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.

REFERENCES AND NOTES

1. E. Cabib, R. Roberts, B. Bowers, *Annu. Rev. Biochem.* **51**, 763 (1982).
2. D. G. Drubin, *Cell* **65**, 1093 (1991).
3. E. M. Shematek and E. Cabib, *J. Biol. Chem.* **255**, 895 (1980).
4. M. S. Kang and E. Cabib, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5808 (1986).
5. P. C. Mol, H.-M. Park, J. T. Mullins, E. Cabib, *J. Biol. Chem.* **269**, 31267 (1994).
6. P. Madaule, R. Axel, A. M. Myers, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 779 (1987).
7. Y. Matsui and A. Toh-e, *Gene* **114**, 43 (1992).
8. W. Yamochi *et al.*, *J. Cell Biol.* **125**, 1077 (1994).
9. Y. Matsui and A. Toh-e, *Mol. Cell. Biol.* **12**, 5690 (1992).
10. J. Drgonová and E. Cabib, unpublished results.
11. B. Errede and D. E. Levin, *Curr. Opin. Cell Biol.* **5**, 254 (1993).
12. H. Nonaka *et al.*, *EMBO J.* **14**, 5931 (1995).
13. D. E. Levin and E. Bartlett-Heubusch, *J. Cell Biol.* **116**, 1221 (1992).
14. T. Roemer, G. Paravicini, M. A. Payton, H. Bussey, *ibid.* **127**, 567 (1994).
15. E. Cabib, unpublished data. These results are similar to those shown in Fig. 1, A and C.
16. E. M. Shematek, J. A. Braatz, E. Cabib, *J. Biol. Chem.* **255**, 888 (1980).
17. C. M. Douglas *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12907 (1994).
18. P. Mazur *et al.*, *Mol. Cell. Biol.* **15**, 5671 (1995).
19. E. Cabib, B. Bowers, R. L. Roberts, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3318 (1983).
20. J. Mulholland *et al.*, *J. Cell Biol.* **125**, 381 (1994).
21. A. Hall, *Annu. Rev. Cell Biol.* **10**, 31 (1994); Y. Takai, T. Sasaki, K. Tanaka, H. Nakahishi, *Trends Biochem. Sci.* **20**, 227 (1995).
22. J. Chant and L. Stowers, *Cell* **81**, 1 (1995).
23. The lipid-modified form of Rho1p was purified from the membrane fraction of *Spodoptera frugiperda* cells (Sf9 cells), which were infected as described with the baculovirus carrying the *RHO1* gene [T. Mizuno *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6442 (1991)].

24. We thank Y. Matsui for strains and antibodies, D. Levin for strains, P. T. Wingfield for help in protein microsequencing, and A. Robbins for a critical reading of the manuscript.

26 December 1995; accepted 20 February 1996

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

Hiroshi Qadota,* Christophe P. Python,* Shunsuke B. Inoue,* Mikio Arisawa, Yasuhiro Anraku, Yi Zheng, Takahide Watanabe, David E. Levin, Yoshikazu Ohya†

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.

H. Qadota, Y. Anraku, Y. Ohya, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan.
C. P. Python and D. E. Levin, Department of Biochemistry, Johns Hopkins University School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205, USA.
S. B. Inoue, M. Arisawa, T. Watanabe, Department of Mycology, Nippon Roche Research Center, 200. Kajiwara, Kamakura, Kanagawa Prefecture 247, Japan.
Y. Zheng, Department of Biochemistry, University of Tennessee, Memphis, TN 38163, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.

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,

which suggests that *RHO1* function is required for GS activity.

Therefore, we tested whether purified, recombinant glutathione-S-transferase (GST)–Rho1p restored GS activity to membrane fractions from the most impaired *rho1* mutant (*rho1-3*). Membranes from this mutant were virtually devoid of activity at 37°C. GS activity was restored fully by the addition of GTP-γ-S–bound GST-Rho1p but not by GST-Cdc42p, another member of the Rho family of GTPases (Fig. 1B). GTP-γ-S could be replaced with GTP, but not with GDP (guanosine diphosphate) (Fig. 1C). These results indicate that the GS-deficient mutant membranes lack only Rho1p function.

We also examined GS activity from yeast cells expressing a constitutively active *RHO1* allele (*RHO1-Q68H*, where Q is Gln and H is His). The analogous mutation in Ras results in a protein that has a reduced capacity to hydrolyze GTP and has transforming potential in mammalian cells (*11*). The GTP requirement of GS activity was examined in membranes obtained from *rho1-3* cells overexpressing *RHO1* or *RHO1-Q68H* under the inducible control of the *GAL1* promoter (Fig. 2). Under inducing conditions (galactose), expression of *RHO1-Q68H* resulted in GS activity that was independent of exogenous GTP. By contrast, GS activity in membranes from cells overexpressing *RHO1* was largely dependent on GTP. Similar results were obtained with another activated allele (*RHO1-G19V*, where G is Gly and V is Val) (*12*). Taken together, these results indicate that GS activity requires functional Rho1p in the GTP-bound state.

To determine if Rho1p is a component of the GS complex, we monitored the amount of Rho1p during purification of GS activity. The enzyme was purified after extraction from membranes by successive product entrapments, which are affinity purification procedures based on the affinity of the enzyme to its own product (*3*). Both Rho1p and Fks1p were enriched in the partially purified GS (Fig. 3). The specific activity of GS was increased approximately 700-fold through purification, whereas Rho1p was enriched approximately 400-fold. GS purified from the *rho1-5* mutant was deficient in GS activity despite normal amounts of both the Rho1p and Fks1p proteins (*13*). To determine if Rho1p copurifies with GS because it physically associates with the GS complex, we immunoprecipitated the partially purified enzyme with either of two monoclonal antibodies to Fks1p. The resultant immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with antibody to Rho1p. Rho1p co-immunoprecipitated with Fks1p (Fig. 4A).

Finally, we examined the localization of

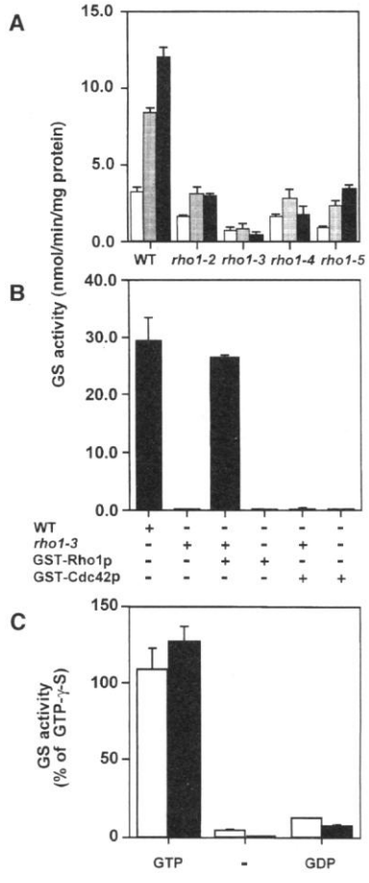

Fig. 1. GS activity from *rho1* mutants (*20*). (**A**) Thermolability of GS activity in *rho1* temperature-sensitive mutants and in the wild type (WT). Extracts were made from cells growing at room temperature and assayed for GS activity at the indicated temperatures (white bars, 23°C; gray bars, 30°C; black bars, 37°C) in the presence of 50 μM GTP-γ-S. (**B**) Reconstitution of GS activity in *rho1-3* membranes with recombinant Rho1p (*21*). GS activity in *rho1-3* membrane fractions was measured at 37°C in the presence of 1 μg of the indicated recombinant GTPase and 50 μM GTP-γ-S (*20*). (**C**) Requirement for GTP in reconstituted GS activity. GS activity in wild-type membranes (open bars) or *rho1-3* membranes with added GST-Rho1p (1 μg) (solid bars) was measured at 37°C in the presence of the indicated guanine nucleotide (20 μM). Results are expressed as a percentage of the activity observed with wild-type membranes in the presence of GTP-γ-S (50 μM). For all GS assays, the mean and standard error for four experiments is shown.

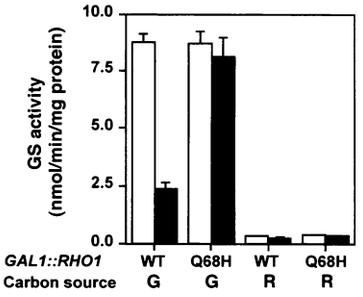

Fig. 2. GS activity independent of GTP in a constitutively active *RHO1* mutant. Cultures of *rho1-3* cells harboring plasmids with either *RHO1* or *RHO1-Q68H* (*8*) under the control of the inducible *GAL1* promoter were grown at room temperature in medium containing 2% raffinose (R) (repressing conditions). Galactose (G) was added (to 2%) to half of each culture, and cells were cultured for an additional 4 hours to induce expression of *RHO1*. GS activity in membrane fractions was assayed at 37°C in the presence (open bars) or absence (solid bars) of GTP-γ-S.

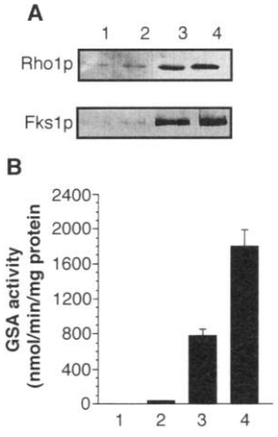

Fig. 3. Enrichment of Rho1p and Fks1p during purification of GS. GS was purified from a wild-type strain (A451) (*3*). (**A**) Immunoblot analysis of Rho1p and Fks1p through purification (*22*). (**B**) GS specific activity through purification. Purification steps were as follows: lane 1, membrane fraction; lane 2, detergent extract; lane 3, first product entrapment; and lane 4, second product entrapment.

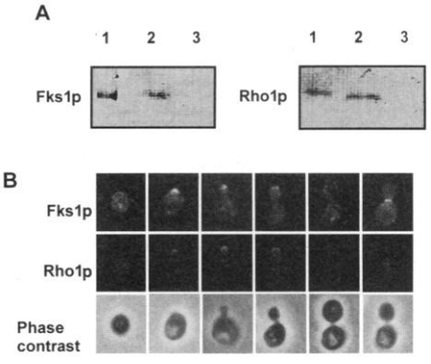

Fig. 4. Association of Fks1p and Rho1p in vivo. (**A**) Co-immunoprecipitation of Rho1p with Fks1p (*23*). Partially purified GS was incubated with monoclonal antibodies to Fks1p, 1A6 (lane 1), and 1F4 (lane 2), and antibody to human endothelin B type receptor (lane 3). Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting. (**B**) Colocalization of Fks1p and Rho1p at sites of cell wall remodeling (*24*). Indirect immunofluorescence microscopy was used to visualize Fks1p and ^{HA}Rho1p in double-stained cells.

Rho1p, tagged at its NH_2-terminus with the influenza hemagglutinin (HA) epitope (^{HA}Rho1p), and of Fks1p in growing yeast cells. Rho1p is located at the bud tip (the site of polarized growth) during bud formation and at the bud neck (the site of septum formation) during cytokinesis (*7*). Indirect immunofluorescence of cells labeled with antibodies to HA and Fks1p revealed that Fks1p colocalizes with ^{HA}Rho1p (Fig. 4B). These results suggest that Rho1p, like Fks1p, is a component of the GS complex. This complex is redistributed through the cell cycle so as to reside at sites of cell wall remodeling.

Rho1p interacts with Pkc1p (protein kinase C) (*14*, *15*), and this interaction activates Pkc1p in vitro (*15*). Like *rho1* mutants, *pkc1* mutants display cell integrity defects that result from a deficiency in cell wall construction. However, several observations indicate that Pkc1p does not take part in the activation of GS. Mutants in *PKC1* displayed no defect in GS activity (*16*). Overexpression of Pkc1p did not restore GS activity to *rho1* mutants (*17*). Finally, Pkc1p was not detected in the purified GS complex (*18*). Therefore, we propose that Rho1p has at least two distinct regulatory roles in the maintenance of cell integrity. One is the activation of GS, and the other is the stimulation of Pkc1p for signal transduction. Rho1p may serve to coordinate, both specially and temporally, several events required for effective cell wall remodeling. Both the GTP requirement for GS activity and the structure of fungal Pkcs are evolutionarily conserved (*6*, *19*), which suggests that the dual function of Rho1p may be conserved as well.

REFERENCES AND NOTES

1. V. J. Cid *et al.*, *Microbiol. Rev.* **59**, 345 (1995); F. M. Klis, *Yeast* **10**, 851 (1994).
2. P. C. Mol, H. M. Parks, J. T. Mullins, E. Cabib, *J. Biol. Chem.* **269**, 31267 (1994).
3. S. B. Inoue *et al.*, *Eur. J. Biochem.* **231**, 845 (1995).
4. C. M. Douglas *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12907 (1994); A. F. J. Ram, S. S. C. Brekelmans, L. J. W. M. Oehlen, F. M. Klis, *FEBS Lett.* **358**, 165 (1995); P. Garrett-Engele, B. Moilanen, M. S. Cyert, *Mol. Cell. Biol.* **15**, 4103 (1995).
5. P. Mazur *et al.*, *ibid.*, p. 5671.
6. P. J. Szaniszlo, M. S. Kang, E. Cabib, *J. Bacteriol.* **161**, 1188 (1985).
7. W. Yamochi *et al.*, *J. Cell Biol.* **125**, 1077 (1994).
8. P. Madaule, R. Axel, A. M. Myers, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 779 (1987).
9. H. Qadota, Y. Anraku, D. Botstein, Y. Ohya, *ibid.* **91**, 9317 (1994).
10. Yeast strains YOC752 (*rho1-2*), YOC729 (*rho1-3*), YOC754 (*rho1-4*), YOC755 (*rho1-5*), and YOC764 (wild type) were used here. YOC752, YOC729, and YOC755 were hypersensitive to Calcofluor white and echinocandin B at 23°C.
11. C. J. Der, T. Finkel, G. M. Cooper, *Cell* **44**, 167 (1986).
12. YPH499 cells carrying plasmids with wild-type *RHO1* (pYO762), *RHO1-G19V* (pYO906) under the control of the *GAL1* promoter, or vector alone (pYO761) were used. Cells were incubated in galactose medium for 10 hours, and GS activity associated with the membrane fraction was measured (*3*). Most of the GS activity from cells with pYO906 was independent of GTP-γ-S, whereas only 15 to 20% of the activity in the control strains was independent of GTP-γ-S.
13. S. B. Inoue, H. Qadota, T. Watanabe, Y. Ohya, unpublished results.
14. H. Nonaka *et al.*, *EMBO J.* **14**, 5931 (1995).
15. Y. Kamada *et al.*, *J. Biol. Chem.* **271**, 9193 (1996).
16. A temperature-sensitive *pkc1* strain (SYT11-12A) and its isogenic wild-type strain (YS3-6D) [S. Yoshida, E. Ikeda, I. Uno, H. Mitsuzawa, *Mol. Gen. Genet.* **231**, 337 (1992)] were grown in YPD (yeast extract–peptone-dextrose) at 23°C. A *pkc1*Δ strain (DL376) and its isogenic wild type (DL100) [D. E. Levin and E. Bartlett-Heubusch, *J. Cell Biol.* **116**, 1221 (1992)] were grown at 23°C in YPD containing 10% sorbitol. GS activities were assayed at 23° and at 37°C.
17. Mutants used were *rho1-3* and *rho1-5* carrying *PKC1* on a multicopy plasmid (pYO910) or vector alone (pYO324).
18. The partially purified enzyme fraction (second product entrapment) was analyzed by immunoblotting with antibody to Pkc1p.
19. T. Toda, M. Shimanuki, M. Yanagida, *EMBO J.* **12**, 1987 (1993); G. Paravicini *et al.*, *Yeast*, in press.
20. Crude extracts were prepared as described [Y. Kamada, U. S. Jung, J. Piotrowski, D. E. Levin, *Genes Dev.* **9**, 1559 (1995)] and stored at −80°C in lysis buffer supplemented with 33% glycerol. Membrane fractions, where indicated, were obtained from crude extracts, and GS activity was measured as described in (*2*) with the following modifications: uridine diphosphate–[^{3}H]glucose was used as the substrate and α-amylase (1 U/40 μl) was added to reaction mixtures to eliminate [^{3}H]glucose incorporation into glycogen.
21. Recombinant GST-Rho1p and GST-Cdc42p were expressed in Sf9 insect cells and purified as described [Y. Zheng, R. Cerione, A. Bender, *J. Biol. Chem.* **269**, 2369 (1994)].
22. A series of protein sample dilutions was analyzed by immunoblotting with guinea pig antiserum to Rho1p or mouse monoclonal antibody to Fks1p (T2B8) (*3*). Each antibody recognized a single band with an apparent molecular size of 25 kD (Rho1p) (*13*) or 200 kD (Fks1p) (*3*) on immunoblots of yeast whole membrane fraction. The amount of antigens was estimated by densitometry.
23. Goat antibody to mouse immunoglobulin G (IgG) coupled to agarose (20 μl; Sigma) was incubated with medium (500 μl) from cultures of cells producing the monoclonal antibody for 5 hours at 37°C. The agarose beads were washed five times with phosphate-buffered saline and twice with buffer A [0.4% CHAPS, 0.08% cholesteryl hemisuccinate, 50 mM tris-Cl (pH 7.5), 1 mM EDTA, 8 μM GTP-γ-S, and 33% glycerol]. Partially purified GS (1.8 μg) was added, and the reaction mixtures were further incubated for 2 hours at 37°C. The beads were washed four times with buffer A, and the bound complexes were analyzed by immunoblotting with antiserum to Rho1p or monoclonal antibody to Fks1p (T2B8).
24. Cells of haploid strain YOC785, which bears *rho1*Δ and the HA-tagged *RHO1* gene (*7*) on a centromere plasmid (pYO904), were double-stained with mouse monoclonal antibody to Fks1p (T2B8) and rabbit antibody to HA (MBL, Nagoya, Japan) as described [J. R. Pringle *et al.*, *Methods Cell Biol.* **31**, 357 (1989)]. Secondary antibodies were fluorescein isothiocyanate–conjugated antibody to mouse IgG (Cappel) and tetramethylrhodamine B isothiocyanate–conjugated antibody to rabbit IgG (Cappel). Control strains (YPH499 for ^{HA}Rho1p and Δ*fks1* for Fks1p) produced no signals in single staining experiments. The secondary antibodies did not cross-react with the heterologous primary antibodies. Some internal punctate staining of Fks1p that did not colocalize with ^{HA}Rho1p may represent the presence of secretory intermediates.

25. We thank J. Mulholland and D. Botstein for facilities to raise antibodies, Y. Kamada and R. Cerione for discussion, A. Myers for plasmids, and B. J. Blacklock for Sf9 cells. Supported by grants from the Ministry of Education, Science, Sports and Culture of Japan (07740581 and 07254203) to Y.O., from NIH (GM48533) and the American Cancer Society (FRA-446) to D.E.L., from the Japan Society for the Promotion of Science for Japanese Junior Scientists to H.Q., and from the Swiss National Science Foundation to C.C.P.

4 December 1995; accepted 20 February 1996