Complementing Yeast rho1 Mutation Groups with Distinct Functional Defects*

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Saccharomyces cerevisiae is a multifunctional molecular switch involved in establishment of cell morphology. We systematically characterized isolated temperature-sensitive mutations in the RHO1 gene and identified two groups of rho1 mutations (rho1A and rho1B) possessing distinct functional defects. Biochemical and cytological analyses demonstrated that mutant cells of the rho1A and rho1B groups have defects in activation of the Rho1p effectors Pkc1p kinase and 1,3-β-glucan synthase, respectively. Heteroallelic diploid strains with rho1A and rho1B mutations were able to grow even at the restrictive temperature of the corresponding homoeallelic diploid strains, showing intragenic complementation. The ability to activate both of the essential Rho1p effector proteins was restored in the heteroallelic diploid. Thus, each of the complementing rho1 mutation groups abolishes a distinct function of Rho1p, activation of Pkc1p kinase or 1,3-β-glucan synthase activity.

After establishment of cell polarity, morphogenesis of plant and fungal cells is determined by organization of the intracellular cytoskeleton and construction of the extracellular cell wall. A Rho-type small GTP-binding protein (Rho1p) in the budding yeast Saccharomyces cerevisiae has been shown to play a pivotal role in cell morphogenesis by regulating its effector proteins. Rho1p binds and activates Fks1p and Fks2p, two closely related catalytic subunits of 1,3-β-glucan synthase (GS), thereby directly controlling cell wall synthesis (1, 2). Rho1p also binds and activates Pck1p, a yeast homolog of mammalian protein kinase C. Through the mitogen-activated protein kinase (MAPK) cascade, Pkc1p regulates organization of the actin cytoskeleton and transcription of several genes involved in cell wall integrity (3–6). Other Rho1p-interacting proteins include Bni1p, Skn7p, and Sec3p (7–10). Gene disruptions analyses revealed that among the five Rho1p effector proteins, Fks1/2p and Pck1p are the most important in yeast cell growth. Δfks1Δfks2 and Δpck1 are both lethal in complete medium (11, 12), whereas Δsec3 shows slow growth in synthetic medium (13), and Δbni1 and Δskn7 display normal growth (7, 15). Thus, Rho1p controls cell morphogenesis by regulating the activities of two essential effector proteins important for cell wall synthesis and actin cytoskeleton organization.

Several conditional lethal mutations (high temperature-sensitive mutations) in the RHO1 gene (rho1-2, rho1-3, rho1-4, rho1-5) have been isolated in our laboratory and characterized for elucidation of Rho1p function. Biochemical analyses of the rho1 mutants greatly contributed to the understanding of the essential pathways downstream of Rho1p (2, 5, 6). However, the rho1 mutants did not always exhibit a single unique phenotype. Helliwell et al. (6) reported that actin morphologies differ among the rho1 mutants: rho1-3 and rho1-4 display normal polarized actin patches, whereas rho1-2 and rho1-5 possess delocalized actin patches. In this study, we investigated what kind of phenotypic differences exist among the rho1 mutants and why. We found that two groups of rho1 mutations show "intragenic complementation." Based on characterization of the complementing rho1 mutations in terms of activation of each Rho1p effector protein, the mechanism of intragenic complementation is discussed in conjunction with multifunctional properties of Rho1p.

EXPERIMENTAL PROCEDURES

Media, Strains, and Genetic Manipulations—Standard procedures were used for DNA manipulations and Escherichia coli transformation (16). The E. coli strain SCS1 (Stratagene, San Diego, CA) was used for propagation of the plasmids used in this study (Table I). The S. cerevisiae strains used are listed in Table II. All strains except Δpck1 Δlsl11 are isogenic derivatives of YPH499 and YPH500. Yeast transformation was carried out using the lithium acetate method (17). Genetic manipulations for yeast were carried out as described (18). Yeast cells were grown either in rich medium (YPD; 1% Bacto-yeast extract (Difco), 2% Bacto-peptone (Difco), and 2% glucose (Wako Chemicals, Osaka, Japan)) or in synthetic growth medium (0.67% yeast nitrogen base (Difco) and 2% glucose) supplemented appropriately.

Isolation of Temperature-sensitive rho1 Mutants—To isolate temperature-sensitive rho1 mutants, we introduced random mutations into the entire region of RHO1 by the error-prone polymerase chain reaction method (19). The RHO1 open reading frame was amplified by AmpliTaq polymerase with 5′-ATT AAC CCT CAC TAA AGA GAT CTC TAT TAC AAG AAG ACA CAC TT-3′ and 5′-GGG GGA ATT CAT CAC ACA AGT TG-3′. The amplified polymerase chain reaction fragment was cloned into the EcoRI-BglII site of pY070. The resulting plasmids were transformed into the YOC706 strain containing pY074 (YcplUG-RHO1) with the RHO1 gene deleted. Transformants were streaked onto YPD plates and incubated at 23 and 37 °C for screening temperature-sensitive mutants. The resultant candidates were selected on plates contain-
The resulting plasmids were digested with resulting fragments containing locus was performed as follows. Plasmids carrying the temperature- \( \rho^{1} \)

**Table I**

| Name               | Parent plasmid | Markers                  | Ref. |
|--------------------|----------------|--------------------------|------|
| pRS314             | TRP1, CEN      |                          | 39   |
| pRS316             | URA3, CEN      |                          | 39   |
| pYO701             | pRS314         | TRP1, RHO1 promoter, RHO1 terminator, CEN | 36   |
| pYO743 (YlpHate63S)| HIS3           |                          | 36   |
| pYO774 (YCpUG-RHO1)| pRS316         | URA3, GAL1 promoter, RHO1, CMK1 terminator, CEN | 36   |

**Table II**

| Strain               | Relevant genotype                  | Ref. |
|----------------------|------------------------------------|------|
| YPH499               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 39   |
| YPH500               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 39   |
| YOC701               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC706               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC729               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC751               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC752               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC754               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC755               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC756               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC757               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC758               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC759               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC760               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC761               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC762               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC763               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC764               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC771               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC772               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC774               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC775               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC776               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC777               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC778               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC779               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC780               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC781               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC782               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC783               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC784               | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC1081              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC1087              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC1943              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2905              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2435              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2867              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2868              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2869              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2870              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2871              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2872              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2873              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |
| YOC2874              | MATa ade2 his3 leu2 lys2 trp1 ura3 | 2    |

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ing 5-fluoroorotic acid to eliminate pYO774. After rescue of the mutated plasmids, the plasmids were re-transformed into YOC706 and checked for the phenotypes. By scanning common mutations in 39 independently isolated \( \rho^{1} \) mutants, we identified 13 single mutations that may result in a temperature-sensitive phenotype. We then made 13 single mutations by site-directed mutagenesis and examined temperature sensitivity in their growth. Finally, 12 temperature-sensitive \( \rho^{1} \) mutations were identified.

Integration of the mutant \( \rho^{1} \) genes into the chromosomal ADE3 locus was performed as follows. Plasmids carrying the temperature-sensitive \( \rho^{1} \) mutations were digested with BamHI and SalI, and the resulting fragments containing \( \rho^{1} \) were cloned into plasmid pYO743. The resulting plasmids were digested with SalII, and the mutant \( \rho^{1} \) genes were integrated into the ADE3 locus (20) of the diploid strain YOC701 (RHO1/rho1::HIS3). From the resultant strains, haploid \( \rho^{1} \) mutants were obtained by tetrad analysis (18).

**Preparation of the Membrane Fraction and Measurement of In Vitro GS Activity**—Cells were grown at 25 °C in 1 liter medium in a 2-liter flask rotating in an air incubator (Innova 4330) at 150 rpm until \( A_{600} = 1.1 \) All the following procedures were carried out at 4 °C, unless otherwise stated. The cells were harvested; washed with 1 mM EDTA; and disrupted by vortexing four times for 2 min each with 5-ml glass beads in 20 ml of breaking solution containing 0.5 M NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 1500 \( \times g \) for 5 min, the supernatant was collected and transferred to 33 PC tubes (Hitachi). The membrane fraction was collected by centrifugation at
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100,000 \times g \text{ for } 30 \text{ min in an RP707 rotor (Hitachi) with Himac CP 65β (Hitachi). The resultant pellet was suspended with membrane buffer containing 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1 mM β-mercaptoethanol, and 33% glycerol and homogenized with a Dounce homogenizer. The GS activity of the membrane fraction was measured according to a previously described procedure (21). As described (21), the assay buffer contained an excess amount of GTPγS.

**Immunoblot Analysis of Mpk1p—**Cells were culture law phase at 25 °C in YPD medium and shifted to 37 °C for 2 h. Cell extracts were prepared as described (22). Protein samples (50 μg) were loaded onto SDS–polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, catalog no. RP32092F), and blotted with either an anti-phospho-p44/42 MAPK(Thr202/Tyr204) antibody (Santa Cruz Biotechnology, catalog no. sc-6802) or an anti-MAPK antibody (New England Biolabs Inc., catalog no. 9101) or an anti-MAPK antibody (Santa Cruz Biotechnology, catalog no. sc-6802).

**Cell Lysis Assay—**Yeast cells were incubated on a YPD plate at 25 °C for 2 days and shifted to 37 °C overnight. The plate was then overlaid with an alkaline phosphatase assay solution as described by Paravicini et al. (3). Colonies containing lysed cells turned blue within 1 h, whereas control colonies remained unstained, even after 2 h.

**Actin Staining—**Actin staining with rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene OR) was carried out as described (23). Cells were observed with a Leica Model DMRE microscope fitted with HXC PL APL0. Images were captured using a CCD camera (Loper MicroMax/OL) and Metamorph Imaging software (Universal Imaging Corp., West Chester, PA). All images presented were processed using Adobe Photoshop software.

**RESULTS**

**Random Mutagenesis of RHO1 Yields a Collection of Temperature-sensitive rho1 Mutants—**To understand the cellular functions of Rho1p, we systematically generated temperature-sensitive rho1 mutants. Random mutations were introduced into the RHO1 gene using error-prone polymerase chain reaction (see “Experimental Procedures”). As shown in Fig. 1, we finally identified 12 temperature-sensitive rho1 mutations. Sequencing of these mutants revealed that all of the substituted amino acids were those conserved in mammalian RhoA protein. Especially the mutation sites of rho1-2 and rho1-10 were located in the switch 1 and switch 2 regions conserved in the Ras-related proteins. These regions are also collectively called the “putative effector recognition domain” (24). They are considered to render Ras-related proteins subject to conformational change in response to GTP/GDP exchange (25–27).

**Genetic Evidence for Distinct Separable Rho1p Functions—**By reciprocal mating between strains carrying 12 different rho1 mutations, we constructed diploid strains and checked their temperature sensitivity (Fig. 2). All diploid strains homozygous for the temperature-sensitive alleles (e.g. rho1-2/rho1-2) failed to grow at the restrictive temperature. On some occasions, diploid cells containing different recessive alleles (heteroallelic diploids) were able to grow at the restrictive temperature. For example, rho1-2/rho1-4 (a heteroallelic diploid) grew well at 37 °C, although rho1-2/rho1-2 and rho1-4/rho1-4 (homoallelic diploids) did not. This occasional phenomenon is called intragenic complementation. The pattern of growth at the restrictive temperature for all combinations of the rho1 alleles revealed two intragenic complementation groups of the rho1 mutations. The rho1A group consisted of the rho1-2 and rho1-5 mutants, whereas the rho1B group included the rho1-3, rho1-4, rho1-10, and rho1-11 mutants. In all combinations between mutant alleles of the rho1A and rho1B groups, intragenic complementation was observed (Table III). The rest of the mutants were not classifiable in either group. These results indicated that Rho1p has at least two different essential functions, one of which is abolished in the rho1A group mutants, and the other in the rho1B group mutants.

**Glucan Synthesis Activity of the rho1B Group Severely Decreases in Vitro—**The membrane fraction was prepared from each group of rho1 mutant cells grown at 25 °C and was either kept at the same temperature or shifted to 37 °C. We found that all of the rho1 mutant strains had less GS activity than the wild-type strain under both conditions (Fig. 3A). The reduced GS activity is not due to the failure to activate the Pkc1p-MAPK cascade because a Δpck1 mutant exhibits no less GS activity than the wild-type strain (2). It is noteworthy that the rho1B group mutants exhibited significantly lower GS activity than the rho1A group mutants under both conditions.

Failure to observe temperature-sensitive GS activities in Fig. 3A may represent irreversible inactivation of the mutant proteins at the restrictive temperature (28). To clarify this point, we isolated the membrane fractions from the rho1 mutant strains grown at 25 °C and measured their GS activities at both 25 and 35 °C. We found that all of the rho1B group mutants exhibited temperature sensitivity for the in vitro GS activities.
whereas the rho1A group mutants did not (Fig. 3B).

We also measured GS activity in the membrane fractions of homoallelic (rho1-2/rho1-2 and rho1-4/rho1-4) and heteroallelic (rho1-2/rho1-4) diploid cells. Under both conditions, the GS activities in the homoallelic rho1-2/rho1-2 and rho1-4/rho1-4 diploid cells were essentially the same as those in the corresponding haploid mutants (Fig. 3C). In heteroallelic rho1-2/rho1-4 cells, the in vitro GS activity was enhanced compared with that in rho1-4/rho1-4 diploid cells, irrespective of the assay conditions used.

**Mutants of the rho1A Group Fail to Activate the Pkc1p-Mpk1p Pathway**—To evaluate activation of the Pkc1p-MAPK cascade in the complementing rho1 mutant groups, we attempted to detect induced signaling through the Pkc1p-Mpk1p pathway in the rho1 mutants. Pkc1p activates the MAPK cascade composed of Bck1p (MAPK kinase kinase), Mkk1/2p (MAPK kinase), and Mpk1p/Slt2p (MAPK) (29–32). Under physiological conditions, tyrosine/threonine phosphorylation of Mpk1p is known to be induced by shifting the growth temperature from 23 to 37 °C (33, 34). Western blot analysis was performed after a heat shock treatment using antibodies that specifically recognize Mpk1p or phosphorylated Mpk1p (Fig. 4A). Comparison of the Mpk1p levels revealed no essential difference between the wild-type strain and any of the rho1 mutants. However, upon heat treatment, phosphorylated Mpk1p was induced in rho1A group cells apparently to a lesser extent than in wild-type cells. On the other hand, the rho1B group exhibited no defect in heat shock-induced Mpk1p phosphorylation. These results indicate that mutants in the rho1A group are defective in activation of the Pkc1p-Mpk1p pathway.

We also examined the levels of phosphorylated Mpk1p in homoallelic (rho1-5/rho1-5 and rho1-10/rho1-10) and heteroallelic (rho1-5/rho1-10) diploid cells upon heat shock (Fig. 4B). Heteroallelic rho1-5/rho1-5 and rho1-10/rho1-10 diploid cells contained the same level of phosphorylated Mpk1p as the cells of the corresponding haploid strains. In contrast, heteroallelic rho1-5/rho1-10 diploid cells contained an increased level of phosphorylated Mpk1p compared with cells of the severely affected rho1A group mutants (rho1-5/rho1-5).

**Phenotypic Analyses of the Complementing rho1 Mutants**—Biochemical analyses of the complementing rho1 mutants mentioned above demonstrated that mutations in the rho1A and rho1B groups result in decreased Pkc1p activation and lowered GS activity, respectively. To determine whether other phenotypic differences exist between the rho1A and the rho1B groups, cytological characterization and phenotypic analysis of the complementing rho1 mutant groups were performed. It is well known that mutants with defects in the Pkc1p-Mpk1p pathway display both actin delocalization and cell lysis phenotypes (3, 5, 6). Therefore, actin localization and cell lysis phenotypes were analyzed in rho1, Δpck1, and temperature-sensitive GS mutants.

Actin morphology was examined in rho1 and other mutant cells incubated for 5 h after being shifted from 25 to 37 °C and then stained with rhodamine-labeled phalloidin. As shown in Fig. 5A, cells of the rho1B group and the fks1-1125 mutants as well as those of the wild-type control displayed normal localization of actin patches at the growing cell surface. Conversely, the rho1A group, Δpck1, and the fks1-1154 mutants exhibited delocalized actin patches at the restrictive temperature. Additionally, the rho1A group and Δpck1 displayed cell enlargement. We also examined actin morphology in homoallelic (rho1-

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**TABLE III**

| Group and allele | Mutation |
|------------------|----------|
| rho1A            | E45V     |
| rho1-2           | G121C    |
| rho1-5           |          |
| rho1B            | L60P     |
| rho1-3           | W104R    |
| rho1-4           | D70G/S165P |
| rho1-4/10        | E102K/K167E |

**Fig. 3.** The rho1B group mutants have a severe defect in GS activity in vitro. **A**, GS activities in the membrane fractions isolated from haploid rho1 mutant and wild-type cells. The membrane fractions were prepared from MATa cells of rho1 mutants and the wild-type strain (Table II) cultured at 25 °C (white bars) or shifted to 37 °C for 2 h (black bars). GS activity was assayed at 25 °C. Each value represents the mean ± S.D. of at least three experiments. **B**, temperature-sensitive GS activities of the rho1B group mutants. The membrane fractions were prepared from cells grown at 25 °C. The GS activities were assayed at 25 or 35 °C. Each value represents the mean ± S.D. of at least three experiments. **C**, enhanced GS activity in heteroallelic diploid cells. Membrane fractions were prepared from YOC2868 (rho1A/rho1B), YOC2898 (rho1-2/rho1-2), YOC2870 (rho1-4/rho1-4), and YOC2871 (rho1-2/rho1-4) cells cultured at 25 °C (white bars) or shifted to 37 °C for 2 h (black bars). GS activity was assayed at 25 °C. The values presented are the means ± S.D. of at least three experiments.
heteroallelic (\textit{rho1-2/rho1-4} and \textit{rho1-5/rho1-10}) diploid cells at the restrictive temperature (Fig. 5B). The \textit{rho1A} group homoallelic diploid cells (\textit{rho1-2/rho1-2} and \textit{rho1-5/rho1-5}) exhibited essentially the same defects in actin morphology as the corresponding haploid cells. In contrast, the heteroallelic diploid cells (\textit{rho1-2/rho1-4} and \textit{rho1-5/rho1-10}) exhibited normal actin localization.

To directly examine the cell lysis phenotype of \textit{rho1} and other mutants, we carried out a simple plate overlay assay, in which leakage of alkaline phosphatase from cells is detected (3). As positive controls, the mutant strains of \textit{\textit{H9004 pkc1/stt1}} and \textit{rho1-5}, which were previously reported to result in cell lysis, were employed (5, 35, 36), whereas as negative controls, the wild-type strain and the \textit{rho1-3} mutant (5) were used. Fig. 6 shows that the \textit{rho1A} group, \textit{\textit{H9004 pkc1}}, and \textit{fks1-1154} mutant cells resulted in cell lysis, whereas the wild-type, \textit{\textit{rho1B}} group, and \textit{fks1-1125} mutant cells did not. Taking this into account, we conclude that the \textit{rho1A} and \textit{rho1B} group mutants exhibit phenotypes similar to those of mutants of Pkc1p (\textit{\textit{H9004 pkc1}}) and GS (\textit{\textit{fks1-1125}}), respectively.

**DISCUSSION**

Implications of Intragenic Complementation in the Multifunctional Protein Rho1p—Intragenic complementation is sometimes observed among mutations in a multifunctional gene. For example, the yeast His4 protein is a multifunctional protein catalyzing three different enzymatic steps in histidine biosynthesis, and a mutation in this protein affects only one of them (37). Our intragenic complementation results are compat-

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**FIG. 4.** The \textit{rho1A} group mutants are defective in activation of the Pklep-Mpk1p pathway. \textit{A}, amounts of phosphorylated Mpk1p in \textit{rho1} mutant cells. Cells of \textit{MATa} strains (Table II) were shifted from 25 to 37 °C for 2 h, and cell extracts were made. Immunoblot analysis was carried out as described under "Experimental Procedures" with anti-phospho-p44/42 MAPK antibody (p44/42; upper panel) or anti-MAPK antibody (lower panel). \textit{B}, heteroallelic diploid cells contain increased levels of phosphorylated Mpk1p. YOC2872 (\textit{rho1-5/rho1-5}), YOC2873 (\textit{rho1-10/rho1-10}), YOC2874 (\textit{rho1-5/rho1-10}), and YOC2868 (\textit{RHO1/RHO1}) cells were cultured at 25 °C and shifted to 37 °C for 2 h. Cell extracts were prepared, and immunoblot analysis was performed as described for \textit{A} using anti-phospho-p44/42 MAPK antibody (upper panel) or anti-MAPK antibody (lower panel).

**FIG. 5.** Actin patches are delocalized in the \textit{rho1A} group mutants. \textit{A}, delocalized actin patches in the \textit{rho1A} group mutants. Cells were cultured at 25 °C, shifted to 37 °C for 5 h, fixed, stained with rhodamine-labeled phalloidin, and observed. The mutant and wild-type \textit{MATa} strains were used (Table II). B, normal actin localization in heteroallelic diploid cells. YOC2869 (\textit{rho1-2/rho1-2}), YOC2870 (\textit{rho1-4/rho1-4}), YOC2871 (\textit{rho1-2/rho1-4}), YOC2872 (\textit{rho1-5/rho1-5}), YOC2873 (\textit{rho1-10/rho1-10}), YOC2874 (\textit{rho1-5/rho1-10}), and YOC2868 (\textit{RHO1/RHO1}) cells were treated as described for \textit{A} and observed.
The results of the intragenic complementation analyses enable us to classify rho1 mutations into three groups: the rho1A group (rho1-2 and rho1-5), the rho1B group (rho1-3, rho1-4, rho1-10, and rho1-11), and the rest. An intragenic complementation study of calmodulin mutations showed that particular Phe residues can be related to several diverse calmodulin functions (38). The fact that Rho1p regulates two essential effectors, Fks1/2p and Pkc1p, suggests that the functions of this protein may also be borne by groups of residues that complement each other. We thus examined whether each of the essential Rho1p functions was abolished in the rho1A or rho1B group mutants. Measurement of two essential downstream activities of Rho1p demonstrated that the rho1A group lacks the ability to activate the Pck1p-Mpk1p pathway and that the rho1B group has severe defects in GS activation. The failure of each mutant group to activate each downstream pathway was consistent with phenotypic differences of the group mutants; the rho1A group mutants showed close phenotypic resemblance to Δpck1, whereas the rho1B group mutants exhibited phenotypic similarity to fks1-1125. Finally, examinations of homoallelic and heteroallelic diploids with rho1 mutations revealed mutual complementation of the effector lesions caused by mutations in the rho1A and rho1B groups. Taking all these observations together, we consider it most likely that the intragenic complementation of the rho1 mutations is ascribable to complementation of decreased effector activities downstream of Rho1p (Fig. 7). This study presents the first biochemical evidence showing the mechanism of intragenic complementation observed in mutations in the RHO1 gene.

**Effect of the Complementing rho1 Mutations on Interaction with Effector Proteins**—One of the possible mechanisms underlying the inability of complementing rho1 mutants to activate Pck1p or GS is that the mutant proteins cannot physically interact with Pkc1p or GS. Indeed, we observed that rho1-2, which belongs to the rho1A group showing a defect in Pck1p activation, compromised an interaction between Pck1p and the GTP-bound form of Rho1p when detected by a two-hybrid assay. However, rho1-5, which also belongs to the rho1A group, did not affect the two-hybrid interaction (data not shown), suggesting that the rho1-5 mutation compromises Pck1p activation without affecting interaction. Similarly, rho1-3, which belongs to the rho1B group, abrogated GS activation without affecting the interaction with GS because GS purified from the rho1-3 mutant contained the same amount of Rho1p as the wild-type strain (data not shown). We speculate that the mutant protein (Rho1-5p or Rho1-3p) cannot induce the conformational change in effector enzymes that is required for their activation.

**Structural Investigation of Rho1p and Its Effector Proteins**—The three-dimensional structure of the active form of the human RhoA protein has been solved (14). Based on the high sequence identity (72%) between the human RhoA protein and yeast Rho1p, it seemed appropriate to map Rho1p mutation points onto the structural model of the human RhoA protein. Mutations belonging to the same complementation group were not mapped in a specific region on the RhoA protein structure, suggesting that some mutated amino acids influence the activation of effector proteins indirectly by perturbing the global structure of Rho1p itself (data not shown). However, we found that Glu45 (rho1-2) and Lys167 (rho1-11) were mapped on the surface of the RhoA protein, with their residues exposed to the outside. Since alteration of such amino acid residues would not affect the global protein structure, they are considered to be directly involved in the recognition of effector proteins. Indeed, they are located within or near the switch 1 and switch 2 regions that are reported to be important for association of the Rho-type GTPase with its effectors (14). Because the rho1-2 and rho1-11 mutations abolished the activation of Pck1p and GS, respectively, Glu45 and Lys167 might be involved in the specificity of effector recognition.

This study provides a clue to understanding the multifunctional properties of Rho1p at the amino acid sequence level. For
further study, it will be effective to systematically generate a number of rho1 mutants that have substitutions at Glu\textsuperscript{145} or Lys\textsuperscript{167} and to test their allele-specific ability to activate the effector proteins. Any amino acids located near Glu\textsuperscript{145} or Lys\textsuperscript{167} in the three-dimensional structure will also be targeted. We expect that investigations on the structure and functions of a multifunctional protein like Rho1p will help to establish a new concept of the signal transduction network in living cells.

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Complementing Yeast rho1 Mutation Groups
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