Signaling toward Yeast 1,3-β-glucan Synthesis

Shunsuke Ben Inoue1, Hiroshi Qadota2, Mikio Arisawa1, Yasuhiro Anraku2, Takahide Watanabe1, and Yoshikazu Ohya2*

1Department of Mycology, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa Prefecture 247, Japan; and 2Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Key words: yeast/cell wall/1,3-β-glucan synthase/rho GTPase/geranylgeranyltransferase I/plasma membrane invagination

ABSTRACT. 1,3-β-glucan synthase catalyzes the synthesis of a 1,3-β-linked glucan polymer which produces the main rigidity of the yeast cell wall. Recent success in purification of this enzyme by product entrapment (21) has provided new insights into the dynamic aspects of the cell wall. This relatively simple procedure made it possible to identify the genes encoding the catalytic subunits of glucan synthase. In addition, the involvement of a rho type GTPase in the regulation of glucan synthase was demonstrated with the purified enzyme. Based on intracellular localization of the glucan synthase subunits, we have proposed a model in which assembly of the subunits is important for the activation of glucan synthase at sites of polarized growth. In this article, we will focus on biochemistry of 1,3-β-glucan synthase and signaling through rho type GTPase.

The yeast cell wall is not a static structure. During cell growth, cell wall precursors are polymerized at the cell surface, then modified and connected to each other. Other materials are made in the endomembrane system, secreted to the cell surface and subsequently assembled into the cell wall. After the primary cell wall is formed, further modification and deposition of wall components frequently occur. Compared with the mature cell wall, the newly-synthesized cell wall is unable to provide full protection from mechanical or chemical stress. However, the synthesis and insertion of new cell wall is not normally deleterious to cell growth. That the new cell wall is not a liability to cell growth suggests that synthesis and assembly of the cell wall are well coordinated and occurs in such a way as to reduce the effects of mechanical and chemical stress. This coordinate synthesis and assembly is most necessary at the onset of the cell surface growth when, in the presence of high turgor pressure, the existing cell wall must be loosened enzymatically prior to cell surface expansion.

Until recently, our understanding of the regulation of cell wall biosynthesis had been quite limited. One reason for this is that the cellular machinery responsible for the coordination of cell wall synthesis had not been characterized well. The other reason is that none of the genes encoding the subunits of the enzyme which catalyzes the synthesis of the major cell wall component had been unequivocally identified. Even in the budding yeast Saccharomyces cerevisiae, which has long proven to be a useful model for the study of cell morphology and development, investigation of the molecular biology of cell wall synthesis began only recently.

A 1,3-β-glucan polymer is the major cell wall component in many fungi and is responsible for cell wall rigidity. Recent discovery of the yeast genes encoding the catalytic subunits and a regulatory subunit of 1,3-β-glucan synthase has produced a more accurate picture of the spatial and temporal control of cell wall assembly. Several recent reviews illustrate progress in the overall understanding of cell wall structure and assembly in yeast (11, 26) and other fungi (48). In this paper, we focus on the regulation of 1,3-β-glucan synthesis in yeast cells and discuss the role of rho-type GTPases in cell wall assembly.

A. Biochemistry of glucan synthesis

1. 1,3-β-glucan synthase in yeast. Yeast cell wall polysaccharides are mainly composed of glucan, mannan and chitin. Glucan, the major filamentous component of the wall architecture, accounts for more than...
half of the dry weight of the yeast cell wall. β-Glucan contains 1,3 and 1,6 linkages, and the former is mainly responsible for the rigidity of the yeast cell wall. The key enzyme for formation of the 1,3 linked β-glucan polymer from UDP-glucose (UDPG) is 1,3-β-glucan synthase (EC 2.4.1.34). 1,3-β-glucan synthase is located on the plasma membrane (50, 51) and a number of biochemical studies of this enzyme has been carried out with membrane preparation from S. cerevisiae. These early studies have shown that the enzyme is composed of at least two subunits, a membrane-bound catalytic subunit and a GTP-binding regulatory subunit (25, 53). 1,3-β-glucan synthase activity has been similarly analyzed in other fungi, such as Candida albicans (42), Hansenula anomala (25), Schizosaccharomyces pombe (47), Neurospora crassa (2, 3, 25), Aspergillus fumigatus (4), as well as in higher plants (14, 23, 36). As in budding yeast, the enzyme activity purified from other fungi separated into soluble and insoluble fractions (25, 47). While comparative biochemical of 1,3-β-glucan synthase has been developed in the last decade, until recently almost no progress had been made on the synthase’s molecular identity, including its structure and subunit stoichiometry, or its regulation.

2. Recent success in purification of 1,3-β-glucan synthase by product entrapment. To purify 1,3-β-glucan synthase, a special procedure, called “product entrapment”, has been developed (24). This method is a kind of affinity purification based on the affinity of the enzyme to its own product. It was first applied to the purification of chitin synthase, and later, to the purification of other enzymes from various sources such as the 1,3-β-glucan synthase of Neurospora crassa (2, 3) and the cellulose synthase of Acetobacter xylinum (54). Despite the usefulness of this clever method, formerly published methods for the yeast glucan synthase assay were not suited for product entrapment. The original method for the enzyme assay included in the reaction mixture two protein factors, BSA and α-amylase (8). BSA was originally included because it reduces the concentration of free fatty acids which inhibit 1,3-β-glucan synthase activity (27). α-amylase was included because it minimizes the effect of glycogen synthase, a frequent contaminant in membrane preparation.

However, we found that the addition of BSA and α-amylase had negative effects on product entrapment. Therefore, we tried several alternative procedures and modifications during preparation of the membrane fractions and finally found suitable conditions. The critical modifications we made were to use 50 mM Tris/Cl pH 7.5, 0.5 M NaCl, 1 mM EDTA as the washing solution and 50 mM Tris/Cl pH 7.5, 1 mM EDTA, 33% glycerol as the suspension solution. This modification greatly improved the purity of our membrane preparation as evidenced by a lowered requirement for BSA in the enzyme assay. Indeed, our membrane preparation essentially has the same specific activity of glucan synthase as that of membranes prepared and assayed by the procedure of Cabib and Kang (8). The effect of α-amylase is also negligible with our preparation.

Since 1,3-β-glucan synthase is a membrane-bound enzyme, the establishment of the stable solubilization condition of the enzyme is the first step for the purification. Although some attempts to solubilize and purify this enzyme were reported, until recently a reproducible solubilization method had not been developed (2-4, 14, 23, 25, 36). We succeeded in using CHAPS and cholesterol hemisuccinate detergent mixture for solubilization of 1,3-β-glucan synthase (21). CHAPS is a zwitterion detergent and is often used for the solubilization of plasma membrane proteins, and cholesterol hemisuccinate is a cholesterol derivative and is thought to mimic cholesterol structures which are rather rich in plasma membrane. With this detergent mixture, we could solubilize, in the presence of 4 μM GTP/S, about 80% of the 1,3-β-glucan synthase activity from the membrane fraction (21). This solubilized enzyme was relatively stable; however, some proteolytic degradation of some components of 1,3-β-glucan synthase has been observed even at 4°C (unpublished observation).

After the addition of UDPG as a substrate for 1,3-β-glucan synthase, the reaction mixture became turbid in a time dependent manner and finally a fine white 1,3-β-glucan aggregate was observed. This 1,3-β-glucan was easily collected by low speed centrifuge and the formation of small amount of glucan is enough to co-precipitate the enzyme (Fig. 1).

One of the most important characteristic of 1,3-β-glucan synthase's molecular identity, including its structure and subunit stoichiometry, or its regulation.

![Fig. 1](image_url) The time course of 1,3-β-glucan formation. The residual activity in the supernatant after low speed centrifugation was measured (closed circle) at the indicated time. The values indicated were as the percentage of original sample. 1,3-β-glucan synthase activity of the low speed supernatant was measured after the spin column treatment to remove UDPG added in the reaction mixture. The turbidity of reaction mixture was measured by the absorbance at 600 nm (open circle) at indicated time.
can synthase is that in the presence of UDPG the enzyme is tightly bound to its product (1,3-β-glucan), and in the absence of UDPG the bound enzyme is released from its product. Non-specific glucan binding proteins are washed out with the buffer containing UDPG and, then, 1,3-β-glucan synthase, which is tightly bound to glucan in the presence of UDPG, is extracted with the buffer without UDPG. By this simple procedure, an over 700-fold purification could be achieved in two cycles of product entrapment (21). We noticed that more than two cycles of product entrapment did not further increase the specific activity of this enzyme. However, it is possible that some proteins, which might be essential for the efficient elongation of 1,3-β-glucan, could not be eluted from the synthesized glucan or could be easily washed out with buffer which contains UDPG. The partially purified enzyme was quite stable when stored at -80°C (Fig. 2).

3. Identification of the catalytic subunit. Using partially purified 1,3-β-glucan synthase as an antigen, several monoclonal antibodies (mAbs) were generated (21). These mAbs were screened for their ability to immunoprecipitate 1,3-β-glucan synthase activity. Among the dozen mAbs tested, several mAbs on Western blot recognized a 200-kDa protein. Enrichment of this 200-kDa protein by product entrapment clearly correlated with increased specific activity for glucan synthesis (21). Furthermore, photoaffinity labeling of the partially purified 1,3-β-glucan synthase activity with 32P-UDPG showed the specific labeling of this 200-kDa protein (unpublished results). Taken together, these results suggest that this 200-kDa protein is the catalytic subunit of 1,3-β-glucan synthase. After determination of partial amino acid sequences of this 200-kDa protein, two very similar genes (GSC1 and GSC2) were cloned (21). The gene products of GSC1 and GSC2 are thought to be integral membrane proteins containing 16 transmembrane domains each. Although a putative UDPG-binding sequence (RXGG) (18, 31) is not located on Gsclp or Gsc2p, both genes have homologous motifs in their putative cytosolic face; for example, RITG at 1532 on Gsclp and RVTG at 1551 on Gsc2p. The deletion of either gene is not lethal, but loss of both genes is lethal. The deletion of GSC1 caused a marked reduction of 1,3-β-glucan synthase activity but in the case of GSC2 deletion, only a slight reduction was observed (21). Interestingly, GSC1 was independently cloned using genetic approaches in conjunction with inhibitors of 1,3-β-glucan synthase or cell wall assembly. The following GSC1 clones were obtained: ETG1 from the echinocandin B derivative (L-733,560) resistant mutant (15, 16), PBR1 from the papulacandin B resistant mutant (10), and CWH53 from the calcofluor white hypersensitive mutant (45). GSC1 was also termed FKS1 (16) and CND1 (19) because it was cloned from FK506 hypersensitive mutant and also as a gene which causes synthetic lethality with calcineurin. Furthermore, GSC2 was also termed FKS2 (35). Hereafter we call them FKS1 and FKS2 in the text.

B. Signaling through rho type GTPase

1. Involvement of a GTP-binding protein in glucan synthesis. As described above, 1,3-β-glucan synthase activity could be separated into two fractions by treating the membrane preparation with detergent and salt (2% Tergitol NP-40 and 2 M NaCl) (25, 37). From the reconstitution experiments, a two component model was proposed: the membrane-bound catalytic subunit and the GTP-binding regulatory subunit which is released from membrane fraction by treatment with the detergent mixture. Recently, the GTP-binding subunit was partially purified by a combination of two column chromatography. The molecular weight of this GTP-binding subunit was estimated by photoaffinity labeling to be 20 kDa (37). Nonetheless, the final purified sample still contained a number of other polypeptides, and was, therefore, not suited for detailed analysis of this regulatory component.

Based on the fact that glucan synthase activity is attached to the plasma membrane, Shematek et al. proposed a model for the glucan synthesis during the yeast cell cycle (50, 51). In this model, the cell surfaces of both the daughter and mother cell contain glucan synthase, but only the glucan synthase of the daughter cell is active. This model explains well why cell surface growth takes place only in a bud, and is consistent with the in vitro studies showing the existence of a regulatory subunit and activation cofactors. However, a more detailed model describing exactly how this spatial activation might occur has had to wait until the molecular
mechanism of regulation of glucan synthesis was actually unveiled.

2. Involvement of GGTase I in glucan synthase activity. Genetic study in Schizosaccharomyces pombe has suggested that geranylgeranyltransferase I (GGTase I) plays some role in the regulation of glucan synthase. Ribas et al. isolated two temperature-sensitive mutants, cwg1-1 and cwg2-1, which also exhibited sensitivities to Calcofluor white, a drug which interferes with cell wall assembly (47). They also found that crude extracts of cwg1-1 and cwg2-1 cells contain reduced 1,3-β-glucan synthase activity at high temperature (47). Cloning and DNA sequencing of the cwg2+ gene (17) have revealed that the gene product has homology of S. cerevisiae Cal1/Cdc43 (40), a β subunit of geranylgeranyltransferase I. Geranylgeranylation is a posttranslational modification which results in the covalent attachment of a C20 isoprenoid to the C-terminal cysteine residue of substrates proteins (12, 49). Functional conservation between cwg2+ and CAL1/CDC43 also is suggested by the fact that expression of S. cerevisiae Cal1/Cdc43 suppresses the growth and glucan synthase defect of the cwg2-1 mutant at the restrictive temperature (17). As in S. cerevisiae, the S. pombe membrane fraction contains at least two components essential for glucan synthase. Detergent extraction of the glucan synthase into a soluble and a particulate fraction and subsequent reconstitution indicates that the cwg2-1 mutant activity is affected in the soluble component (47). These results suggest that geranylgeranylation of a GTPase is responsible for the activity in the soluble component of glucan synthase.

S. cerevisiae GGTase I has been well characterized biochemically by using the recombinant prenyltransferase (9, 34). Analysis of bacterially expressed Cal1/Cdc43 and Ram2, the α subunit of the enzyme has revealed that the yeast GGTase I is a Mg2+-requiring, Zn2+-metalloenzyme (34). This is consistent with the results from studies of the mammalian FTase (46) and GGTase I (56) which demonstrated a requirement for Mg2+ in isoprenoid transfer and for Zn2+ in binding of protein substrate. Yeast GGTase I differs from mammalian FTase, since GGTase I can also function with Ca2+ as the only divalent cation (34). Ca2+ likely binds to both Mg2+ and Zn2+ binding sites of the yeast GGTase I to mediate both isoprenoid transfer and substrate binding. Involvement of Ca2+ in the regulation of the GGTase I function in vivo is suggested by the fact that one allele of the CAL1/CDC43 gene, cal1-1, shows a Ca2+-dependent growth phenotype (39).

Among prenyltransferase mutants, a mutation in the GGTase I β-subunit gene was the first to be isolated and characterized in S. cerevisiae. call-1 was identified originally as a mutation resulting in a Ca2+-dependent phenotype (39). The call-1 mutant simultaneously exhibits a homogeneous terminal phenotype with a G2/M nucleus and a small bud at 37°C (39). Independent screening of yeast cell cycle mutants which accumulated enlarged unbudded cells identified six other alleles, cdc43-2~cdc43-7 (1). Detailed analyses of the call1/cdc43 mutants have suggested that substrate specificity of the mutant GGTase I accounts for the different terminal phenotypes of call1 and cdc43 (41). Yeast GGTase I is essential for yeast cell growth, since deletions of the CAL1 gene result in a lethal phenotype (40). As easily speculated from the functional conservation of GGTase I between the two yeasts, involvement of GGTase I in glucan synthesis in budding yeast was recently confirmed (Inoue et al., submitted for publication).

Although GGTase I normally is an essential gene for growth, it can be made non-essential, when the dosage of the two GTPases, Rho1p (30) and Cdc42p (22), are artificially elevated (41). Since the yeast GGTase I prenylates these two GTPases, Cdc42p and Rho1p are implicated genetically as the two essential substrates of GGTase I (41). Qadota et al. proposed the possibility that rho GTPases act as regulators of GGTase I (43). RHO1 and RHO2 genes were isolated as allele-specific high dosage suppressors of call-1. The ability of rho GTPase overproduction to suppress call-1 may be due in part to the fact that Rho1p and Rho2p are substrates of GGTase I. However, rho GTPase also appears to enhance protein modification of another substrate by GGTase I in vivo suggesting that rho GTPases carry out positive feedback regulation of GGTase I activity (43).

3. Implication of rho GTPase in glucan synthesis. rho GTPases comprise one subfamily of ras-related GTP-binding proteins. The GTPases are in general thought to act as molecular switches by cycling between the "active" GTP-bound form and the "inactive" GDP-bound form (7, 20). Transitions between these two states are controlled by regulator molecules, including GTPase-activating proteins (GAPs), guanine-nucleotide-exchange factors (GEFs), and guanine-nucleotide-dissociation inhibitors (GDIs) (6).

Impairment of glucan synthase activity in the GGTase I mutant suggested that one of the substrates of yeast GGTase I is involved in the regulation of 1,3-β-glucan synthase (17, 47). In addition, since many of the rho GTPases are substrates of GGTase I, it is a reasonable speculation that a rho GTPase is the regulatory component of the glucan synthase. In S. cerevisiae, five members of the rho subfamily (RHO1, RHO2, RHO3, RHO4, and CDC42) have been identified (22, 30, 32, 33). RHO1 and CDC42 are essential genes (22, 30). RHO3 and RHO4 are related functionally to each other and loss of both results in growth failure above 30°C (32, 33). Assuming that GGTase I modifies proteins ending with a Cys-Ali-Ali-Leu (Ali: aliphatic) sequence, Rho1p, Rho2 and Cdc42p are predicted to be substrates.
of GGTase I. There is one more substrate for GGTase I, Rsr1p, which is not essential for growth, but required for bud site selection (5).

Recently we found evidence that Rho1p is a regulatory component of 1,3-β-glucan synthase. Based on phenotypic analyses of conditional-lethal mutations in the RHO1 gene, we and others have suggested that the normal function of Rho1p is to maintain cell integrity (44, 55). Involvement of Rho1p in cell wall assembly is further suggested by the observation that many rho1 strains exhibit hyper-sensitivity to echinocandin B, an antifungal glucan synthase inhibitor (Fig. 3). Biochemical examination of the rho1 mutants have revealed that the membrane fractions of temperature-sensitive rho1

Fig. 3. Sensitivities of wild-type and rho1 mutants to echinocandin B. YPD soft agar (2 ml) was warmed at 55°C, mixed with culture of wild-type or rho1 strains, and poured onto YPD agar plate with a paper disk containing 60 µg echinocandin B. The plates were incubated at 23°C for 3 days. The diameters of the halos were 1.5 (A: YOC764, wild-type), 2.0 (B: YOC752, rho1-2), 1.9 (C: YOC729, rho1-3) and 2.2 cm (D: YOC755, rho1-5).
mutant cells exhibit a dramatic reduction of 1,3-β-glucan synthase activity. Partially purified glucan synthase contains Rho1p, and Rho1p is enriched during the enzyme purification. Although the wild-type enzyme requires GTP-γS for its maximum activity, the enzyme from a dominant active RHO1 mutant cells has full activity even in the absence of GTP-γS. These results indicate that the GTP/GDP cycling of yeast Rho1p regulates the 1,3-β-glucan synthase activity (44a).

The subcellular location of glucan synthase has been determined with immunofluorescence microscopy and has provided further insights on the spatial regulation of glucan synthase. We located a catalytic subunit of the glucan synthase (Fks1p) mainly at the sites of cell surface growth (44a). Several dots of signal are also seen in the cells, probably representing premature subunits in secretory organelle. It has been known that Rho1p is located at the tips of polarized growth sites (55). Because GTP-bound Rho1p is required to activate glucan synthase, our model (Fig. 4) predicts that active glucan synthase is located only at the growing portion of the bud. Glucan synthase in secretory organelles is inactive, because of the absence of the regulatory subunit in these organelles. Both catalytic and regulatory subunits of glucan synthase are not present on the surface of the nongrowing mother cells, probably because of the short half-life of the subunits. Thus, assembly and polarized localization of the glucan synthase subunit likely accounts for the spatial regulation of glucan synthase.

4. Role of cortical actin patches in glucan synthesis. The yeast plasma membrane is not a smooth structure. Growing daughter cells contain finger-like plasma membrane invaginations which have been shown to be associated with cortical actin patches (38). Mulholland et al. have proposed, based primarily on biophysical considerations, that these actin-associated, fingerlike invaginations are the sites of cell wall synthesis (38). Yeast cells, like other walled organism, maintain a large osmotic gradient across their plasma membrane which results in turgor pressure. While this turgor pressure creates the force needed for expansion of the cell surface, it also can be expected to create a difficult situation for insertion of new cell wall materials. The presence of a large turgor pressure at the sites of new wall synthesis, where the existing wall must be loosened prior to growth, may cause the cell to lyse. To prevent this catastrophe, Mulholland et al. have proposed that the yeast invaginate the plasma membrane where the cell wall is synthesized. Such an invagination would provide a localized reduction of the surface tension normally produced by cellular turgor pressure (38). 1,3-β-linked glucan is polymerized on the plasma membrane with cytosolic UDP-glucose. Other cell wall components, such as mannanprotein and at least a portion of 1,6-β-linked glucan are synthesized and processed in secretory organelle. In Mulholland's model, these cell wall precursors and components would be assembled and connected together in the exocytoplasmic space of the actin-associated, plasma membrane invagination. This model can also be applied to cell wall synthesis in the regenerating protoplast (28).

The Rho GTPase, Cdc42p is located on the plasma membrane, especially in areas of active cell growth which contain cortical actin patches (58). Cdc42p is essential for bud assembly and bud growth as well as for the selection of the proper site for bud emergence (22). Other studies have indicated that Cdc42p activates Ste20p and Ste20p-like kinases which are essential for bud assembly and mating signal transduction (13, 52, 57). Since many secretory vesicles are seen in the vicinity of the Cdc42p localization, Cdc42p has been implicated in regulation of membrane fusion of the secretory vesicles (58). Recently, an in vitro assay for assembly of the cortical actin cytoskeleton in permeabilized cells has recently been developed (29). Using this assay Li et al. have shown that the incorporation of actin into the bud is stimulated by GTP-γS and that this stimulation is absent if a CDC42 mutant is used in the assay. These results suggest that Cdc42p is involved in the regulation of cortical actin assembly, particularly at areas of polarized growth.

We found that Rho1p activates 1,3-β-glucan synthase. Although the localization of Rho1p has not been determined by immunoelectron microscopy, our immunofluorescence microscopy analysis of Fks1p and Rho1p indicates that the glucan synthase complex is located at sites of polarized growth. Thus, Fks1p and
Rho1p, like Cdc42p, appear to be located at the same, or adjacent to the same area where cortical actin patches, which contain finger-like invaginations of plasma membrane, are observed. Insights into the activation mechanism of Rho1p and Cdc42p as well as on interaction of these GTPases with the cortical actin cytoskeleton will provide a more complete understanding of the regulation of cell wall synthesis.

Acknowledgments. We thank Jon Mulholland for critical reading of the manuscript. This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan.

Signaling toward Yeast 1,3-β-glucan Synthesis

REFERENCES


29. Li, R., Zheng, Y., and Drubin, D. 1995. Regulation of cor-


