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Vectorial synthesis of a polysaccharide by isolated plasma membranes

(chitin/chitin synthase/*Saccharomyces cerevisiae*)

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ABSTRACT **To ascertain the directionality of chitin synthesis by yeast plasma membranes, the external surface of *Saccharomyces cerevisiae* protoplasts was labeled with ferritin–concanavalin A. After protoplast lysis, plasma membranes were isolated and treated with trypsin to activate chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxy-D-glucosyltransferase, EC 2.4.1.16). The membranes were then enrobed in agar and allowed to synthesize chitin from UDP-*N*-acetylglucosamine. After fixation and embedding in Epon, thin sections were stained for chitin with wheat germ agglutinin–colloidal gold complexes. The chitin marker was found near the ferritin-labeled external face of the membrane—i.e., the polysaccharide was located on the outside of the membrane, as it is in the intact cell. Chitin synthase activity was not detected in intact protoplasts before or after treatment with trypsin. The enzyme became available to trypsin activation after lysis of the protoplasts. Together with similar, previously reported experiments on the inactivation of chitin synthase by glutaraldehyde, these results indicate that the enzyme faces the interior of the cell. We conclude that, both *in vivo* and *in vitro*, the synthase receives *N*-acetylglucosamine residues from UDP-*N*-acetylglucosamine at the cytoplasmic face of the membrane and transfers them vectorially to a growing chain of chitin that is concomitantly extruded to the outside.**

In bacteria, fungi, and plants, the structural polysaccharides of the cell wall are found outside the plasma membrane. Their precursors, usually sugar nucleotides, are formed in the cytoplasm. Little is known about the mechanisms by which the glycosyl units cross the plasma membrane. In most cases, it has not even been established whether assembly of the sugar chains precedes, accompanies, or follows extrusion through the membrane. The hydrophilic character of sugars and the insolubility of most cell wall polysaccharides pose formidable obstacles to passage through the membrane lipid layer. For some polysaccharides of bacterial cell walls, such as peptidoglycan, teichoic acid, and the lipopolysaccharide of enteric bacteria, the problem seems to have been solved by attachment of oligosaccharide intermediates to lipid carriers able to cross the membrane (1). Another possibility is preassembly within the cell followed by exocytosis of secretory vesicles as seems to occur with yeast mannoproteins (2). In other cases, however, such as for chitin (3) or β(1-3)glucan (4), no evidence could be found for involvement of a lipid carrier or for exocytosis. Progress in understanding the mechanism of assembly and translocation of glycosyl residues has been hampered by the lack of cell-free systems able to perform both functions. We report here the availability of such a system. In it, isolated plasma membranes from the yeast *Saccharomyces cerevisiae* can both synthesize and translocate to the outside the polysaccharide chitin, the main component of the yeast primary septum (5).

MATERIALS AND METHODS

Growth of Yeast and Preparation of Protoplasts. *S. cerevisiae* X2180 (ATCC 26109) was grown either in minimal medium (6) or in YEPD (3) to the middle of logarithmic phase. Yeast cells were converted into protoplasts as described (3, 7). Protoplasts were stored in 0.8 M sorbitol.

Preparation of Plasma Membranes. The method was a modification of a published procedure (8). To 0.7 ml of protoplast suspension, obtained from 0.7 g (wet weight) of yeast cells, was added 9.1 ml of a solution containing 0.8 M sorbitol, 50 mM Tris·HCl at pH 7.5, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $MnSO_4$, and 1.89 mg of ferritin–concanavalin A (Con A) (Miles Laboratories; containing 0.117 mg of Con A according to the manufacturer). After incubation for 20 min at room temperature, 0.21 ml of Con A (20 mg/ml) was added and the mixture was incubated for an additional 10 min. The flocculated protoplasts were harvested by centrifugation for 1 min at 2°C in a swinging bucket rotor at 250 × *g*. The pellet was carefully resuspended in 9.1 ml of the sorbitol/Tris/Mg^{2+}/Ca^{2+}/Mn^{2+} mixture described above but lacking Con A. After centrifugation for 3 min at 250 × *g*, the pellet was washed a second time in the same way. Finally, the pellet was resuspended in 11.9 ml of 1 mM EDTA at room temperature, rapidly transferred to a Lourdes Multi-mix homogenizer, and stirred for 3 min at 40% of maximum speed while being cooled in ice. After incubation of the lysate for 15 min at 30°C, plasma membranes were isolated by centrifugation in linear gradients of Renografin as described (8). The membrane band (4.6 ml, pooled from three 14-ml tubes) was diluted with 7 ml of 50 mM Tris·HCl (pH 7.5) and centrifuged at 8,000 × *g* for 10 min. The sedimented membranes were washed once with 10 ml of 50 mM Tris, pH 7.5/2 mM $MgSO_4$ and resuspended in the same buffer to a final volume of 0.5 ml.

Chitin Synthesis with Plasma Membrane Preparations. Aliquots of the membrane suspensions were incubated with the necessary amount of trypsin to obtain optimal activation of chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxy-D-glucosyltransferase, EC 2.4.1.16) (3). After the reaction was stopped with soybean trypsin inhibitor (3), the membranes were enrobed in agar (9). Cylindrical agar blocks (1 × 1 mm) were incubated at 30°C in a standard reaction mixture for chitin synthase assay (ref. 3; see also legend of Table 1) with UDP-*N*-acetylglucosamine as substrate. Fixation and embedding followed published procedures (9).

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Abbreviations: Con A, concanavalin A; WGA, wheat germ agglutinin; WGA-gold, wheat germ agglutinin–colloidal gold complexes.

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Staining of Chitin with Wheat Germ Agglutinin–Gold Complexes (WGA-gold). The complex was prepared as described (10). Thin sections were stained with the complex according to Horisberger and Vonlanthen (11). The sections were then stained for 5 min in 1% uranyl acetate and for 5 min in 3% lead citrate before examination with the electron microscope.

Preparation of a Membrane Fraction from Untreated or Trypsin-Treated Protoplasts. For the experiment of Table 2, 0.6 ml of protoplast suspension obtained from 0.6 g (wet weight) of cells was incubated for 15 min at 30°C with 0.2 or 0.4 mg of trypsin in a total volume of 1 ml and at a final sorbitol concentration of 0.8 M. The reaction was stopped by addition of 0.3 or 0.6 mg, respectively, of soybean trypsin inhibitor in a volume of 10 μl. A control was incubated without trypsin. The protoplasts were disrupted by homogenization in 8.5 ml of 1 mM EDTA as described above and the lysate was centrifuged at 105,000 × *g* for 30 min. The membrane pellet was washed with 8 ml of 25 mM Tris·HCl, pH 7.5/5 mM $MgSO_4$ and resuspended in the same buffer to a final volume of 0.83 ml.

RESULTS

Unidirectional Synthesis of Chitin by Yeast Plasma Membranes. By isolating intact plasma membranes from *S. cerevisiae*, we had shown that chitin synthase, in a zymogen form, is associated with the membranes (8). In the present study, plasma membranes were isolated from Con A-coated protoplasts, essentially as reported (8), but some ferritin-Con A was included in the treatment of the intact protoplasts to provide a label for the external surface of the membranes. After activation of chitin synthase zymogen with trypsin, the membranes were enrobed in agar to prevent diffusion of the reaction product in the subsequent incubation. When the enrobed membranes were incubated with UDP-*N*-acetyl[^{14}C]glucosamine under the conditions of the chitin synthase assay, an insoluble product accumulated in time-dependent fashion (Table 1). The reaction was inhibited by polyoxin D, a specific inhibitor of chitin synthase (12).

In parallel experiments, enrobed membranes were incubated with or without unlabeled UDP-*N*-acetylglucosamine and with or without polyoxin D. After fixation with glutaraldehyde and osmium tetroxide, the membranes were embedded in Epon. Thin sections cut from these preparations were stained with WGA-gold to ascertain the distribution of chitin (11). In the absence of substrate (Fig. 1*e*) or in the presence of polyoxin D (Fig. 1*d*), only a few gold grains remained attached to the section. After incubation with the complete system, numerous gold particles were seen in close juxtaposition to the membrane profiles (Fig. 1 *a–c*). Almost invariably, the grains were found near the outer face of the membranes, as identified by the ferritin-Con A marker (arrowheads in Fig. 1). Occasionally, ambiguous situations resulted from the closeness of membrane fragments with opposing polarity, as seen for example in the cluster of membranes in the lower portion of Fig. 1*b*.

Table 1. Synthesis of chitin by agar-enrobed membranes

Incubation conditions	*N*-Acetylglucosamine incorporated, nmol
Standard mixture, 1 hr at 30°C	7.0
Standard mixture, 3 hr at 30°C	15.1
Standard mixture plus 0.27 mM polyoxin D, 3 hr at 30°C	0.3

Each mixture contained five agar blocks (1 × 1 mm) containing trypsin-treated membranes, 33 mM Tris·HCl at pH 7.5, 35 mM *N*-acetylglucosamine, 1.8 mM $MgSO_4$, and 1.1 mM UDP-*N*-acetyl[^{14}C]glucosamine (New England Nuclear; 4×10^5 cpm/μmol) in a total volume of 45 μl. After incubation, agar blocks were washed with 1 ml of the water three times; the blocks were allowed to soak in water for 10 min each time. Finally, 0.5 ml of water was added and the agar was melted by heating for 5 min in a boiling water bath. The tube contents were transferred quantitatively to scintillation vials and their radioactivity was measured after addition of Hydrofluor (National Diagnostics).

The purity of the plasma membrane preparation is indicated by the presence of ferritin on almost all of the visible profiles. Only one side of each membrane profile was labeled with ferritin, as expected from exclusive binding of ferritin-Con A to the external surface of the protoplasts. The label also shows that most membranes are curled inside out and that some smaller fragments appear to have given rise to inverted vesicles. The very rare finding of a primary septum fragment, probably originated in a bud scar (Fig. 1*d*), provided an additional internal control for the staining procedure; the fragment is covered with gold particles as expected from its chitin content (10).

These results show that chitin is laid down on the external side of the plasma membranes but do not provide information about the orientation of chitin synthase in the membrane.

Sidedness of Chitin Synthase on Plasma Membrane. We have reported (8) that chitin synthase is unaffected by treatment of the external face of the plasma membrane, as in intact protoplasts, with glutaraldehyde but is rapidly and irreversibly inactivated when the inner side of the membrane is exposed to glutaraldehyde. These results indicate that the synthase is exposed on the cytoplasmic side of the membrane. In another approach to this problem, the activity of the enzyme and its sensitivity to trypsin were measured in intact and lysed protoplasts.

Experiments with intact protoplasts required the presence of 0.8 M sorbitol as osmotic stabilizer. In a preliminary trial with a membrane preparation it was ascertained that sorbitol did not interfere significantly either with proteolytic activation or with activity of chitin synthase (not shown). Intact protoplasts did not exhibit synthase activity (Table 2). After trypsin treatment, a small amount of activity was detected. A few lysed protoplasts were observed in the preparation by phase-contrast microscopy and probably were responsible for this activity. No additional lysis occurred as a result of trypsin treatment. When untreated or trypsin-treated protoplasts were disrupted by osmotic shock and the particulate fraction was isolated, chitin synthase activity did not increase. Further treatment of both preparations with trypsin, however, elicited a large increase in

Table 2. Chitin synthase in intact protoplasts and in particulate fraction before and after trypsin treatment

Fraction used	*N*-Acetylglucosamine incorporated into chitin, nmol
Untreated protoplasts:	
Intact protoplasts	0.1
Membranes before trypsin	0.2
Membranes after trypsin	5.1
Trypsin-treated protoplasts:	
Intact protoplasts	0.4
Membranes before trypsin	0.6
Membranes after trypsin	4.6

All incubations with intact protoplasts included 0.8 M sorbitol. Incubation mixtures for chitin synthase activity determination were as described in Table 1. An amount of protoplasts or of membranes from 6 mg (wet weight) of yeast cells was used per incubation mixture. When membranes were treated with trypsin, the amount of protease was 2 μg per reaction mixture. After incubation for 30 min at 30°C, the activity of chitin synthase was determined by measuring incorporation of radioactivity into insoluble material by a filtration method (4).

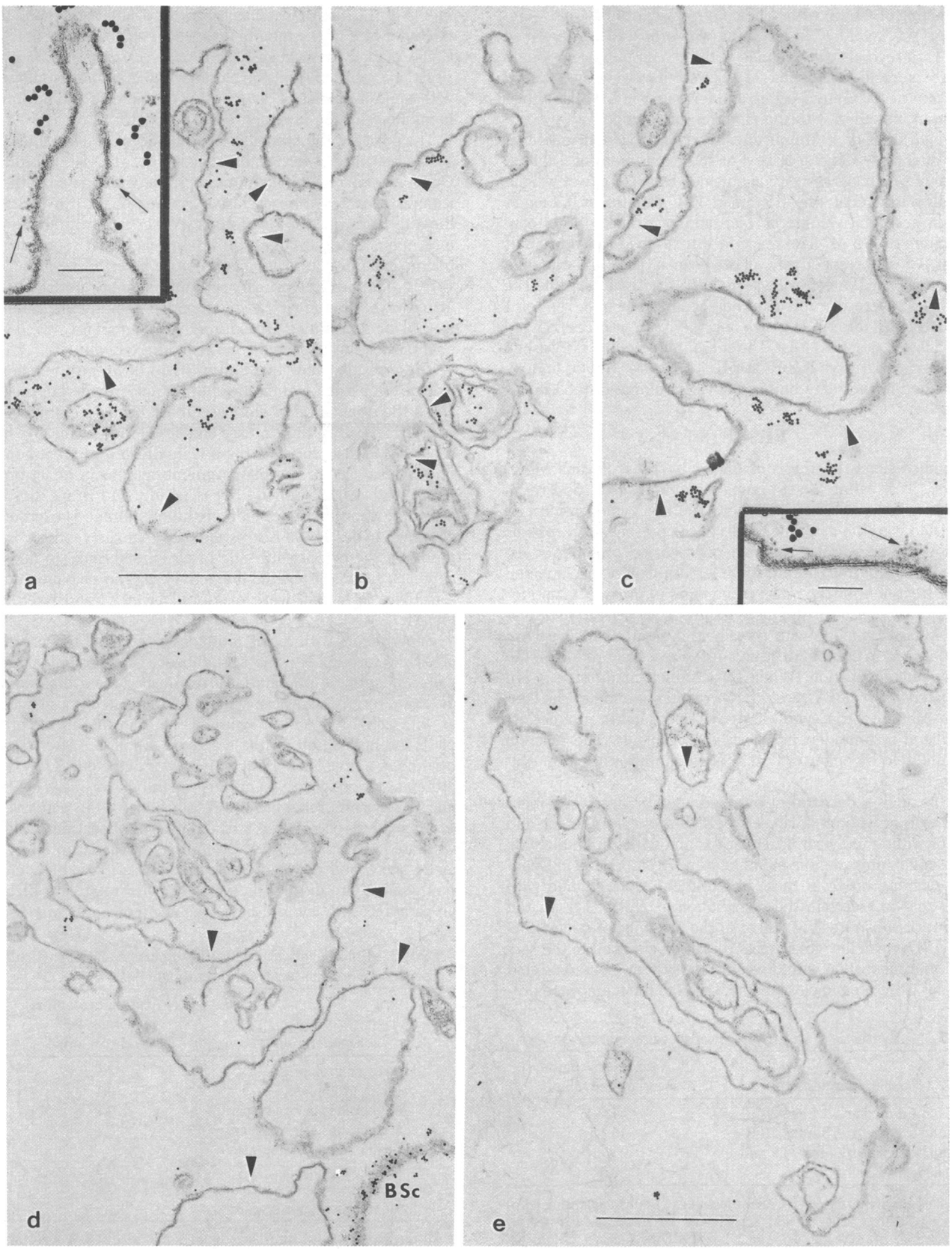

FIG. 1. Location of chitin synthesized by isolated plasma membranes. Gold grains indicate the distribution of chitin, whereas ferritin molecules (see arrows on high-magnification *Insets* in *a* and *c*) identify the outer face of membranes, which is marked with arrowheads. The thin filaments in the background come from the agar matrix. (*a–c*) Complete incubation mixture, 1-hr incubation at 30°C. Note that smaller membrane fragments also clearly derive from the plasma membrane, as indicated by the ferritin label. (*d*) Complete mixture plus 0.27 mM polyoxin D. BSc, fragment of bud scar that consists of chitin and is heavily labeled with gold particles. (*e*) Incubation mixture lacking UDP-*N*-acetylglucosamine. (Bars: *a* and *e*, 1 μm; *Insets*, 0.1 μm. *a–c*, ×32,700; *d* and *e*, ×24,400; *Insets*, ×79,800.)

activity (Table 2). Doubling the amount of trypsin used for the treatment of protoplasts did not alter the results (data not shown). The data show that the zymogen of chitin synthase becomes available for proteolytic activation only after disruption of the protoplasts.

DISCUSSION

In a previous study (9), we showed that chitin synthesized by a yeast plasma membrane preparation remained closely associated with the membranes. In those experiments, neither the orientation of the membranes nor the precise location of the chitin could be determined. In the present work, the outer face of the plasma membrane was unambiguously identified with ferritin-Con A and the position of the synthesized chitin was pinpointed by staining with WGA-gold. The identification of the material located by WGA-gold as chitin is supported by several lines of evidence. The only polysaccharide synthesized by yeast membrane preparations from UDP-*N*-acetylglucosamine is chitin, as shown by digestion of the product by purified chitinase (12). The membranes do not contain or make any product that binds WGA-gold in the absence of substrate (Fig. 1*e*). No staining was observed when polyoxin D, a specific competitive inhibitor of chitin synthase (12, 13), was included in the reaction mixture (Fig. 1*d*). Furthermore, WGA binds specifically to β-(1-4)-linked *N*-acetylglucosamine groups (14) and WGA-gold has been shown to be a specific label for chitin in yeast cell wall preparations (10). From the distribution of the gold grains we therefore conclude that the synthesized chitin was located near the outer face of the membrane—i.e., in the same position as it is found *in vivo* (6).

Whereas the reaction product is found on the outer side of the plasma membrane, the enzyme that catalyzes the reaction, chitin synthase, appears to be exposed on the cytoplasmic side: the enzyme is not susceptible to inactivation by glutaraldehyde or to activation by trypsin unless the protoplasts are lysed and the inner face of the membrane is exposed. Different and more intricate interpretations of the results are conceivable—e.g., that the synthase zymogen is exposed at the outer membrane surface and that both glutaraldehyde and trypsin can act on it only from a transmembrane position, not directly. There is no evidence, however, for such peculiar effects, and at present the straightforward conclusion that the synthase faces the cytoplasm seems to be the best way to explain our data.

The results also show that only membranes, and not small particles such as chitosomes (15, 16), are involved in chitin synthesis in our system. The absence of activation of chitin synthase by trypsin in intact protoplasts indicate that, if present, chitosomes are intracellular. The position of synthesized chitin at the outer face of the membrane, however, would require the organelles to move *en bloc* to the outside of the membrane after protoplast lysis, which seems an unlikely event.

The enzyme responsible for the synthesis of UDP-*N*-acetylglucosamine, UDP-*N*-acetylglucosamine pyrophosphorylase, is a soluble (i.e., presumably cytoplasmic) protein in yeast (17). Together with this knowledge, our results strongly suggest that *N*-acetylglucosamine residues are transferred from the nucleotide sugar in the cytoplasm to the synthase located at the inner face of the plasma membrane. The sugar residues are then joined by the enzyme to a growing chitin chain which is extruded through the membrane as it is synthesized. The complete process may require other components, such as proteins and phospholipids, but the entire machinery is localized in the plasma membrane. Furthermore, no source of energy other than the free energy of hydrolysis of UDP-*N*-acetylglucosamine is required for the synthesis and extrusion. The membranes, which were thoroughly washed during preparation, were able to sustain chitin synthesis for hours (Table 1) without addition of an exogenous energy source.

It is tempting to speculate about the mechanisms of translocation of the sugar residues through the membrane. A lipid carrier is unlikely because no such intermediate was found either in solubilized chitin synthase fractions (3) or in much more extensively purified preparations of the enzyme recently obtained in our laboratory (unpublished data). A possible alternative is the formation of a pore structure either by the synthase itself, if it happens to span the membrane, or in conjunction with other proteins, as postulated by Haigler *et al.* (18) for the formation of cellulose in *Acetobacter xylinum*. When homogeneous preparations of chitin synthase become available, it will be possible to explore this problem further by inserting the enzyme, with or without other membrane components, into artificial liposomes and then examining its ability to function. What we know now is that we do not have to look outside the plasma membrane for any essential component of the system.

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