In Vitro Activity of 1,3-\(\beta\)-D-Glucan Synthase Requires the GTP-binding Protein Rho1*  

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In the yeast Saccharomyces cerevisiae, the family of RHO genes are implicated in the control of morphogenetic events although the molecular targets of these GTP-binding proteins remain largely unknown. The activity of 1,3-\(\beta\)-D-glucan synthase, the product of which is essential for cell wall integrity, is regulated by a GTP-binding protein, which we here present evidence to be Rho1p. Rho1p was found to copurify with Fks1p, a glucan synthase subunit, in preparations of the enzyme purified by product entrapment and was also shown to be depleted by a detergent extraction procedure known to remove the GTP-binding regulatory component. Specific ADP-ribosylation of Rho1p by exoenzyme C3 inactivates glucan synthase activity specified by FKS1 and FKS2 as demonstrated in membrane preparations from fks2 and fks1 deletion strains, respectively, and in the purified enzyme containing Fks1p. Rho1p and Fks1p were co-immunoprecipitated from purified glucan synthase under conditions that maintained enzyme activity in the immunoprecipitate. Putative Rho homologs were also identified and implicated in the regulation of glucan synthase activity from Candida albicans, Aspergillus nidulans, and Cryptococcus neoformans by ribosylation studies. The regulation of 1,3-\(\beta\)-D-glucan synthase activity by Rho1 is consistent with its observed role in morphogenetic control and osmotic integrity.

The cell wall of Saccharomyces cerevisiae and other yeast and fungi is an essential structural element, providing osmotic support and defining cell morphology. It is also a dynamic component(s), respectively. The fractions are inactive individually but yield active enzyme when recombined (4). Stimulation by GTP and the requirement for a GTP-binding protein appear to be a conserved feature of glucan synthases to the extent that the regulatory component is functionally interchangeable in a number of yeast and fungi (4, 5). The partial purification and characterization of the GTP-binding/GTPase properties of the glucan synthase activating component from S. cerevisiae have been reported (6). A GTPase activating protein affecting the level of GTP stimulation has also been identified (6).

Many regulatory GTPases have a functional requirement for posttranslational prenylation. In Schizosaccharomyces pombe, 1,3-\(\beta\)-D-glucan synthase activity is reduced as a result of a mutation in the cwg2 gene, which encodes the \(\beta\)-subunit of geranylgeranyltransferase I (7). It was proposed that this activity prenylates the GTP-binding component of glucan synthase. In S. cerevisiae, the substrates of geranylgeranyltransferase I include the small GTPases encoded by RH01 and CDC42 (8), members of the RHO family implicated in the control of yeast morphogenetic processes (1). RHO1 is an essential gene (9) required for maintaining osmotic integrity and also required for yeast budding and cell surface growth, results suggesting a possible role in the control of cell wall formation and cytoskeletal organization (10, 11). Moreover, it was recently observed that rho1-ts mutants resulted in temperature-sensitive glucan synthase enzyme activity, which could be remedied by the addition of recombinant Rho1p, further implicating Rho1p as the regulatory subunit of glucan synthase (12, 37).

Recent progress has been made in identifying other genes affecting 1,3-\(\beta\)-D-glucan synthase (13-18); in this regard, multiple approaches have led to the identification of FKS1 (CND1, CWH53, ETG1, GSC1, PBR1) and FKS2 (GSC2) (13, 18–25). The simultaneous disruption of FKS1 and FKS2 is lethal, suggesting essential overlapping function of the gene products (25), which are large (≈215 kDa) integral membrane proteins. Several lines of evidence indicate that Fks1p and the Fks2p homolog (88% sequence identity) are required, possibly catalytic, functionally redundant subunits of glucan synthase including: 1) the identification of Fks1p and Fks2p as the target of the glucan synthase inhibitors echinocandins and papulacandins (13, 19–21, 25), 2) the reduction of glucan synthase specific activity in membranes prepared from fks1-deleted strains (19, 20, 24, 25), 3) the co-purification of Fks1p with glucan synthase activity (24), and 4) the immunodepletion of glucan synthase activity with monoclonal and polyclonal anti-Fks antibodies (24, 25).

The specific ADP-ribosylation of members of the Rho family with Clostridium botulinum exoenzyme C3 (ADP-ribosyltransferase) has proven to be a useful tool in identifying Rho proteins and in characterizing both their in vivo and in vitro (26-29) biological function(s). In light of these observations, we investigated the presence and function of Rho1p in glucan synthase utilizing ribosylation and immunochemical studies. We report results which suggest that Rho1p is the regulatory component of 1,3-\(\beta\)-D-glucan synthase, and we also demonstrate the in vitro association of Rho1p with Fks1p, the putative catalytic subunit of the enzyme.
with Tris-glycine gels (Novex) run with prestained molecular weight standards (SeeBlue from Novex or high range from Bio-Rad). Samples were prepared in SDS-sample application buffer (Novex) containing 1% (v/v) β-mercaptoethanol; Rho protein samples were denatured by boiling for 2 min, while Fks protein samples were denatured at room temperature for 2 min. Western blotting was performed as described previously (25), with the exception that blots were dried by chemiluminescence with Renaissance reagents (DuPont NEN).

Exoenzyme C3 Ribosylation—Exoenzyme C3 from C. botulinum type C (ATCC 17784) was obtained from Calbiochem. Recombinant exoenzyme C3 (derived from C. botulinum type D-1873) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). For identification of exoenzyme C3 and ribosylation, the final reaction was incubated at room temperature in the following medium (Difco nitrogen base with amino acids, 10 mM potassium fluoride, GTP, and 20 mM potassium phosphate, pH 8.0, 1.0 mM MgCl₂, 1.0 mM AMP, and 0.25 mM [³²P]InAD (2.5 C/mmol; Amersham). A typical reaction was performed in 10 μl with 25 μg of membrane protein. Reactions were incubated with recombinant exoenzyme C3 at 20-25 °C for 1 h. Reactions were quenched with cold 10% trichloroacetic acid, and proteins were resolved by SDS-PAGE on 14 or 16% gels. Following autoradiography, bands were quantitated on a PhosphoImager with ImageQuant software (Molecular Dynamics). To determine the effects of ribosylation on glucan synthase activity, samples were diluted into a final labeling reaction containing 50 mM HEPES, pH 8.0, 1.0 mM MgCl₂, 1.0 mM AMP, and 2.0 mM NaD. Reactions were initiated by the addition of exoenzyme C3 (1 μg/ml) to the indicated final concentration and incubated at 30 °C (identical to other conditions unless otherwise noted). The final reaction was performed in 70 μl with 100 to 400 μg of membrane protein. Portions were removed at the indicated time points and used immediately to initiate a glucan synthase activity assay (sample diluted 4-fold in the assay), performed as described above. Non-ribosylated control samples were similarly prepared, except that exoenzyme C3 was omitted, and portions assayed at the same time points.

Tergitol Nonidet P-40/NaCl Fractionation—Microsomal membranes from YFF2714-5B (fks2Δ) were subjected to ribosylation with exoenzyme C3 (20 μg/ml) and [³²P]InAD as described above, except that the incubation (30 °C) was limited to 10 min. The sample was then adjusted to 2% Nonidet P-40 and 2 M NaCl to inhibit the activity of exoenzyme C3. The final reaction was performed in 70 μl with 100 to 400 μg of membrane protein. Portions were removed at the indicated time points and used immediately to initiate a glucan synthase activity assay (sample diluted 4-fold in the assay), performed as described above. Non-ribosylated control samples were similarly prepared, except that exoenzyme C3 was omitted, and portions assayed at the same time points.

Immunochromatographic Studies with Anti-Fks2p—Immunoprecipitations were performed with the following materials: glucan synthase purified by product entrapment (present work), anti-Fks2p (228) antibodies generated and affinity-purified (as described previously (25), and Staphylococcus aureus cells (Pansorb Inc., Pansorbin cells; Calbiochem) washed and resuspended in glucan synthase activity assay buffer. The membrane and soluble fractions were assayed for glucan synthase activity separately and in combination as described above.

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RESULTS

Rho1p Co-purifies with Glucan Synthase—We have observed that optimal solubilization of membrane bound S. cerevisiae glucan synthase activity is achieved with 0.2% CHAPS and that enzyme from the fks2-deleted strain is more readily solubilized than that from the fks1-deleted strain under these conditions. Solubilized Fks1p glucan synthase was purified approximately 300-fold to a specific activity of 2 mol of glucose incorporated per min/mg of protein (at 25 °C) by product entrapment and resolubilization in 0.4% CHAPS, 0.08% cholesterol hemisuccinate by a modification of a recently reported procedure (24). Similar to the results of Inoue et al. (24), a significant enrichment of Fks1p and a number of smaller polypeptides was observed by Western blot and silver-stained SDS-PAGE (data not shown).

Efforts to label and identify a small molecular weight GTP-binding protein in the purified sample by photoaffinity labeling with azido-[32P]GTP were unsuccessful (6). The presence of Rho1p in glucan synthase samples was assessed by Rho-specific ADP-ribosylation with C. botulinum exoenzyme C3 and [32P]NAD. Previous studies (11, 26) have demonstrated that in yeast, Rho1p is the sole target of ribosylation by exoenzyme C3 from both C and D-type strains of C. botulinum. We verified the Rho1p specificity of the exoenzyme C3 employed in this work with a rho1N46S mutant strain in which the site of Rho1p ribosylation (Asn46) is abolished (11). A labeled Rho protein band was not detected in autoradiographs of ribosylation reactions with membrane preparations from the rho1N46S mutant (Fig. 1A). A Rho1p band at approximately 23 kDa was observed following ribosylation (Fig. 1A) of both crude microsomal membrane fractions (WT, fks2a, and fks1Δ) and purified glucan synthase prepared from the fks2Δ strain. Approximately equal amounts of glucan synthase activity from the crude and purified fks2Δ glucan synthase preparations were analyzed in Fig. 1A, lanes 4 and 5.

Glucan Synthase Is Inactivated by Ribosylation of Rho1p—Incubation of wild-type microsomal membrane fractions with exoenzyme C3 resulted in a loss of glucan synthase activity in subsequent enzyme assays (Fig. 1B). Exoenzyme C3 dose-dependent inactivation was observed; maximal inactivation was 75–80% at ≅10 μg/ml exoenzyme C3. However, subjection of membrane preparations from the rho1N46S mutant strain to the same ribosylation reaction had no effect on glucan synthase activity (Fig. 1C). The presence of the mutant Rho1N46Sp did not have a significant effect on the glucan synthase specific activity (data not shown). Fks1p and Fks2p are alternative glucan synthase subunits with overlapping function (25). To determine the potential dependence on Rho1p of glucan synthase containing either Fks1p or Fks2p, microsomal membranes prepared from fks2-deleted or fks1-deleted strains were subjected to ribosylation and assayed. Enzyme activity of both Fks1p and Fks2p glucan synthase was rapidly inactivated to the same extent (−80%) as found with the wild type (Fig. 1C).

In addition, glucan synthase in membrane preparations from wild-type cells grown under conditions that induce expression of Fks2p and repress that of Fks1p (10 mM CaCl2 or acetate as the carbon source) (25) was similarly inactivated by −80% following ribosylation (data not shown). The extent of inactivation of membrane bound Fks1p glucan synthase was not altered by the presence or absence of GTP-γS or GTP during preparation of the membrane samples. Inactivation of purified soluble Fks1p glucan synthase by Rho1p ribosylation was also observed, although to a lesser extent (55%) and at a slower rate (Fig. 1C). We suspected that the detergents present in the purified glucan synthase might be retarding the ribosylation reaction; however, a 2–10-fold dilution of the enzyme sample during ribosylation did not alter the rate nor extent of inactivation.

Extraction of Rho1p from Microsomal Membranes—An initial indication that glucan synthases contain a peripherally bound GTP-binding protein was the ability to extract the required stimulatory component from membrane preparations with detergent and salt (4). We analyzed the distribution of Rho1p as a function of this fractionation procedure. Microsomal membranes prepared from the fks2-deleted strain were C3-ribosylated and subjected to extraction with Nonidet P-40 and NaCl. As shown in Fig. 2A, Rho1p is essentially quantitatively extracted by this treatment. Control studies that omitted the ribosylation demonstrated that glucan synthase activity of each fraction is significantly depleted and can be partially recovered (−55%) by recombining the fractions (Fig. 2B), as reported previously (4). Efforts to ribosylate Rho1p in the supernatant fraction following detergent extraction were unsuccessful, presumably due to effects of the Nonidet P-40 (data not shown).

Co-immunoprecipitation of Fks1p and Rho1p—We and others have suggested previously that Fks1p and Fks2p are alternative subunits of glucan synthase (19, 24, 25), possibly comprising the catalytic component of the enzyme, based in part on the nearly quantitative immunodepletion of glucan synthase activity from 0.2% CHAPS solubilized membrane extracts from an fks1-deleted strain with anti-Fks2p antibodies (25). The presence of Fks2p in the immunoprecipitated fractions was verified by anti-Fks2p Western blots and immunoprecipitation
were subjected to ribosylation with exoenzyme C3 (20 μg/ml) and [32P]NAD and extracted with 2% Nonidet P-40 and 2 M NaCl as described under “Materials and Methods.” The samples were analyzed by SDS-PAGE, autoradiography, and PhosphorImager analysis. Lanes: 1, detergent treated membrane prior to centrifugation; 2, extracted supernatant fraction; 3, extracted membrane fraction. B, glucan synthase activity was determined for the soluble and membrane fractions (similarly obtained except that exoenzyme C3 and the 32P isotope were omitted) separately and in combination as described under “Materials and Methods.” Results in B are plotted as the percent glucan synthase activity relative to membranes (plus Nonidet P-40) and are the mean ± S.D. for 2 determinations. Lanes: 1, extracted supernatant fraction; 2, extracted membrane fraction; 3, combined supernatant and membrane fractions.

experiments with 35S-labeled cell extracts. We have found that while this antibody is specific for Fks2p under denaturing conditions (i.e. Western blot), it will also cross-react with Fks1p under non-denaturing conditions and can immunodeplete Fks1p-derived glucan synthase activity in a manner specifically blockable by the peptide (pks2-1) against which the antibody was generated (Fig. 3). Although the homology between the FKS2-derived peptide, pks2-1, and the corresponding FKS1-derived sequence is limited (38% identity), it would appear that the anti-Fks2p antibodies are recognizing an epitope common to both native proteins. Furthermore, immunoprecipitation of soluble purified glucan synthase with anti-Fks2p antibodies and S. aureus cells results in an immunoprecipitate fraction that substantially retains glucan synthase activity (Fig. 3, sample 1). It is observed that formation of the Fks2p-antibody-S. aureus complex is moderately inhibitory (Fig. 3, compare samples 2 and 5); if the activity in the anti-Fks2p immunoprecipitate is compared with that in the noncentrifuged ternary immunocomplex as the relevant control (sample 2), the relative recovery of activity in the immunoprecipitate fraction (samples 1P and 7P) represents 81 and 94%, respectively. The observed recovery of activity prompted us to ask if Fks1p and Rho1p co-immunoprecipitate under the same conditions. Immunoprecipitation of purified glucan synthase with anti-Fks2p and subsequent determination of the distribution of Rho1p and Fks1p by C3 ribosylation (Fig. 4A) and Western blot (Fig. 4B), respectively, indicates substantial co-immunoprecipitation of Rho1p with Fks1p under conditions demonstrated to maintain nearly full enzymatic activity.

Rho Homologs in Other Organisms—Previous studies on the fractionation and exchange of the regulatory and catalytic components of glucan synthase from different fungi and yeast have suggested that the presence of a GTP-binding regulatory subunit is a common feature of glucan synthases (4, 5). ADP-ribosylation by exoenzyme C3 is highly selective for members of the Rho family (11, 30); therefore, we employed ribosylation assays to determine if Rho homologs might be implicated in glucan synthase regulation in other organisms. Exoenzyme C3 ribosylation and autoradiography of microsomal membranes from S. cerevisiae by product entrapment and have demonstrated that Rho1p-specific ADP-ribosylation by exoenzyme C3 significantly inhibits glucan synthase activity. That the protein co-purifying with glucan synthase and implicated in its function is definitely Rho1p was confirmed by studies with a rho1ΔN4665 mutant strain expressing a functional mutant Rho1p that cannot be ribosylated (Fig. 1A) (11). While ribosylation of RHO1 membrane preparations results in a 80% loss of glucan synthase activity, similar treatment of rho1ΔN4665 membrane preparations with exoenzyme C3 had no effect. Since glucan synthase activity has a nearly complete requirement for the regulatory component (6), the incomplete inactivation observed likely reflects either incomplete ribosylation or partial function of the ribosylated Rho1p. However, the possibility that an alternate stimulatory component is present cannot be discounted. ADP-ribosylation of Rho proteins occurs at an asparagine residue (Asn46 in Rho1p) in the putative effector domain, but affects neither GTP binding nor hydrolysis (31). Presumably, the consequences of ribosylation reflect altered interactions with the effector protein, although we observed that Rho1p ribosylation did not affect the co-immunoprecipitation of Rho1p and Fks1p (data not shown).

These results, and those discussed below, suggest that in vitro glucan synthase activity requires Rho1p. This conclusion is consistent with the recent observations of Ohya and co-workers who also observed co-purification and co-immunopre-
cell growth (10). These studies suggested that two populations of Rho1p are present; one localized to a saturable binding site(s) in the plasma membrane, located specifically at sites of adherence to the cytosol and the other localized to a saturable binding site in the cytosol. Recent results indicate a second function of Rho1p and suggest that the cell lysis phenotype accompanying a rho1 deficiency results, at least in part, from a reduction in 1,3-β-D-glucan synthase activity.

The existing biochemical evidence for a GTP-binding regulator subunit of glucan synthase rests largely on the detergent extraction of the regulatory component from microsomal membranes accompanied by a reversible loss of enzyme activity (4) and the partial purification and characterization of the regulatory activity from the detergent-soluble fraction (6). Consistent with these observations we have shown that the same extraction of an fks2-deleted membrane preparation results in the nearly complete depletion of Rho1p from the membrane fraction. As a glucan synthase component, and quite possibly the catalytic subunit itself, Fks1p (or Fks2p) would be expected to be associated with the regulatory GTP-binding protein. Here we have demonstrated the nearly complete co-immunoprecipitation of Fks1p and Rho1p from purified glucan synthase by a cross-reacting anti-Fks2p antibody. The fact that Fks1p and Rho1p are associated in an immunocomplex retaining nearly all of the initial soluble glucan synthase activity strongly implicates both proteins as subunits of the enzyme.

Recent results suggest that the yeast cell has two glucan
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synthase isozymes, one containing Fks1p and the other containing Fks2p, and that each isozyme is normally involved in distinct cell wall synthesizing roles (25). Fks1p is implicated in vegetative bud growth on glucose where it is the predominant isoform expressed, while Fks2p is implicated in sporulation and the response to mating pheromone. Fks1p and Fks2p are highly homologous (88% sequence identity) except for a notable dissimilarity at the N termini. This region, predicted to be cytoplasmic and unusually acidic (19, 25), might be important in differentiating the two isoforms, and hence glucan synthase activities, by localization and/or regulation. We were especially intrigued by the finding that the simultaneous deletion of RHO3 and RHO4 results in a phenotype similar to that of rho1 mutants (33), suggesting that a different RHO homolog might also be involved in the control of glucan synthase. However, our present results show glucan synthase activity in both fks2Δ and fks1Δ membranes prepared to be equally sensitive to inactivation by Rho1p-specific ribosylation, suggesting that Rho1p is the required, or at least the predominant, regulatory component of glucan synthase containing either Fks1 isoform.

It is, however, possible that in cells lacking FKS1 or grown under conditions which induce FKS2, Fks2p is largely forced into a compensating role requiring the regulation or perhaps localization of glucan synthase by Rho1p, whereas in its normal specialized role(s) glucan synthase containing Fks2p might be regulated by an alternate RHO homolog.

Ribosylation of membrane extracts from Candida, Aspergillus, and Cryptococcus identified putative Rho protein homologs in these organisms, based on the presumption that the Rho selectivity of exoenzyme C3 observed in S. cerevisiae and human cell extracts (11, 30) is maintained in these yeast and fungal species. The partial, albeit variable, glucan synthase inactivation in man cell extracts (11, 30) is maintained in these yeast and fungi. The partial, albeit variable, glucan synthase inactivation by Rho1p-specific ribosylation, suggesting that Rho1p is the required, or at least the predominant, regulatory component of glucan synthase containing either Fks1 isoform. Inactivation by Rho1p-specific ribosylation, suggesting that Rho1p is required, or at least the predominant, regulatory component.

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