

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 morphogenesis. 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 homoallelic 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),¹ thereby directly controlling cell wall synthesis (1, 2). Rho1p also binds and activates Pkc1p, 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 disruption

analyses revealed that among the five Rho1p effector proteins, Fks1p and Pkc1p are the most important in yeast cell growth. $\Delta fks1\Delta fks2$ and $\Delta pkc1$ are both lethal in complete medium (11, 12), whereas $\Delta sec3$ shows slow growth in synthetic medium (13), and $\Delta bni1$ and $\Delta skn7$ 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 characterizations 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 $\Delta pkc1/stt1$ 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 AAC AAG ACA CAC TT-3' and 5'-GGG GGA ATT CAT GTC ACA ACA AGT TG-3'. The amplified polymerase chain reaction fragment was cloned into the *EcoRI*-*Bgl*III site of pYO701. The resulting plasmids were transformed into the YOC706 strain containing pYO774 (YCpUG-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-

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¹ The abbreviations used are: GS, 1,3- β -glucan synthase; MAPK, mitogen-activated protein kinase; GTP γ S, guanosine 5'-O-(3-thiotriphosphate).

TABLE I
Plasmids used in this work

Name	Parent plasmid	Markers	Ref.
pRS314		<i>TRP1</i> , <i>CEN</i>	39
pRS316		<i>URA3</i> , <i>CEN</i>	39
pYO701	pRS314	<i>TRP1</i> , <i>RHO1</i> promoter, <i>RHO1</i> terminator, <i>CEN</i>	36
pYO743 (YIpHade3S)		<i>HIS3</i>	36
pYO774 (YCpUG-RHO1)	pRS316	<i>URA3</i> , <i>GAL1</i> promoter, <i>RHO1</i> , <i>CMK1</i> terminator, <i>CEN</i>	36

TABLE II
Strains used in this work

Strain	Relevant genotype	Ref.
YPH499	<i>MATa ade2 his3 leu2 lys2 trp1 ura3</i>	39
YPH500	<i>MATa ade2 his3 leu2 lys2 trp1 ura3</i>	39
YOC701	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 RHO1/rho1Δ::HIS3</i>	2
YOC706	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3</i> (pYO774)	2
YOC729	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::LYS2 ade3::rho1-3::HIS3</i>	2
YOC751	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-1::LEU2</i>	2
YOC752	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-2::LEU2</i>	2
YOC754	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-4::LEU2</i>	2
YOC755	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-5::LEU2</i>	2
YOC756	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rhoC112R::LEU2</i>	2
YOC757	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-6::LEU2</i>	2
YOC758	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-7::LEU2</i>	2
YOC759	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-8::LEU2</i>	2
YOC760	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-9::LEU2</i>	2
YOC761	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-10::LEU2</i>	2
YOC762	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-11::LEU2</i>	2
YOC763	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rhoA::LEU2</i>	2
YOC764	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::RHO1::LEU2</i>	2
YOC771	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-1::LEU2</i>	2
YOC772	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-2::LEU2</i>	2
YOC774	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-4::LEU2</i>	2
YOC775	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-5::LEU2</i>	2
YOC776	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rhoC112R::LEU2</i>	2
YOC777	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-6::LEU2</i>	2
YOC778	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-7::LEU2</i>	2
YOC779	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-8::LEU2</i>	2
YOC780	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-9::LEU2</i>	2
YOC781	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-10::LEU2</i>	2
YOC782	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-11::LEU2</i>	2
YOC783	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rhoA::LEU2</i>	2
YOC784	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::RHO1::LEU2</i>	2
YOC1081	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 fks1Δ::HIS3 fks2Δ::LYS2 ade3::fks1-1125::TRP1</i>	^a
YOC1087	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 fks1Δ::HIS3 fks2Δ::LYS2 ade3::fks1-1154::TRP1</i>	^a
YOC1943	<i>MATa ade2 his3 leu2 lys2 trp1 ura3</i>	35
YOC2005	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 stt1Δ::HIS3</i>	35
YOC2435	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-3::LEU2</i>	This study
YOC2867	<i>MATa ade2 his3 leu2 lys2 trp1 ura3 rho1Δ::HIS3 ade3::rho1-3::HIS3</i>	This study
YOC2868	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::RHO1::LEU2/ade3::RHO1::LEU2</i>	This study
YOC2869	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-2::LEU2/ade3::rho1-2::LEU2</i>	This study
YOC2870	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-4::LEU2/ade3::rho1-4::LEU2</i>	This study
YOC2871	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-2::LEU2/ade3::rho1-4::LEU2</i>	This study
YOC2872	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-5::LEU2/ade3::rho1-5::LEU2</i>	This study
YOC2873	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-10::LEU2/ade3::rho1-10::LEU2</i>	This study
YOC2874	<i>MATa/α ade2/ade2 his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho1Δ::HIS3/rho1Δ::HIS3 ade3::rho1-5::LEU2/ade3::rho1-10::LEU2</i>	This study

^a M. Abe, M. Minemura, T. Utsugi, K.-M. Sekiya, A. Hirata, H. Qadota, K. Morishita, T. Watanabe, and Y. Ohya, unpublished data.

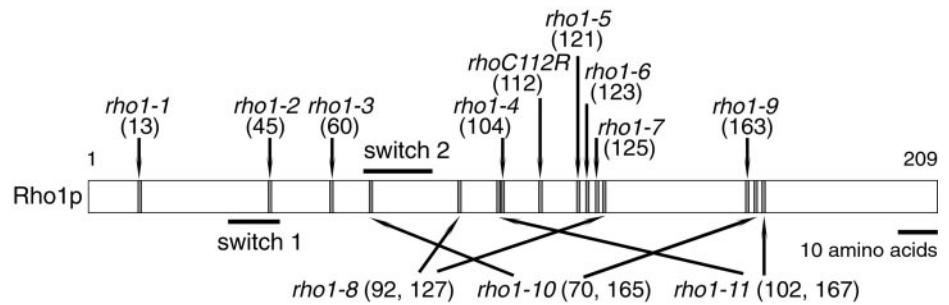
ing 5-fluoroorotic acid to eliminate pYO774. After rescue of the mutagenized plasmids, the plasmids were re-transformed into YOC706 and checked for the phenotypes. By scanning common mutations in 39 independently isolated *rho1* mutants, we identified 13 single mutations that may result in a temperature-sensitive phenotype. We then made 13 single mutations by site-directed mutagenesis and examined temperature sensitivity in their growth. Finally, 12 temperature-sensitive *rho1* mutations were identified.

Integration of the mutant *rho1* genes into the chromosomal *ADE3* locus was performed as follows. Plasmids carrying the temperature-sensitive *rho1* mutations were digested with *Bam*HI and *Sal*I, and the resulting fragments containing *rho1* were cloned into plasmid pYO743. The resulting plasmids were digested with *Sac*II, and the mutant *rho1*

genes were integrated into the *ADE3* locus (20) of the diploid strain YOC701 (*RHO1/rho1Δ::HIS3*). From the resultant strains, haploid *rho1* 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 $A_{600} = 1$. 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

FIG. 1. Mutation sites in temperature-sensitive *rho1* mutants. Each of the 12 *rho1* mutants obtained has one or two mutated amino acids. Switch 1 (residues 33–49) and switch 2 (residues 33–49) constitute the putative effector recognition regions. Shaded bars indicate the mutation points.



100,000 $\times g$ for 30 min in an RP70T rotor (Hitachi) with Himac CP 65 β (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 GTP γ S.

Immunoblot Analysis of Mpk1p.—Cells were cultured to log 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 μ g) were loaded onto SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, catalog no. RPN2020F), and blotted with either an anti-phospho-p44/42 MAPK(Thr²⁰²/Tyr²⁰⁴) 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 HCX PLA PLAPO. 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 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 bearing 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*

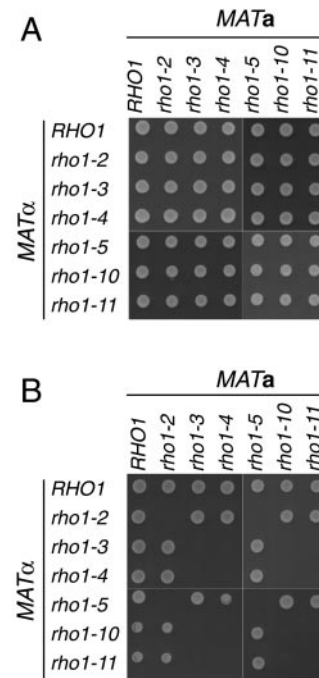


FIG. 2. Intragenic complementation among temperature-sensitive *rho1* mutations. Diploids selected with complementary auxotrophic markers of the strains were inoculated with a multipoint inoculation device onto YPD plates. The plates were incubated at 25 °C (A) or 37 °C (B) for 3 days.

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 Δ *pkc1* 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 reversible inactivation of the mutant proteins at the restrictive temperature (28). To clarify this point, we isolated the membrane fractions from the *rho1* mutants 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,

TABLE III

Group and allele	Mutation
<i>rho1A</i>	
<i>rho1-2</i>	E45V
<i>rho1-5</i>	G121C
<i>rho1B</i>	
<i>rho1-3</i>	L60P
<i>rho1-4</i>	W104R
<i>rho1-10</i>	D70G/S165P
<i>rho1-11</i>	E102K/K167E

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/Slp2p (MAPK) (29–32). Under physiological conditions, tyrosine/threonine phosphorylation of Mpk1p is known to be induced by shifting the growth temperature from 23 to 37–39 °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). Homoallelic *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*, Δ *pkc1*, 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

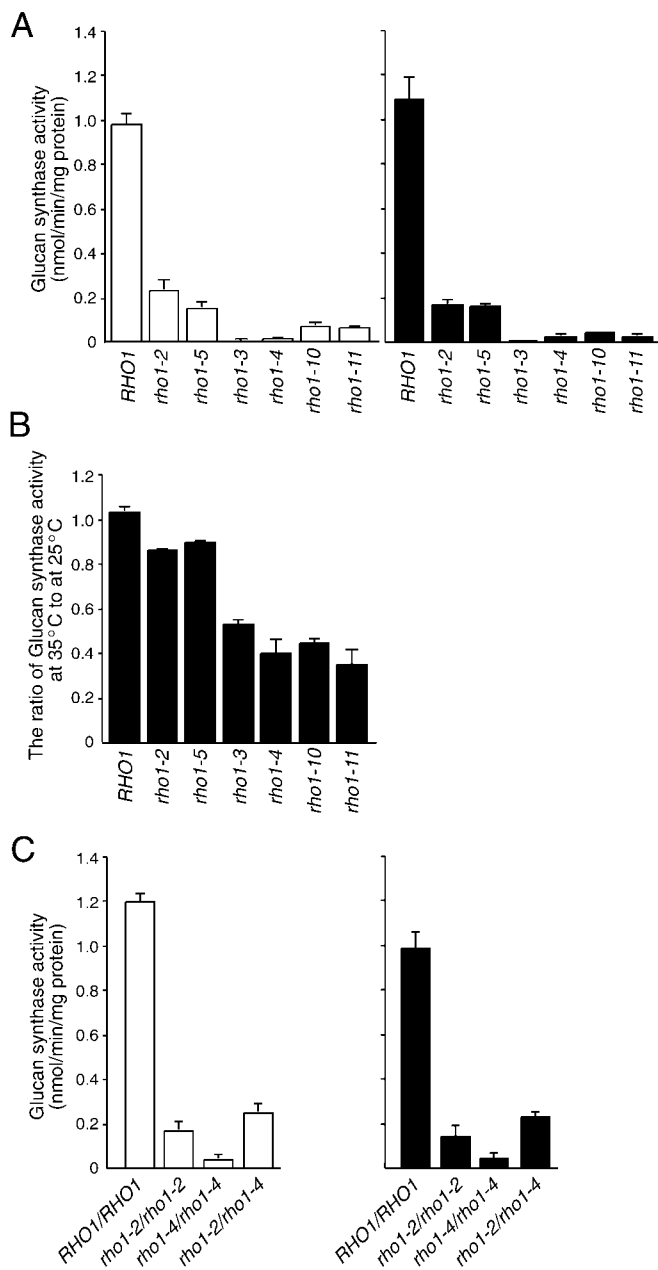


FIG. 3. The *rho1B* group mutants have a severe defect in GS activity *in vitro*. A, GS activities in the membrane fractions isolated from haploid *rho1* mutant and wild-type cells. The membrane fractions were prepared from *MAT α* cells of *rho1* mutants and the wild-type strain (Table II) cultured 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 \pm 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 \pm S.D. of the ratio of GS activities at 35 °C to those at 25 °C from at least three experiments. C, enhanced GS activity in heteroallelic diploid cells. Membrane fractions were prepared from YOC2868 (*RHO1/RHO1*), YOC2869 (*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 \pm S.D. of at least three experiments.

well as those of the wild-type control displayed normal localization of actin patches at the growing cell surface. Conversely, the *rho1A* group, Δ *pkc1*, and the *fks1-1154* mutants exhibited delocalized actin patches at the restrictive temperature. Additionally, the *rho1A* group and Δ *pkc1* displayed cell enlargement. We also examined actin morphology in homoallelic (*rho1-*

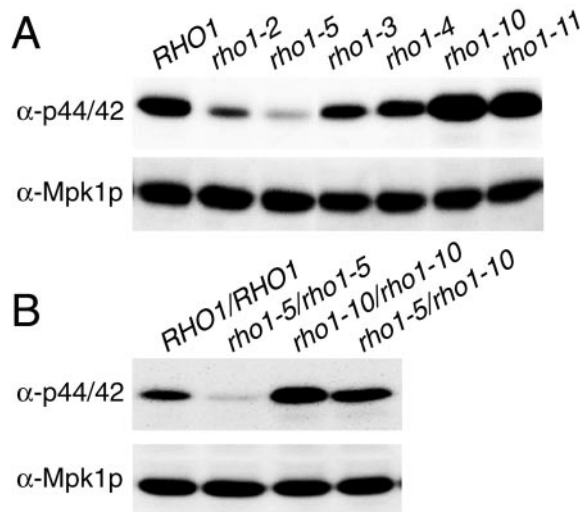


FIG. 4. The *rho1A* group mutants are defective in activation of the Pkc1p-Mpk1p pathway. A, amounts of phosphorylated Mpk1p in *rho1* mutant cells. Cells of *MAT α* strains (Table II) were shifted from 25 to 37 °C for 2 h, and cell extracts were made. Immunoblot analysis was carried out as described under "Experimental Procedures" with anti-phospho-p44/42 MAPK antibody (p44/42; upper panel) or anti-MAPK antibody (lower panel). B, heteroallelic diploid cells contain increased levels of phosphorylated Mpk1p. YOC2872 (*rho1-5/rho1-5*), YOC2873 (*rho1-10/rho1-10*), YOC2874 (*rho1-5/rho1-10*), and YOC2868 (*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 A using anti-phospho-p44/42 MAPK antibody (upper panel) or anti-MAPK antibody (lower panel).

2/*rho1-2*, *rho1-4/rho1-4*, *rho1-5/rho1-5*, and *rho1-10/rho1-10*) and heteroallelic (*rho1-2/rho1-4* and *rho1-5/rho1-10*) diploid cells at the restrictive temperature (Fig. 5B). The *rho1A* group homoallelic diploid cells (*rho1-2/rho1-2* and *rho1-5/rho1-5*) exhibited essentially the same defects in actin morphology as the corresponding haploid cells. In contrast, the heteroallelic diploid cells (*rho1-2/rho1-4* and *rho1-5/rho1-10*) exhibited normal actin localization.

To directly examine the cell lysis phenotype of *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 Δ *pkc1/stt1* and *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 *rho1-3* mutant (5) were used. Fig. 6 shows that the *rho1A* group, Δ *pkc1*, and *fks1-1154* mutant cells resulted in cell lysis, whereas the wild-type, *rho1B* group, and *fks1-1125* mutant cells did not. Taking this into account, we conclude that the *rho1A* and *rho1B* group mutants exhibit phenotypes similar to those of mutants of Pkc1p (Δ *pkc1*) and GS (*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-

A

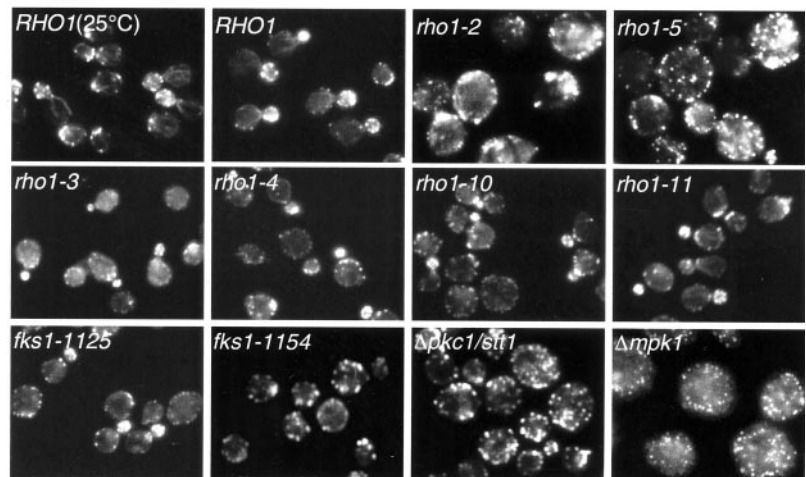
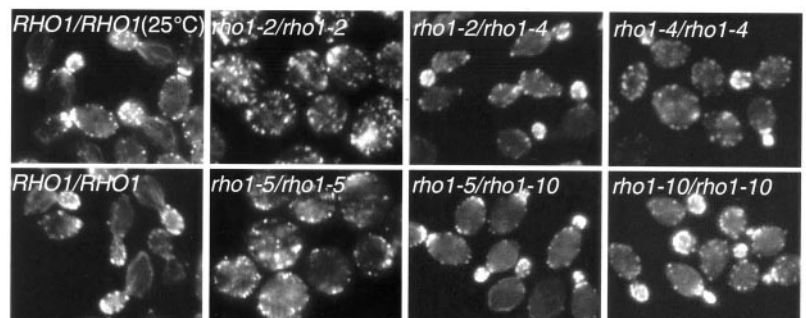


FIG. 5. Actin patches are delocalized in the *rho1A* group mutants. A, delocalized actin patches in the *rho1A* group mutants. Cells were cultured at 25 °C, shifted to 37 °C for 5 h, fixed, stained with rhodamine-labeled phalloidin, and observed. The mutant and wild-type *MAT α* strains were used (Table II). B, normal actin localization in heteroallelic diploid cells. YOC2869 (*rho1-2/rho1-2*), YOC2870 (*rho1-4/rho1-4*), YOC2871 (*rho1-2/rho1-4*), YOC2872 (*rho1-5/rho1-5*), YOC2873 (*rho1-10/rho1-10*), YOC2874 (*rho1-5/rho1-10*), and YOC2868 (*RHO1/RHO1*) cells were treated as described for A and observed.

B



ible with this view, although Rho1p may not be as easily separable into structural domains as the His4 product. Of the alternative mechanisms that might account for intragenic complementation, those that involve interactions of identical subunits seem unlikely since yeast Rho1p is thought to be present *in vivo* as a monomer.

The results of the intragenic complementation analyses enable us to classify *rho1* mutants 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 mutations. Measurement of two essential downstream activities of Rho1p demonstrated that the *rho1A* group lacks the ability to activate the Pkc1p-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 Δ *pkc1*, 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 Pkc1p 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 Pkc1p activation, compromised an interaction between Pkc1p 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 Pkc1p 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 Pkc1p 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

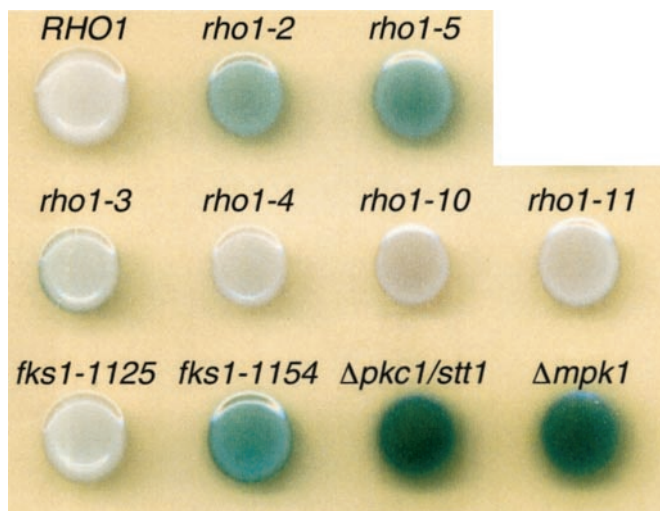
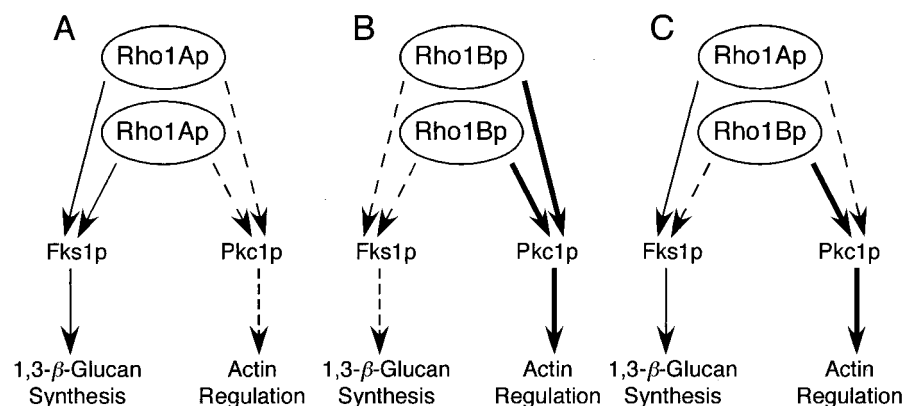


FIG. 6. The *rho1A* group mutants exhibit cell lysis. Cells were incubated on YPD plates at 25 °C for 3 days. After overnight incubation at 37 °C, alkaline phosphatase released from the cells as a result of lysis was detected by 5-bromo-4-chloro-3-indolyl phosphate. The *MAT α* strains of the *rho1* mutants were used (Table II).

FIG. 7. Characteristics of two intragenic complementation groups. The *rho1* mutation groups, *rho1A* and *rho1B*, abolish the essential functions of Rho1p, activation of Pkc1p and GS, respectively. A, homoallelic *rho1A* group mutants; B, homoallelic *rho1B* group mutants; C, heteroallelic *rho1A/rho1B* mutants. Thin and thick arrows represent affected and unaffected control steps, respectively.



further study, it will be effective to systematically generate a number of *rho1* mutants that have substitutions at Glu⁴⁵ or Lys¹⁶⁷ and to test their allele-specific ability to activate the effector proteins. Any amino acids located near Glu⁴⁵ or Lys¹⁶⁷ 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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REFERENCES

1. Drgonová, J., Drgon, T., Tanaka, K., Kollár, R., Chen, G.-C., Ford, R. A., Chan, C. S. M., Takai, Y., and Cabib, E. (1996) *Science* **272**, 277–279
2. Qadota, H., Python, C. P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E., and Ohya, Y. (1996) *Science* **272**, 279–281
3. Paravicini, G., Cooper, M., Friedli, L., Smith, D. J., Carpentier, J.-L., Klig, L. S., and Payton, M. A. (1992) *Mol. Cell. Biol.* **12**, 4896–4905
4. Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A., and Takai, Y. (1995) *EMBO J.* **14**, 5931–5938
5. Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohya, Y., and Levin, D. E. (1996) *J. Biol. Chem.* **271**, 9193–9196
6. Helliwell, S. B., Schmidt, A., Ohya, Y., and Hall, M. N. (1998) *Curr. Biol.* **8**, 1211–1214
7. Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Fujita, Y., Hotta, K., Qadota, H., Watanabe, T., Ohya, Y., and Takai, Y. (1996) *EMBO J.* **15**, 6060–6068
8. Evangelista, M., Blundell, K., Longtine, M. S., Chow, C. J., Adames, N., Pringle, J. R., Peter, M., and Boone, C. (1997) *Science* **276**, 118–122
9. Alberts, A. S., Bouquin, N., Johnston, L. H., and Treisman, R. (1998) *J. Biol. Chem.* **273**, 8616–8622
10. Guo, W., Tamanoi, F., and Novick, P. (2001) *Nat. Cell Biol.* **3**, 353–360
11. Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., and Thorner, J. (1990) *Cell* **62**, 213–224
12. Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Cemas, J. A., Nielsen, J. B., and Foor, F. (1995) *Mol. Cell. Biol.* **15**, 5671–5681
13. Haarer, B. K., Corbett, A., Kweon, Y., Petzold, A. S., Silver, P., and Brown, S. S. (1996) *Genetics* **144**, 495–510
14. Ihara, K., Muraguchi, S., Kato, M., Shimizu, T., Shirakawa, M., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1998) *J. Biol. Chem.* **273**, 9656–9666
15. Morgan, B. A., Bouquin, N., Merrill, G. F., and Johnston, L. H. (1995) *EMBO J.* **14**, 5679–5689
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
18. Kaiser, C. S., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Cadwell, R. C., and Joyce, G. F. (1992) *PCR Methods Applications* **2**, 28–32
20. Ohya, Y., and Botstein, D. (1994) *Genetics* **138**, 1041–1054
21. Inoue, S. B., Takewaki, N., Takasuka, T., Mio, T., Adachi, M., Fujii, Y., Miyamoto, C., Arisawa, M., Furuichi, Y., and Watanabe, T. (1995) *Eur. J. Biochem.* **231**, 845–854
22. Martin, H., Rodriguez-Pachón, J. M., Ruiz, C., Nombela, C., and Molina, M. (2000) *J. Biol. Chem.* **275**, 1511–1519
23. Pringle, J. R., Peterson, R. A., Adams, A. E. M., Sterns, T., Drubin, D. G., Haarer, B. K., and Jones, E. W. (1989) *Methods Cell Biol.* **31**, 357–436
24. Marshall, M. S., Davis, L. J., Keys, R. D., Mosser, S. D., Hill, W. S., Scolnick, E. M., and Gibbs, J. B. (1991) *Mol. Cell. Biol.* **11**, 3997–4004
25. Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., and Scolnick, E. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 4725–4729
26. Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) *EMBO J.* **9**, 2351–2359
27. Milburn, M. V., Prive, G. G., Milligan, D. L., Scott, W. G., Yeh, J., Jancarik, J., Koshland, D. E., and Kim, S. H. (1991) *Science* **254**, 1342–1347
28. Drgonová, J., Drgon, T., Roh, D.-H., and Cabib, E. (1999) *J. Cell Biol.* **146**, 373–387
29. Lee, K. G., and Levin, D. E. (1992) *Mol. Cell. Biol.* **12**, 172–182
30. Costigan, C., Gehrung, S., and Snyder, M. (1992) *Mol. Cell. Biol.* **12**, 1162–1178
31. Irie, K., Takase, M., Lee, K. S., Levin, D. E., Araki, H., Matsumoto, K., and Oshima, Y. (1993) *Mol. Cell. Biol.* **13**, 3076–3083
32. Lee, K. S., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K., and Levin, D. E. (1993) *Mol. Cell. Biol.* **13**, 3067–3075
33. Kamada, Y., Jung, U. S., Piotrowski, J., and Levin, D. E. (1995) *Genes Dev.* **9**, 1559–1571
34. Zarrov, P., Mazzoni, C., and Mann, C. (1996) *EMBO J.* **2**, 83–91
35. Yoshida, S., Ikeda, E., Uno, I., and Mitsuzawa, H. (1992) *Mol. Gen. Genet.* **231**, 337–344
36. Qadota, H., Anraku, Y., Botstein, D., and Ohya, Y. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9317–9321
37. Fink, G. R. (1966) *Genetics* **53**, 445–459
38. Ohya, Y., and Botstein, D. (1994) *Science* **263**, 963–966
39. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19–27