Prenylation of Rho1p Is Required for Activation of Yeast 1,3-β-Glucan Synthase

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One of the essential protein substrates of geranylgeranyl transferase type I in the budding yeast Saccharomyces cerevisiae is a rho-type GTPase, Rho1p, which is a regulatory subunit of 1,3-β-glucan synthase. Previous studies have indicated that modification of Rho1p is significantly reduced in a mutant of the β subunit of geranylgeranyl transferase type I called cal1-1. Here we present genetic and biochemical evidence showing that modification of Rho1p is required for activity of 1,3-β-glucan synthase. The 1,3-β-glucan synthase activity of the cal1-1 membrane was significantly reduced compared with that of the wild-type membrane. The impaired activity was partly due to the reduced amount of Fks1p, a putative catalytic subunit of 1,3-β-glucan synthase, but also partly due to reduced affinity between unmodified Rho1p and Fks1p. Glutathione S-transferase (GST)-Rho1 protein with or without the C-terminal motif required for the modification were purified and used to analyze the interaction. The modified form of GST-Rho1p was specifically able to restore the 1,3-β-glucan synthase of the rho1-3 membrane. Gel overlay analysis indicated that an unmodified form of GST-Rho1p fails to interact with Fks1p. These results indicated that the geranylgeranylation of Rho1p is a prerequisite to the assembly and activation of 1,3-β-glucan synthase in vivo. Increased cytoplasmic levels of divalent cations such as Ca²⁺ restored both Rho1p modification and the 1,3-β-glucan synthase activity of cal1-1, suggesting that cytoplasmic levels of the divalent cations affect geranylgeranyl transferase type I activity in vivo.

Post translational modification of proteins is essential for the precise targeting and function of many proteins. One of the well-known modifications, protein prenylation, has been observed at the C terminus of a wide variety of cellular proteins, including members of Ras and Rho small GTPase, the γ subunit of G-protein, nuclear lamin, and yeast a-factor. Three distinct enzymes catalyze these reactions. Farnesyl transferase transfers the C-15 farnesyl, and geranylgeranyl transferase type I (GGTase I)¹ and type II (GGTase II) transfer the C-20 geranylglycereryl. Among these enzymes, farnesyl transferase and GGTase I have similar properties; they are composed of an α/β heterodimer with a common α subunit, and their recognition motif is highly conserved. Proteins that are prenylated by these enzymes contain a C-terminal CAAX recognition sequence, where C is cysteine, A is an aliphatic amino acid, and X is any amino acid. The cysteine residue is the site of prenylation, and the last amino acid is the primary determinant of each enzyme. Farnesyl transferase preferentially prenylates the CAAX-containing proteins, whose X is methionine, serine, cysteine, glutamine, or alanine, whereas GGTase I preferentially prenylates them if X is leucine (1-3). Cross-specificity, however, has also been reported both in vitro (4-6) and in vivo (7, 8), probably because of the similarity in subunit composition. Both farnesyl transferase and GGTase I are Mg²⁺-requiring, Zn²⁺-metalloenzymes that require Mg²⁺ for isoprenoid transfer and Zn²⁺ for binding of the protein substrate (9, 10). In addition, the yeast GGTase can function with only Ca²⁺ (11). GGTase II shows slightly different properties. GGTase II modifies Rab/Ypt proteins at the C-terminal first or second cysteine. Although GGTase II is composed of catalytic α/β subunits, this catalytic core lacks the ability to bind Rab/Ypt proteins efficiently and therefore the Rab escort protein is also necessary for the activation (12, 13). In the budding yeast Saccharomyces cerevisiae, genes that encode these proteins had already been identified. RAM2 encodes the common α subunit of farnesyl transferase and GGTase I (14). CAL1 (15), also known as CDC43 (16), and DPR1 (17), also known as RAM1, encode the distinct β subunit of GGTase I and farnesyl transferase, respectively. Yeast α and β subunits of GGTase II are encoded by BET4 and BET2, and yeast Rab escort protein is encoded by MSI4/MSR6 (18-21).

S. cerevisiae contains two essential rho-type small GTPases, Rho1p and Cdc42p, which are modified by GGTase I. It is noted that two mutants of β subunit of GGTase I, cal1-1 and cdc43-5, exhibited different substrate specificity. In cal1-1 cells, the proportion of Rho1p in a soluble fraction increased dramatically, but the localization of Cdc42p was not affected by cal1-1. On the other hand, in cdc43-5 cells, Cdc42p but not Rho1p was observed in a soluble fraction (22). In addition, the ts lethality of cal1-1 was suppressed by the overexpression of RHO1, and that of cdc43-5 was suppressed by the overexpression of CDC42 (22). These results suggested that modification of Rho1p and Cdc42p are specifically impaired in cal1-1 and cdc43-5, respectively.

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¹ The abbreviations used are: GGTase I, geranylgeranyl transferase type I; GGTase II, geranylgeranyl transferase type II; GS, 1,3-β-glucan synthase; GTPase, guanine 5’-y-triphosphate; YPD, yeast extract/peptone/dextrose; CHAPS, 3-[3-cholamidopropyl]dimethyammonio]-1-propanesulfonic acid; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; Mes, 4-morpholineethanesulfonic acid.

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Rh1p, a yeast homolog of mammalian rhoA, regulates cell morphogenesis and cell wall integrity through the transfer of specific signals via effector proteins (23). Four effectors (or potential effectors) of Rh1p were identified so far (Pck1p, 1,3-β-glucan synthase, Bni1p, and Skn7p). Pck1p, which is a yeast homolog of mammalian protein kinase C, was first identified as a protein that interacts with Rh1p (24, 25). Pck1p regulates the cell wall integrity by activating the mitogen-activated protein kinase cascade. Rh1p was identified as the regulator of 1,3-β-glucan synthase (GS) (26–28). In the fission yeast Schizosaccharomyces pombe, GS is also regulated by Rh1p (29). Bni1p also interacts with Act1p, yeast actin, and Pfy1p, yeast profilin; thus suggesting that Rh1p regulates yeast morphogenesis through Bni1p (30). Skn7p is a transcription factor that has recently been shown to bind to Rh1p (31).

In this study, we genetically investigate the roles of geranylgeranylation in Rh1p function with a conditional lethal mutant of GGTase I. We also biochemically examine the activity of modified and unmodified forms of Rh1p directly by using recombinant proteins expressed in insect cells. Our results indicate that geranylgeranylation of Rh1p is required for both assembly and activity of GS.

**EXPERIMENTAL PROCEDURES**

*Strains and Growth Conditions—Escherichia coli* DH5α (Toyobo) was used for the preparation of plasmids. DH10Bac (Life Technologies, Inc.) was used for the recombination and the preparation of bacmids containing the baculo virus genome with recombinant RHO1. Yeast strains used in this study are YPH500 (MATα ade2 his3 leu2 lys2 trp1 ura3), YOT435–1A (MATα ade2 his3 leu2 lys2 trp1 ura3 cdc43-5) (22), YOC729 (MATα ade2 his3 leu2 lys2 trp1 ura3 rho1::LYS2 ade2::pRHO1-rho-1.3-HIS3) (26), YOC788 (MATα ade2 can1 leu2 trp1 ura3::TRP1), YOC2371 (MATα ade2 his3 leu2 lys2 trp1 ura3::TRP1), YOC2372 (MATα ade2 his3 leu2 lys2 trp1 ura3 cal1::URA3), YOC2373 (MATα ade2 his3 leu2 lys2 trp1 ura3 vma1::TRP1), and YOC2374 (MATα ade2 his3 leu2 lys2 trp1 ura3 vma1::URA3). All strains were cultured in YPD containing 2% glucose (Wako Chemicals), 2% b-glucan synthase activity, the membrane fraction was prepared as described previously (33).

### Experimental Procedures

#### Preparation of Recombinant Rho1p—RHO1 open reading frame fragment of pYOT702 was cloned into pVL1393. The resulting pVL-RHO1 was used for the preparation of bacmids containing the baculo virus genome with recombinant RHO1. Yeast strains used in this study are YPH500 (MATα ade2 his3 leu2 lys2 trp1 ura3), YOT435–1A (MATα ade2 his3 leu2 lys2 trp1 ura3 cdc43-5) (22), YOC729 (MATα ade2 his3 leu2 lys2 trp1 ura3 rho1::LYS2 ade2::pRHO1-rho-1.3-HIS3) (26), YOC788 (MATα ade2 can1 leu2 trp1 ura3::TRP1), YOC2371 (MATα ade2 his3 leu2 lys2 trp1 ura3::TRP1), YOC2372 (MATα ade2 his3 leu2 lys2 trp1 ura3 cal1::URA3), YOC2373 (MATα ade2 his3 leu2 lys2 trp1 ura3 vma1::TRP1), and YOC2374 (MATα ade2 his3 leu2 lys2 trp1 ura3 vma1::URA3). All strains were cultured in YPD containing 2% glucose (Wako Chemicals), 2% b-glucan synthase activity, the membrane fraction was prepared as described previously (33).

Briefly, yeast cells were grown at 23 °C to mid-log phase, harvested, suspended in 0.1 ml of lysis buffer (0.8 M sorbitol, 1 ml EDTA, 10 ml Heps, pH 7.0, 2 ml phenylmethylsulfonyl fluoride), and lysed on ice by vortexing with glass beads. Greater than 80% lysis was confirmed by light microscopy. After the addition of 0.4 ml of lysis buffer, lysates were spun at 800 × g for 10 min at 4 °C, and the supernatant was adjusted to 0.6% by the addition of TEDM buffer, and centrifuged at 100,000 × g for 60 min at 4 °C. The CHAPS concentration of the supernatant was adjusted to 0.6% by the addition of TEDM buffer, and the solution was applied to a column of Mono-Q (Amersham Pharmacia Biotech) that had been equilibrated with TEDMC₉₀ buffer. The flow rate was 0.5 ml/min. After the sample was applied to the column, it was washed with 10 ml of TEDMC₉₀ buffer and eluted with a linear gradient of NaCl (0–500 mM) in TEDMC₉₀ buffer. The fractions containing Rh1p were identified by both Western blotting, using rabbit anti-Rho1p antisera, and by measurement of the GS activation using the rho-1 membrane fraction.

The GST fusion proteins were prepared as described below. The EcoRI–BglII fragment of pYOT70 containing the RHO1-G68L open reading frame was inserted into pBacGST, the resulting pBacGST-RHO1Q68L was transformed into DH10Bac (Life Technologies, Inc.), and the bacmid containing the baculo virus genome was prepared according to the manufacturer’s manual. The pBacGST-RHO1Q68L/LC2068S was constructed by polymerase chain reaction using UTma DNA polymerase (Perkin-Elmer). Two primers (primer1, 5′-GGGAAATTCATGTCACACAAATGTAACTAAGTACGGG-3′, and primer 2, 5′-GGGGTCGACTGATACAAAGATGAAGTTGG-3′), in which underlines show the EcoRI and SfuI site, respectively, were used. Rho1pQ68L was prepared from the detergent extract of the membrane fraction, and Rho1pQ68L/LC2068S was prepared from the soluble fraction. Both recombinant proteins were purified by chromatography with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manual. The eluted fraction was further purified by Mono-Q column chromatography.

#### Northern Blot Analysis—Total RNAs from YPH500, cal1-1, and rho-1-3 cells were isolated as described previously (35). Electrophoresis and hybridization were performed as described previously (36). The probe for FK51 was prepared by polymerase chain reaction with primers 5′-GGTGCGGCAACAATTGCAAAAGATGAAGTTGG-3′ and 5′-GGGGTCGACTGATACAAAGATGAAGTTGG-3′. The probes for YEF3 used as an internal control was the EcoRI–XhoI fragment of p99-YEF3 (37).

#### Cell Fractionation Experiments—A cell fractionation experiment was performed as described previously (22). Briefly, yeast cells were grown at 23 °C to mid-log phase, harvested, suspended in 0.1 ml of lysis buffer (0.8 M sorbitol, 1 ml EDTA, 10 ml Heps, pH 7.0, 2 ml phenylmethylsulfonyl fluoride), and lysed on ice by vortexing with glass beads. Greater than 80% lysis was confirmed by light microscopy. After the addition of 0.4 ml of lysis buffer, lysates were spun at 800 × g for 10 min at 4 °C, and the supernatant was then spun at 436,000 × g for 20 min at 4 °C, and the pellet was resuspended in the same volume (0.5 ml) of lysis buffer. Samples (10 μl) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The Western blot analysis was performed with rabbit anti-Rho1p antisera.

#### The Ligand Overlay Experiments—The ligand overlay experiment was performed as described previously (38, 39) with some modifications. Briefly, the partially purified 1,3-β-glucan synthase (2 μg) of *S. cerevisiae* was subjected to SDS-PAGE on a 5 to 20% gradient gel and then transferred to a polyvinylidene difluoride membrane (Millipore). The proteins on the membrane were denatured by agitating the membrane for 10 min in buffer C (25 mM Mes-NaOH, pH 6.5, 0.5 mM MgCl₂, 0.05 mM ZnCl₂, 0.05% Triton X-100) containing 6 μl guanidium hydrochloride, which was then diluted with an equal volume of buffer C. The denatured proteins on the membrane were reanimated by agitating the membrane overnight in phosphate-buffered saline containing 0.1% bovine serum albumin, 0.5 mM MgCl₂, 0.05 mM ZnCl₂, 0.1% Triton X-100, 5 mM dithiothreitol, 0.1% phosphatidylcholine, and 0.1% CHAPS. The membrane was washed three times with 25 ml Tris-HCl buffer, 0.1 mM MgCl₂, 0.1 mM NaCl, 0.05% Tween 20, 5 mM dithiothreitol, and 2 ml EDTA. The membrane was then reacted at room temperature for 30 min and then at 4 °C for 10 min with 10 μl of GST-Rho1p with [35S]GTP–S (1.25 pmol, 1,000 Ci/mmol; NEN Life Science Products) for 60 min at 30 °C. The reacted membrane was washed four times with 25 ml Mes-NaOH, pH 6.5, 50 mM NaCl, 5 mM MgCl₂, and 0.05% Triton X-100, dried, exposed to an imaging plate, and analyzed with a Bio Imaging Analyzer, model BAS 1000 Mac (Fuji Photo Film Co. Ltd.).
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TABLE I

The echinocandin B sensitivity of some mutants

| Strain          | IC₅₀ (μg/ml) |
|-----------------|-------------|
| Wild type (YPH500) | 2.20 ± 0.15 |
| cal1-1          | 0.49 ± 0.05 |
| cdc43-5         | 1.60 ± 0.23 |
| rho1-3          | 1.04 ± 0.23 |
| Δfks1           | 0.24 ± 0.09 |

RESULTS

The GS Activity of cal1/cdc43 Mutants—Rho1p, a regulatory subunit of GS, contains a consensus motif that can be modified by GGTase I (15). Modification of Rho1p is impaired in cal1-1, a temperature-sensitive mutant of the β subunit of GGTase I (22) that accumulates unmodified Rho1p in the cytosolic fraction. As the first step toward examining whether posttranslational modification of Rho1p is important for GS activity, we measured the GS activity of the membrane prepared from the cal1-1 strain. We found that the membrane fractions of cal1-1 cells exhibited a dramatic reduction of GS activity (Fig. 1). Another allele of cal1-1, cdc43-5 resulted in impairment of the Cdc42p modification but has preserved normal modification activity of Rho1p (22). As a result, no significant reduction of the GS activity was observed in the cdc43-5 membrane (Fig. 1). We next examined whether wild-type, cal1-1, and cdc43-5 strains were sensitive to echinocandin B, which is a known inhibitor of GS (Table I). The sensitivity of cal1-1 was 4–5-fold higher than that of the wild type. In contrast, cdc43-5 showed similar sensitivity to the inhibitor. These results suggested that unmodified Rho1p results in decreased GS activity in vivo.

To examine whether impaired GS activity in cal1-1 was due to decreased activity of unmodified Rho1p, we examined whether recombinant Rho1p restored the reduced GS activity of the cal1-1 membrane. As Qadota et al. (26) showed previously, impaired GS activity of the rho1-3 membrane is fully restored by the addition of recombinant Rho1p expressed by insect cells (Fig. 2A) (26). However, when recombinant Rho1p was added to the membrane fraction of cal1-1, only partial restoration (up to half) the amount of the wild type was observed (Fig. 2A). This suggested that components other than Rho1p are irreversibly damaged in cal1-1. Since the only GS component thus far known is the putative catalytic subunit, Fks1p, we next analyzed the protein level of Fks1p in the wild type and cal1-1. Western blot analysis indicated that the amount of Fks1p in cal1-1 was significantly reduced (Fig. 2C). The rho1-3 mutant exhibited a normal amount of Fks1p. Northern blot analysis of the FKS1 transcript showed that no significant differences were observed between the wild-type and cal1-1 cells (Fig. 2B). Taken together, these results imply that impaired GS activity in cal1-1 was partially due to decreased protein stability of the catalytic subunit, Fks1p.

Geranylgeranylation of Rho1p Is Necessary for the GS Activity in Vitro—Reduced GS activity in the cal1-1 cells suggested that geranylgeranylation of Rho1p is required for the GS activity in vivo. To examine whether the modification of Rho1p is directly required for the activation of GS, we analyzed the ability of modified and unmodified form of Rho1p to restore impaired GS activity of the rho1-3 membrane. C-terminal modified (Rho1pQ68L) and unmodified (Rho1pQ68LC206S) forms of Rho1p were expressed in insect cells as GST fusion proteins. The modified (Rho1pQ68L) and unmodified (Rho1pQ68LC206S) proteins were predominantly recovered in the membrane and cytosolic fraction, respectively, of infected cells. Both proteins were then purified by glutathione-Sepharose 4B and Mono-Q column chromatography (Fig. 3A). A Q68L mutation was used because this mutation results in constitutively active Rho1p. We found that the addition of unmodified (Q68LC206S) Rho1p in amounts of up to 8.0 μg of protein showed no ability to restore the impaired GS activity (data not shown). The addition of either 0.06 μg of Rho1pQ68L or 0.06 μg of Rho1pQ68L and Rho1pQ68LC206S proteins, however, resulted in full activation of GS, indicating that this amount of Rho1pQ68LC206S contained no inhibitor activity (Fig. 3B). Thus these results implied that unmodified Rho1p fails to activate GS in vitro.

A ligand overlay assay with Candida albicans Rho1p has shown that yeast Rho1p can directly bind to the Candida homolog of Fks1p (39). In this experiment, we analyzed the ability of unmodified Rho1p to bind Fks1p. Rho1pQ68L and Rho1pQ68LC206S were radiolabeled with [35S]GTPγS and used for blotting on the polyvinylidene difluoride membrane onto which purified GS had been immobilized following SDS-PAGE. Binding of modified Rho1p (Rho1pQ68L) was observed at the position of Fks1p, but no signal was observed when the unmodified Rho1p (Q68LC206S) was used (Fig. 4). These results indicated that unmodified Rho1p fails to interact with Fks1p.

Intracellular Ca²⁺ Concentration Affects the cal1-1 Phenotype—The cal1-1 mutant was originally isolated as a temperature-sensitive mutant resulting in a Ca²⁺-dependent phenotype; it grows well in Ca²⁺-rich medium containing 100 mM CaCl₂ but not in YPD containing 200 mM CaCl₂ at 37 °C (40). It was also able to grow in a medium containing 3 mM MnCl₂ (Fig. 5A). Biochemical analysis of GGTase I indicated that the enzyme requires Zn²⁺ for its activity, and Ca²⁺ and Mn²⁺ can substitute for Zn²⁺. We therefore examined whether membrane localization of Rho1p and the GS activity in the cal1-1 cells were perturbed by divalent cations in the medium. When cal1-1 cells were cultured in YPD at 23 °C, Rho1p was mainly accumulated in the soluble fraction with modified and unmodified forms. But when these cells were cultured in YPD with 100 mM CaCl₂ or YPD with 3 mM MnCl₂ at 23 °C, Rho1p was observed both in soluble and insoluble fraction, and most Rho1p was observed in as the modified form (Fig. 5B). Moreover, cal1-1 cells cultured in YPD with 3 mM MnCl₂ showed a significant recovery of GS activity (Fig. 5C). These results are consistent with early observations that external divalent cations can affect the cal1-1 phenotype. Wild-type and cal1-1 cells
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Fig. 2. The effect of Rho1p addition to the cal1-1 membrane. A, samples (1 μl) of Rho1p-containing fractions (solid bars) or buffer (open bars) were added into the membrane fractions prepared from wild-type, cal1-1, and rho1-3 cells, respectively, and the GS activity was measured. The values represent the means and S.D. of six experiments. B, the Northern blot analysis of wild-type (lane 1), cal1-1 (lane 2), and rho1-3 (lane 3) total RNAs (10 μg). The filter was hybridized with FKS1 (upper) and YEF3 (lower) probes. C, Western blot analysis of membrane fractions (10 μg) prepared from wild-type (lane 1), cal1-1 (lane 2), and rho1-3 (lane 3). The Western blot analysis was performed with anti-Fks1p monoclonal antibody (T2B8).

DISCUSSION

In this report, we show that the geranylgeranylation of Rho1p has a critical role in the activation of GS. First, the GS activity was dramatically impaired in cal1-1 mutant, which is defective in modification of Rho1p. Second, in the reconstitution experiments with GST-Rho1 proteins, the modified (Q66L) but not the unmodified (Q68LC206S) Rho1p could restore the

Fig. 3. Rho1p geranylgeranylation is necessary for the GS activation. A, GST-Rho1pQ66L (lane 1) and GST-Rho1pQ68LC206S (lane 2) were prepared from insect cells and purified to approximate homogeneity (see “Experimental Procedures”). Samples containing 1 μg of each protein were separated by SDS-PAGE (12.5% gel) and stained with Coomassie Brilliant Blue. B, samples containing 0.06 μg of GST-Rho1pQ66L (solid bars), GST-Rho1pQ68LC206S (open bars), or both proteins (hatched bar) were added into the membrane fraction prepared from rho1-3. Then the GS activities were measured in either the presence or absence of GTPγS. The values represent the means and S.D. of three experiments.

Cultured in YPD with 100 mM CaCl2, however, exhibited significantly decreased GS activity (Fig. 5C). This may be because high Ca²⁺ concentrations would cause unknown damages to GS or to the plasma membrane (see “Discussion”).

Since cytoplasmic Ca²⁺ is sequestered into vacuole (41), a vma1 mutation of a catalytic subunit of vacuolar H⁺-ATPase resulted in an elevated concentration of intracellular Ca²⁺ (33, 42). We showed here that the vma1 mutation suppressed cal1-1. The temperature-sensitive growth phenotype of cal1-1 was suppressed by vma1. Also, population of Rho1p in the membrane fraction increased in the cal1-1/vma1 cells (data not shown). The cal1-1/vma1 cells showed a significant restoration (from ~35~70%) in GS activity (Fig. 6). These results suggest that impaired Rho1p geranylgeranylation in cal1-1 was restored by the increase of cytoplasmic Ca²⁺ concentration.

GS activity of rho1-3 membrane. Third, in the ligand overlay experiments with GST-Rho1 proteins, the modified Rho1p could specifically bind to Fks1p, a putative catalytic subunit of GS, but the unmodified Rho1p could not. Taken together, these results suggest that the geranylgeranylation of Rho1p would be prerequisite to both binding to Fks1p and the activation of GS. Recently, Illenberger et al. (45) demonstrate that the geranylgeranylation of mammalian Cdc42Hs and Rac1 is prerequisite for the stimulation of phospholipase C-β. It is also shown that the geranylgeranylation of rhoA is necessary for the association with phospholipase C-α and the resulting activation of AP-1 transcription (44). These findings clearly demonstrated that the geranylgeranylation of rho-type small GTPase is prerequisite to the activation of effector proteins. There has been indirect evidence suggesting that unmodified Rho1p has less activity toward GS in fission yeast (45). Our results reported here show the importance of Rho1p geranylgeranylation for both the binding to Fks1p and the activation of GS. Therefore, GS is the first effector of rho GTPase, which shows that the geranylgeranylation of rhoGTPase is prerequisite to both binding and activation.

So far, Pck1p, Bni1p, and Skn7p were also identified as protein targets of Rho1p (24, 25, 30, 31). The interaction between Rho1p and each protein was shown by two-hybrid assays with an unmodified form of Rho1p (24, 30, 31). The in vitro interaction between Rho1p and Pck1p was also shown by immunoprecipitation (25) with geranylgeranylated Rho1p. Moreover, the associations of other rho-type small GTPase, Cdc42p, to its effectors, Ste20p and Cla4p, were also revealed.
The values represent the means and S.D. of six experiments.

by the yeast two-hybrid assay (46, 47). It is likely that no geranylgeranylation is necessary for the binding to these effectors. It is still unknown, however, whether the geranylgeranylation would facilitate the binding to effectors and activate effectors or not. We tried to identify the interaction site between Rho1p and Fks1p by the yeast two-hybrid assay with unmodified Rho1p but could detect no obvious interactions (data not shown). Our present results suggest that the geranylgeranylation of Rho1p is prerequisite to both the association to Fks1p and the activation of GS. Thus, the yeast two-hybrid assay could not elucidate all protein-protein interactions, especially for proteins that are modified posttranslationally, although this method is still powerful for the detection of many protein-protein interactions. Therefore, some proteins may be detected as rho GTPase effectors because their interactions could not be detected by the yeast two-hybrid assay. Furthermore, it is a remaining possibility that the geranylgeranyl residue of Rho1p would have an important role in the efficient binding to effectors and the activation of effectors.

The impaired GS activity of cal1-1 would result from the reduced amount of Fks1p (Fig. 2C) but not the decreased mRNA level of FKS1 (Fig. 2B). These results imply that Fks1p is easily degraded when it does not bind Rho1p. Fks1p is synthesized on the membrane-bound ribosome and transported to the plasma membrane through the secretory pathway. Therefore, it is possible that Rho1p-unbound Fks1p is targeted to vacuole instead of the plasma membrane in the cal1-1 cells and is degraded in vacuole. It is also likely that the degradation of Rho1p-unbound Fks1p is facilitated. It is known that some plasma membrane proteins are subject to rapid degradation triggered by the change of environmental conditions. For instance, the yeast zinc transporter Zrt1p is internalized and degraded in the vacuole when cells are exposed to excess Zn\(^{2+}\) (48). The uracil permease Fur4p is also degraded in the vacuole by the signal of nutrient starvation (49). Likewise, Rho1p-unbound Fks1p may be internalized and degraded in the vacuole. Previous observations showed that Rho1p and Fks1p were co-localized at the site of cell wall remodeling in a cell cycle-dependent manner (26). If degradation of Fks1p is facilitated by dissociation of Rho1p, this could further assist in the restricted localization of Fks1p on the plasma membrane.

In this report, we observed impaired GS activities when yeast cells were cultured in YPD with 50 mM or 100 mM CaCl\(_2\), (Fig. 5). These results might be observed as a consequence of the higher extracellular Ca\(^{2+}\) concentration. The higher Ca\(^{2+}\) concentration would cause the unknown critical damage to the plasma membrane and/or GS itself. Besides the effect of relatively high concentrations of extracellular Ca\(^{2+}\), intracellular Ca\(^{2+}\) in the submicromolar range has been known to have various critical roles for living cells. In budding yeast, previous genetic and biochemical studies have indicated that many gene products are involved in the protein families that are regulated by Ca\(^{2+}\). The \(\beta\) subunit of GGTase I, Cal1p/Cdc43p, is one of these proteins. According to our results, Cal1p/Cdc43p regulates the GS activity through Rho1p geranylgeranylation. Thus, Ca\(^{2+}\) may regulate the cell wall remodeling. Furthermore, Ohya et al. (50) isolated several calcium-sensitive (cls) mutants. Among them, CLS4 is identical to CDC24, which encodes the guanine-nucleotide exchange factor for Cdc42p (51), and CLS5 is identical to PFY1, which encodes yeast profilin. Calmodulin plays central roles especially in the mating morphogenetic signaling process (52, 53). These proteins are involved in determining the cell polarity and in cell wall remodeling. Taken together, the intracellular concentration of Ca\(^{2+}\) may regulate yeast cell morphology mediated by these proteins.

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**Fig. 5. Effects of Ca\(^{2+}\) and Mn\(^{2+}\) upon cal1-1.** A, wild-type (WT) and cal1-1 cells (1 \(\times\) 10\(^5\) cells) were spotted onto YPD, YPD + 100 mM CaCl\(_2\), or YPD + 3 mM MnCl\(_2\) media and cultured for 2 days each at 23 °C and 37 °C. B, yeast cells were grown in YPD, YPD + 100 mM CaCl\(_2\), or YPD + 3 mM MnCl\(_2\) at 23 °C to mid-log phase and lysed. The whole cell lysates (L) were then fractionated to soluble (S) and insoluble (P) fractions (see “Experimental Procedures”). The Western blot analysis was carried out with guinea pig anti-Rho1p serum to samples prepared from the wild-type strain (upper panel) and the cal1-1 strain (middle panel). The Western blot analysis of the whole cell lysates of YPH500 (WT) was compared with those of cal1-1 (cal1) cultured in YPD (lower panel). Modified (m) and unmodified (u) forms of Rho1p are indicated. C, yeast cells were cultured in the indicated media, and GS activities were measured in the membrane fractions prepared from wild-type (open bars) and cal1-1 (solid bars) cells. The values represent the means and S.D. of six experiments.
