septa (14) had shown weak labeling with WGA-Au, mainly restricted to the peripheral area of the septum. As the bud scar preparation used in those experiments had a high content of mannan (2), it may be that this polysaccharide was partially shielding chitin from the WGA-Au particles.

Although the WGA- or chitinase-labeled gold particles serve only as markers of the material on the surface, the complete dissolution of septa by purified chitinase show that the septa consist of chitin throughout. Together, these findings indicate that both the initial ring, which ends up as the primary septum "ridge," and the central portion of the septum are made up of chitin, as previously proposed (5).

The very heavy labeling of septa with WGA-Au contrasts with the sparse binding to cell ghosts, even in the bud scar areas. This result suggests that the primary septa in the bud scar are protected by a layer of residual glucan from secondary septa (4, 7), that the gold particles cannot penetrate. A similar conclusion was reached from direct observations of sections of intact cells and cell ghosts by electron microscopy (3, 4). Furthermore, the texture of the surface of metal-shadowed preparations is very different in intact bud scars and septal disks (4), another indication that the exposed material is of a different nature.

In contrast with the gold particles, the much smaller FITC derivatives of WGA and of the chitinases can freely diffuse through the intact ghost cell walls, thus imparting to the bud scar region an intense fluorescence. With FITC-WGA, the fluorescence also extended to the lateral walls in agreement with the sparse distribution of WGA-Au on cell ghosts (cf. also reference 15). Although the lateral wall WGA-binding material is a minor component, it seemed important to establish its nature, both because of possible physiological implications and because of its interference with the use of the lectin as a probe for the formation of the chitin septum.

Several lines of evidence indicate that this material is also chitin. Firstly, the binding of the WGA-Au particles appears to be specific, inasmuch as it was severely inhibited by chitin oligosaccharides; furthermore, albumin-coated particles did not attach to the ghosts. Secondly, treatment with *Streptomyces* chitinase under conditions that remove 97% of the radioactivity from ^{14}C-glucosamine-labeled ghosts eliminated the FITC-WGA fluorescence as well as the WGA-Au binding from lateral walls, although fluorescence remained in the bud scar area. Thirdly, the same apparent result was obtained by prolonged incubation with a purified, but chitinase-contaminated preparation of β-(1 $\rightarrow$ 6)-glucanase. In this case only ~7% of the total radioactivity was released from [^{14}C]glucosamine labeled ghosts. The coincidence between this amount and that liberated by complete digestion of the lateral walls with snail glucanase suggests that the same material is solubilized in both cases. This conclusion is reinforced by the similarity in the chromatographic pattern of the radioactive material in both hydrolysates[3] (Fig. 5 *D* and *F*). In both digests it was shown that all the compounds belonged to the chitin oligosaccharide series, because they were all converted into diacetylchitobiose (plus a little *N*-acetylglucosamine) by *Streptomyces* chitinase.

Because the chitin of lateral walls amounts to <10% of the total, it follows that the bulk of the polysaccharide is in the primary septa.

An unexplained result is the relatively strong labeling of the bud scar area by both WGA-Au and FITC-WGA, after incubation with chitinase (Figs. 3 *e* and 4 *b*). It would seem as if the accessibility of the residual chitin to the markers had been enhanced by this treatment. It may be that on removal of the thick chitin "filling," the thin glucan layer that covers the bud scar partially collapses and permits easier access to the remaining chitin. Nevertheless, no evidence is as yet available on this point.

The liberation of chitin oligosaccharides by β-(1 $\rightarrow$ 6)-glucanase treatment, added to the correlation between fluorescence decrease and chitinase content of glucanase preparations (see Results), strongly suggest that both glucanase and chitinase are required for efficient removal of the radioactive and WGA-binding material. Furthermore,

[3] On the basis of this result, one would have expected to find some chitinase activity in the snail glucanase, as was the case for the β-(1 $\rightarrow$ 6)-glucanase. Nevertheless, we were unable to detect such activity in the chitin-treated snail enzyme. Perhaps the amount present was below the sensitivity of our assay. The alternative possibility, that the small chitin oligosaccharides were preformed in the walls and retained through hydrogen bonding by the β-(1 $\rightarrow$ 6)-glucan, seems remote in consideration of the drastic treatment used for conversion of cells into ghosts.

complete elimination of the residual fluorescence in the bud scar area of chitinase-treated ghosts was obtained by incubation with β-(1 → 6)-glucanase. All these results point to a strong association between chitin and β-(1 → 6)-glucan, a relatively minor component of the yeast cell wall (17). Whether this association is simply physical or includes covalent linkages, it is difficult to decide at the present time. The finding that *Streptomyces* chitinase by itself could eliminate almost completely the FITC-WGA fluorescence, albeit with difficulty, supports the notion that chitin and β-(1 → 6)-glucan are not covalently linked. Furthermore, examination of the radioactive di- and trisaccharide resulting from β-(1 → 6)-glucanase digestion failed to reveal any glucose at the reducing end. The higher oligosaccharides could not, however, be analyzed in the same way because sufficient material was not available. The existence of a few *N*-acetylglucosamine-glucose linkages remains therefore a possibility.

Finally, it is worth noting that before reacetylation half or more of the radioactive material liberated from lateral walls by either β-(1 → 6)-glucanase or snail glucanase was retained on an ion exchange resin. In contrast, the radioactivity solubilized by *Streptomyces* chitinase, which comprises ~97% of the total and is mainly derived from bud scars, was almost completely uncharged, i.e. acetylated. It seems probably that initially all chitin chains were fully acetylated, but some were deacetylated by the alkali treatments used in the preparation of cell ghosts. The bud scar chitin forms a large, tightly packed agglomerate, as shown by the ability of primary septa to maintain their shape after being freed of surrounding structures. It may therefore be more resistant to alkali attack than the relatively more isolated chains or fibrils of lateral walls. This would also explain why *Streptomyces* chitinase can eliminate the fluorescence from lateral walls much more easily than from bud scars. We have recently shown (19) that isolated chitin chains are very susceptible to the attack of chitinases.

The conclusions of the previous discussion are summarized in the drawing of Fig. 7. In this scheme, chitin is present at a high density in the bud scar and at a very low density on the lateral wall. β-(1 → 6)-Glucan is pictured as closely associated with the chitin. FITC-WGA, because of its strong affinity for β-(1 → 4)-*N*-acetylglucosaminyl residues, would bind to chitin both at the bud scars and on the lateral wall, thus yielding almost uniform fluorescence. On the other hand, the FITC-chitinase derivatives would yield fluorescence only in the bud scar region, because of their weaker binding. The poorer attachment of the chitinases may actually be an asset when it is desired to study specific events occurring during chitin deposition in the septal area, without interference from the lateral wall material.

In the outline of Fig. 7, it is assumed that chitinase can eliminate practically all of the lateral wall chitin, despite its association with β-(1 → 6)-glucan, because of the greater susceptibility of the isolated fibrils and of their surface location. On the other hand, the chitinase does not succeed in hydrolyzing all of the more deeply buried polysaccharide inside the bud scar. From the results with ^{14}C-labeled ghosts, the remaining chitin cannot be >3% of the total. Yet, the bud scars are still strongly fluorescent with FITC-WGA (Fig. 4*b*), a result that illustrates the extreme sensitivity of this method. Attack with β-(1 → 6)-glucanase (Fig. 7, lower left) eliminates the β-(1 → 6)-glucan and, thanks to the presence of traces of chitinase, also the chitin from lateral walls. The same treatment, when performed on chitinase-treated ghosts (Fig. 7, lower right) now eliminates the remaining bud scar chitin together with the associated β-(1 → 6)-glucan.

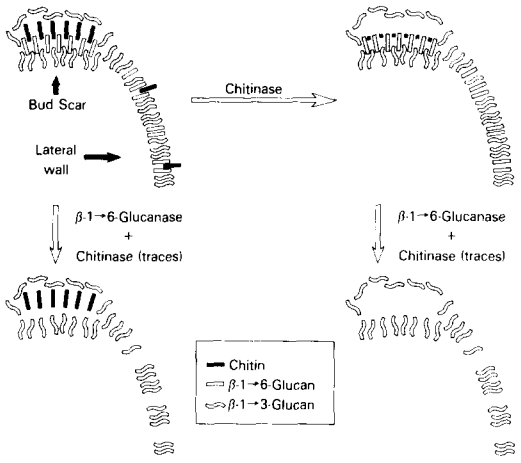

FIGURE 7 Scheme of the distribution of chitin and other polysaccharides in cell ghosts before and after action of different enzymes. The scheme is highly simplified and does not attempt to portray the real situation in detail. Fibrils of β-(1 → 3)-glucan have been laid horizontally over the bud scar to suggest a thin layer, not to signify that they have that orientation. The relative density of chitin in bud scar and lateral wall is not necessarily the real one.

The origin of the chitin interspersed in the lateral wall is unknown. It may result from occasional activation of some molecules of chitin synthetase zymogen or by movement of active chitin synthetase along the plasma membrane (8, 10) from the septum site to random positions over the cell surface. Alternatively, it may be ascribable to an incomplete evolutionary loss of the insertion of chitin in the lateral cell wall which occurs in filamentous fungi.

Aside from the physiological significance of the results, this study illustrates the potentialities, for the study of cell wall structure, of a combination of methods, such as fluorescence and electron microscopy of specific ligands attached to different carriers, action of enzymes, and use of radioactive labeling of specific polysaccharides.

The authors are grateful to Dr. H. J. Phaff (University of California, Davis) for a strain of *Bacillus circulans* and to Dr. F. Rombouts (Agricultural University, Wageningen, The Netherlands) for a sample of purified β-(1 → 6)-glucanase. They also wish to express their thanks to Drs. G. Ashwell, J. Correa, A. Duran, C. Marcus, I. Polacheck, E. Shematek, and M. Slater for useful discussions and criticisms.

Received for publication 10 August 1979, and in revised form 17 December 1979.

REFERENCES

1. BACON, J. S. D., E. D. DAVIDSON, D. JONES, and I. F. TAYLOR. 1966. The location of chitin in the yeast cell wall. *Biochem. J.* **101**:36C–38C.
2. BAUER, H., M. HORISBERGER, D. A. BUSH, and E. SIGARLAKIE. 1972. Mannan as a major component of the bud scars of *Saccharomyces cerevisiae*. *Arch. Mikrobiol.* **85**:202–208.
3. BOWERS, B., G. LEVIN, and E. CABIB. 1974. Effect of polyoxin D on chitin synthesis and septum formation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **119**:564–575.
4. CABIB, E., and B. BOWERS. 1971. Chitin and yeast budding. Localization of chitin in yeast bud scars. *J. Biol. Chem.* **246**:152–159.
5. CABIB, E., and B. BOWERS. 1975. Timing and function of chitin synthesis in yeast. *J. Bacteriol.* **124**:1586–1593.
6. CABIB, E., and V. FARKAS. 1971. The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2052–2056.
7. CABIB, E., R. ULANE, and B. BOWERS. 1974. A molecular model for morphogenesis: the primary septum of yeast. *Curr. Top. Cell. Regul.* **8**: 1–32.
8. DURAN, A., B. BOWERS, and E. CABIB. 1975. Chitin synthetase zymogen is attached to the yeast plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3952–3955.
9. DURAN, A., and E. CABIB. 1978. Solubilization and partial purification of yeast chitin synthetase. Confirmation of the zymogenic nature of the enzyme. *J. Biol. Chem.* **253**:4419–4425.
10. DURAN, A., E. CABIB, and B. BOWERS. 1979. Chitin synthetase distribution on the yeast plasma membrane. *Science (Wash. D. C.).* **203**:363–365.
11. FLEET, G. H., and H. J. PHAFF. 1974. Lysis of yeast cell walls: glucanases from *Bacillus circulans* WL-12. *J. Bacteriol.* **119**:207–219.
12. GEOGHEGAN, W. D., and G. A. ACKERMAN. 1977. Absorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: A new method, theory and application. *J. Histochem. Cytochem.* **25**:1187–1200.
13. HAYASHIBE, M., and S. KATOHDA. 1973. Initiation of budding and chitin-ring. *J. Gen. Appl. Microbiol.* **19**:23–29.
14. HORISBERGER, M., and J. ROSSET. 1976. Localization of wheat germ agglutinin receptor sites on yeast cells by scanning electron microscopy. *Experientia (Basel).* **32**:998–1000.
15. HORISBERGER, M., and M. VONLANTHEN. 1977. Location of mannan and chitin on thin sections of budding yeasts with gold markers. *Arch. Microbiol.* **115**:1–7.
16. JEUNIAUX, C. 1966. Chitinases. *Methods Enzymol.* **8**:644–650.
17. MANNERS, D. J., A. J. MASSON, J. C. PATTERSON, H. BJÖRNDAL, and B. LINDBERG. 1973. The structure of a β-(1 → 6)-D-glucan from yeast cell walls. *Biochem. J.* **135**:31–36.
18. MOLANO, J., A. DURAN, and E. CABIB. 1977. A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* **83**:648–656.
19. MOLANO, J., I. POLACHECK, A. DURAN, and E. CABIB. 1979. An endochitinase from wheat germ. Activity on nascent and performed chitin. *J. Biol. Chem.* **254**:4901–4907.
20. RINDERKNECHT, H. 1960. A new technique for the fluorescent labeling of proteins. *Experientia (Basel).* **16**:430.
21. SEICHERTOVA, O., K. BERAN, Z. HOLAN, and V. POKORNY. 1973. The chitin-glucan complex of *Saccharomyces cerevisiae*. II. Location of the complex in the encircling region of the bud scar. *Folia Microbiol.* **18**: 207–211.
22. TREVELYAN, W. E., and J. S. HARRISON. 1952. Studies of yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. *J. Biochem.* **50**:298–303.