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Rho1p, a Yeast Protein at the Interface Between Cell Polarization and Morphogenesis

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The enzyme that catalyzes the synthesis of the major structural component of the yeast cell wall, β(1→3)-D-glucan synthase (also known as 1,3-β-glucan synthase), requires a guanosine triphosphate (GTP) binding protein for activity. The GTP binding protein was identified as Rho1p. The *rho1* mutants were defective in GTP stimulation of glucan synthase, and the defect was corrected by addition of purified or recombinant Rho1p. A protein missing in purified preparations from a *rho1* strain was identified as Rho1p. Rho1p also regulates protein kinase C, which controls a mitogen-activated protein kinase cascade. Experiments with a dominant positive *PKC1* gene showed that the two effects of Rho1p are independent of each other. The colocalization of Rho1p with actin patches at the site of bud emergence and the role of Rho1p in cell wall synthesis emphasize the importance of Rho1p in polarized growth and morphogenesis.

Little is known at the molecular level about the mechanisms involved in morphogenesis, a fundamental process in growth and differentiation. To study those mechanisms, we have used the cell wall of the yeast *Saccharomyces cerevisiae* (*1*). In the yeast budding cycle, synthesis of a new cell wall starts at bud emergence and continues until the daughter cell completes its maturation, after cytokinesis and septum formation (*1*). Temporal controls must be in place to ensure synchronization between cell wall growth and the cell cycle. Spatial regulation is also required to determine the site where the new bud will emerge (*2*) and to direct growth of the cell wall in an orderly manner that will result in the correct shape for the new cell. To gain information about such controls, we have studied the biosynthesis of β(1→3)glucan, the major structural component of the yeast cell wall (*1*). β(1→3)Glucan synthase (GS), a membrane-bound enzyme, is stimulated by submicromolar concentrations of GTP (*3*). A proteinaceous component that interacts with GTP has been extracted from membranes of several fungi (*4*).

Recently, two fractions, A and B, both essential for GS activity, were solubilized from yeast membranes and a GTP binding protein was purified from fraction A (*5*). To identify this protein, we tested mutants in known small GTP binding proteins. Of the approximately 20 such proteins found thus far in yeast, most participate in intracellular traffic. However, defects in the *RHO1* (*6*) and *RHO3* or *RHO4* (*7*) genes result in arrest of the cell cycle at the small bud stage with concomitant cell lysis (*8*, *9*), a phenotype consistent with a defect in the initiation of cell wall growth caused by impaired glucan synthesis.

Here, we measured GS activity in the absence or presence of guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S) in membrane preparations from strains containing a temperature-sensitive mutation in *RHO1*, *rho1-104* (*8*). Strains with a conditional mutation in *RHO3* and a disruption in *RHO4* (or vice versa) were also tested. Enzymatic activity and GTP stimulation of the synthase from *rho3*/*rho4* mutants differed little from that in the wild type (*10*). On the other hand, synthase from the *rho1* mutant showed a decrease in activity, which was not remedied by the addition of GTP-γ-S (Fig. 1A). The defect was evident even when *rho1* cells were grown at the permissive temperature and was exacerbated by incubation of the cells at 37°C. Fraction A (*5*) restored both activity and GTP-γ-S stimulation to membrane preparations from the *rho1-104* strain (Fig. 1B). The wild-type

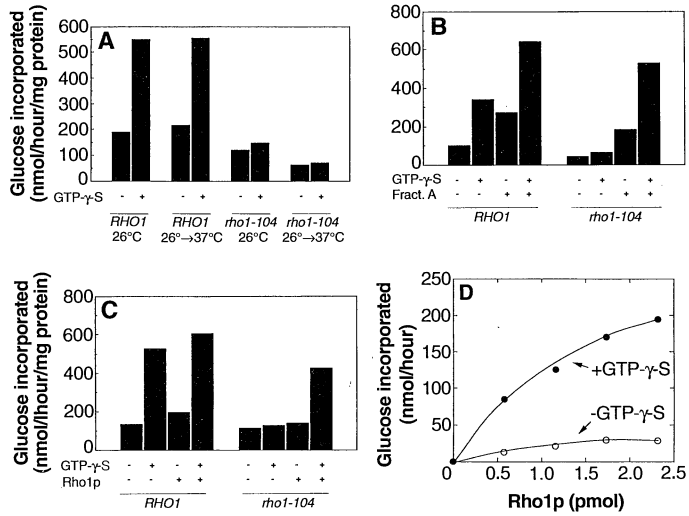

Fig. 1. β(1→3)Glucan synthase defect in *rho1* mutants and reconstitution of the system. In all cases, cells were grown and membrane fractions were prepared and assayed for GS activity as described (*5*). Results are the average of duplicate determinations that differed less than 10%. The wild-type *RHO1* strain used was OHNY1 (*MAT* **a** *ura3 leu2 trp1 his3 ade2*), and the *rho1* mutant strain was HNY21 (*MAT* **a** *ura3 leu2 trp1 his3 ade2 rho1-104*). (**A**) GS activity in wild-type and *rho1-104* membrane fractions. Cells were grown at 26°C continuously (26°C) or transferred to 37°C for 2 hours (26°→37°C) before harvesting. (**B**) Reconstitution of GS activity of *rho1-104* with fraction A. The amount of added fraction A was 20 μg. (**C**) Reconstitution of GS activity in *rho1-104* with recombinant Rho1p (*23*). The amount of added Rho1p was 0.7 pmol per reaction mixture. (**D**) Reconstitution of GS activity with Rho1p and fraction B from strain GS1-36. Fraction B (7.2 μg of protein) was added to all reaction mixtures.

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enzyme was also somewhat stimulated by fraction A, but the percent activation by GTP-γ-S was unchanged (Fig. 1B). Purified preparations (5) of the GTP binding protein yielded similar results but with less stimulation by GTP-γ-S, because the protein was partially bound to GTP (5) during purification (10).

Fraction A was purified from strains OHNY1 (wild type) and HNY21 (*rho1-104*) by MonoQ and Sephacryl S-300 chromatography (5). Determinations of [^{35}S]GTP-γ-S binding by the purified material yielded 14.3 pmol/μg of protein in the wild type but only 1.6 pmol/μg of protein in the mutant. This result suggested that Rho1p is the previously studied (5) GTP binding protein and that our purified preparations contain only one GTP binding protein (5). After SDS–polyacrylamide gel electrophoresis (SDS-PAGE), the material from the *RHO1* strains OHNY1 and GS1-36 (5) showed essentially the same protein pattern, except for a band at 14.4 kD, which was missing in the OHNY1 fraction (Fig. 2A). The patterns yielded by OHNY1 and HNY21 differed only in one major band at 24 kD (Fig. 2A), which is missing in the mutant fraction. Its molecular size is similar to that calculated for Rho1p (23 kD).

To verify that Rho1p was indeed the GS complementary factor, we used recombinant Rho1p in reconstitution experiments.

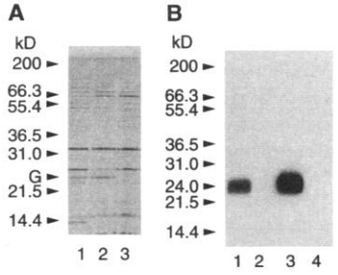

Fig. 2. Identification of a band at 24 kD as Rho1p. (**A**) SDS-PAGE of proteins from *RHO1* strains GS1-36 (lane 1) and OHNY1 (lane 2) and *rho1-104* strain HNY21 (lane 3), after purification of fraction A by MonoQ and Sephacryl 300 columns (5) and Coomassie blue staining. The amount of protein added to each lane was 7.8 μg. The 24-kD band present in preparations from GS1-36 and OHNY1, but absent from HNY21, is labeled G. (**B**) ADP (adenosine diphosphate) ribosylation of GTP binding proteins. Reaction mixtures contained 20 mM Hepes (pH 8), 0.3 mM magnesium acetate, 0.3 mM 5′-AMP (adenosine monophosphate), 10 μM [^{32}P]nicotinamide adenine dinucleotide phosphate (54 × 10^6 cpm for GS1-36 purified protein and 2.7 × 10^6 cpm for recombinant Rho1p), and 13 ng of *C. botulinum* C-3 exoenzyme in a total volume of 15 μl. The C-3 exoenzyme was omitted from the mixtures of lanes 2 and 4. Mixtures in lane 1 and 2 contained 0.2 μg of purified GTP binding protein from *RHO1* strain GS1-36. Mixtures in lanes 3 and 4 contained 3.5 pmol of recombinant Rho1p. Incubation was for 1 hour at 37°C, followed by SDS-PAGE and autoradiography.

Table 1. Glucan synthase activity in *pkc1* mutants. The strains used were DL1783 (*MAT* **a** *leu2-3,112 ura3-52 trp1-1 his4*) and DL376 (*MAT* **a** leu2-3,112 ura3-52 trp1-1 his4 pkc1::LEU2). Cells were grown in the presence of 0.5 M potassium acetate. The other components of growth medium as well as the preparation of membrane and solubilized fractions and enzyme assays were as described (5).

Strain	Specific activity of membranes (nmol hour^{-1} mg^{-1})	Stimulation by GTP-γ-S (fold)	Specific activity of fractions (nmol hour^{-1} mg^{-1})	
			A	B
DL1783 (wild type)	744	5.6	6,560	13,340
DL376 (*pkc1Δ*)	504	4.7	6,240	6,860

Addition of the recombinant protein to membrane preparations from the *rho1-104* mutant restored stimulation of the synthase by GTP-γ-S (Fig. 1C). Guanosine diphosphate (GDP) had no effect (10), in agreement with the finding that GDP-bound Rho1p is inactive in the GS assay (5, figure 10). We reconstituted active GS by adding Rho1p to fraction B (Fig. 1D). This result indicates that the latter fraction contains all proteins required for glucan synthesis other than Rho1p.

Both recombinant Rho1p and purified fraction A from *RHO1* strain GS1-36 were subjected to adenosine diphosphate (ADP) ribosylation with *Clostridium botulinum* C-3 exoenzyme, followed by SDS-PAGE and autoradiography. In both cases, a band at 24 kD was labeled (Fig. 2B). No other radioactive band appeared. We conclude that the GTP binding protein in fraction A is Rho1p.

Protein kinase C, the first member of a regulatory protein kinase cascade that participates in the maintenance of cell wall integrity (11), is under the control of *RHO1* (12). The terminal phenotypes of temperature-sensitive *rho1* and *pkc1* mutants are similar—that is, mother cells with small buds that lyse (8, 13)—although *pkc1* null mutants are rescued in media of high osmolarity (13), whereas *rho1* null mutants are not (8). The effects of the *rho1-104* defect are negated in the presence of 1 M sorbitol (8), but only partially (10). Membrane preparations from a *pkc1* null mutant had less GS activity than the wild type, but no impairment in stimulation by GTP-γ-S (Table 1). Fractional solubilization of the membranes showed that the activity defect resided in fraction B, which contains the catalytic activity (Table 1). This defect in the catalytic subunits may explain the somewhat smaller amount of β(1→3)glucan found in the cell walls of *pkc1* mutants (14).

To separate the effect of Rho1p on glucan synthesis from its effect on protein kinase C, we transformed strain HNY21 (*rho1-104*) with a plasmid [YCp50-PKC1 (R398P), where R is Arg and P is Pro] containing a *PKC1* mutation that results in a constitutively active protein kinase C (PKC) (12). A strain transformed with the YCp50 vector served as a control. As in untransformed cells, preparations of GS from both transformants had little activity, which was stimulated by addition of recombinant Rho1p (15). In regard to lysis and terminal phenotype at the nonpermissive temperature, the phenotypes of both transformed strains were also similar (15). We conclude that the effects of Rho1p on GS and on Pkc1p are independent and that the lytic phenotype is

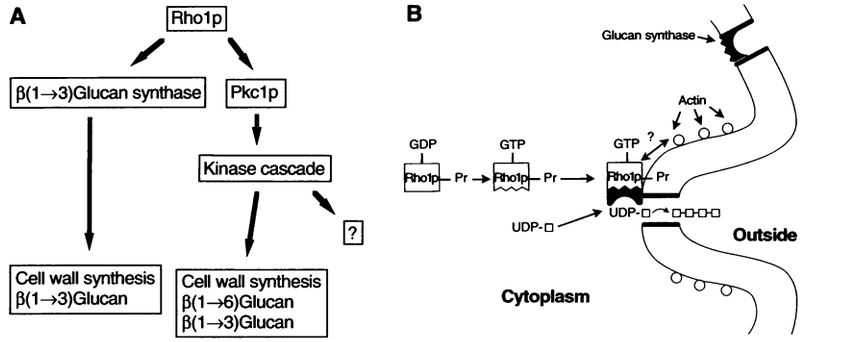

Fig. 3. Functions of Rho1p. (**A**) Rho1p regulation of glucan synthesis and of the MAP (mitogen-activated protein) kinase cascade controlled by protein kinase C. For the interaction between the Pkc1p cascade and β(1→6)glucan, see (14). (**B**) Scheme for the localized regulation of cell wall synthesis at bud emergence. For convenience, the GTP-GDP exchange in Rho1p is shown to occur in the cytoplasm but may take place while Rho1p is attached to the plasma membrane. The small squares attached to UDP represent glucosyl units. Pr, prenyl group.

still present even after correction of the protein kinase C defect. Thus, Rho1p acts both on a morphogenetic enzyme, of which it is an essential subunit, and on a regulatory cascade. Both pathways have an effect on cell wall synthesis (Fig. 3A).

The identification of Rho1p as an activator of GS is important in understanding the spatial regulation of new wall initiation. The distribution of Rho1p in the cell closely mimics that of actin patches (8), and the protein is prenylated (8). GS is bound to the plasma membrane (16), where it must be colocalized with at least a fraction of Rho1p, without which it would be inactive (Fig. 1D). The scheme of Fig. 3B is based on these facts. An inactive GS complex, with only the components of fraction B, including the hydrophobic *FKS1* (17) and *FKS2* (18) gene products, spans the plasma membrane at upper right. When a prenylated Rho1p exchanges its GDP for GTP, a concomitant change in conformation would enable it to bind to the GS complex. This binding, combined with the affinity of the prenyl group for the lipid environment of the membrane, may open a "gate," allowing the substrate (uridine diphosphate–glucose) to bind to the now-exposed catalytic site. Simultaneous synthesis and extrusion of glucan would ensue, as in the case of chitin (19). The active complex may be surrounded by actin filaments (8, 20). In Fig. 3B, a double-headed arrow indicates a possible interaction, direct or indirect, between actin and Rho1p. The Rho proteins function in the organization of the cytoskeleton in animal cells (21), although in yeast Cdc42p may be the operative Rho (22) and the localization of Rho1p may depend on actin rather than the opposite. Thus, Rho1p is poised at a critical boundary between cell polarization [as represented by the cytoskeleton together with the bud site localization and bud site organization machinery (2)] and morphogenesis, as represented by cell wall synthesis.

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Identification of Yeast Rho1p GTPase as a Regulatory Subunit of 1,3-β-Glucan Synthase

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1,3-β-D-Glucan synthase [also known as β(1→3)glucan synthase] is a multi-enzyme complex that catalyzes the synthesis of 1,3-β-linked glucan, a major structural component of the yeast cell wall. Temperature-sensitive mutants in the essential Rho-type guanosine triphosphatase (GTPase), Rho1p, displayed thermolabile glucan synthase activity, which was restored by the addition of recombinant Rho1p. Glucan synthase from mutants expressing constitutively active Rho1p did not require exogenous guanosine triphosphate for activity. Rho1p copurified with β(1→3)glucan synthase and associated with the Fks1p subunit of this complex in vivo. Both proteins were localized predominantly at sites of cell wall remodeling. Therefore, it appears that Rho1p is a regulatory subunit of β(1→3)glucan synthase.

The cell wall of the budding yeast *Saccharomyces cerevisiae* is required to maintain cell shape and integrity (1). Vegetative proliferation requires that the cell remodels its wall to accommodate growth, which during bud formation is polarized to the bud tip. The main structural component responsible for the rigidity of the yeast cell wall is 1,3-β-linked glucan polymers with some branches through 1,6-β-linkages. The biochemistry of the yeast enzyme that catalyzes the synthesis of 1,3-β-glucan chains has been studied extensively (2, 3), but little is known at the molecular level about the genes that encode subunits of this enzyme. Only a pair of closely related proteins (Fks1p and Fks2p) are known to be subunits of the β(1→3)glucan synthase (GS) (3–5). GS activity in many fungal species, including *S. cerevisiae*, requires guanosine triphosphate (GTP) or a nonhydrolyzable analog, such as guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) as a cofactor, which suggests that a GTP binding protein stimulates this enzyme (2, 3, 6). Here, we demonstrate that the Rho1p GTPase is an essential regulatory component of the GS complex.

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The *Saccharomyces RHO1* (Ras homologous) gene encodes a small GTPase that is localized at sites of growth (7) and whose function is essential for viability (8). On the basis of phenotypic analyses of conditional *rho1* mutants, normal function of Rho1p is thought to maintain cell integrity (7, 9). Conditional *rho1* mutants are hypersensitive to Calcofluor white and echinocandin B, drugs that interfere with cell wall assembly. This hypersensitivity suggests that this gene is involved in wall construction (10).

To determine if Rho1p was required for glucan synthesis, we measured GS activity in extracts of temperature-sensitive *rho1* mutants grown at a permissive temperature. GS activity from wild-type cells increased as a function of assay temperature from 23°C to 30°C to 37°C (Fig. 1A). All of the *rho1* mutants tested displayed reduced activity at each temperature relative to the activity of the wild type. Moreover, the enzymes from all but one mutant (*rho1-5*) exhibited some amount of thermolability,