

# The GTP-binding Protein Rho1p Is Required for Cell Cycle Progression and Polarization of the Yeast Cell

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**Abstract.** Previous work showed that the GTP-binding protein Rho1p is required in the yeast, *Saccharomyces cerevisiae*, for activation of protein kinase C (Pkc1p) and for activity and regulation of  $\beta(1\rightarrow3)$ glucan synthase. Here we demonstrate a hitherto unknown function of Rho1p required for cell cycle progression and cell polarization. Cells of mutant *rho1<sup>E45I</sup>* in the G1 stage of the cell cycle did not bud at 37°C. In those cells actin reorganization and recruitment to the presumptive budding site did not take place at the nonpermissive temperature. Two mutants in adjacent amino acids, *rho1<sup>V43T</sup>* and *rho1<sup>F44Y</sup>*, showed a similar behavior, although some budding and actin polarization occurred at the nonpermissive temperature. This was also the case for *rho1<sup>E45I</sup>* when placed in a different genetic background. Cdc42p and Spa2p, two proteins that normally also move to the bud site in a process independent from actin organization, failed to localize properly in *rho1<sup>E45I</sup>*. Nuclear division did not occur in the mutant

at 37°C, although replication of DNA proceeded slowly. The *rho1* mutants were also defective in the formation of mating projections and in congregation of actin at the projections in the presence of mating pheromone. The in vitro activity of  $\beta(1\rightarrow3)$ glucan synthase in *rho1<sup>E45I</sup>*, although diminished at 37°C, appeared sufficient for normal in vivo function and the budding defect was not suppressed by expression of a constitutively active allele of *PKC1*. Reciprocally, when Pkc1p function was eliminated by the use of a temperature-sensitive mutation and  $\beta(1\rightarrow3)$ glucan synthesis abolished by an echinocandin-like inhibitor, a strain carrying a wild-type *RHO1* allele was able to produce incipient buds. Taken together, these results reveal a novel function of Rho1p that must be executed in order for the yeast cell to polarize.

**Key words:** *Saccharomyces cerevisiae* • G proteins • cell polarity • actin • cell cycle

**Y**EAST cells undergo polarization during the vegetative cycle, just before budding, and in the sexual cycle, before conjugation. This polarization is manifested in both cases by the reorganization of the actin cytoskeleton and by the localization of certain proteins at the budding site or at the mating projection (for reviews see Chant, 1996; Drubin and Nelson, 1996; Leberer et al., 1997; Cabib et al., 1998). Those proteins appear to form one or more large complexes that participate in the organization of the bud or mating projection site, while the actin filaments are supposed to guide secretory vesicles to the growing area. One of the proteins involved in bud site and mating projection organization is the Rho-type GTP-binding protein Cdc42p, as well as its activator Cdc24p. Temperature-sensitive mutations in the corresponding genes cause cell cycle arrest at the stage of an unbudded

cell, whereas DNA synthesis and nuclear division can continue, thus generating bi- and tetranucleated cells (Adams et al., 1990). In these mutants actin remains dispersed in cortical patches rather than congregating at the presumptive budding site. The direct target of Cdc42p is still controversial, although Bni1p, a protein that interacts with both profilin and actin, is a possible candidate. Another Rho-type protein involved in these processes is Rho1p. Its mammalian homologue RhoA is clearly involved in actin organization in the formation of stress fibers and focal adhesion complexes (Hall, 1998). In budding yeast Rho1p, like Cdc42p, interacts with Bni1p and has been proposed as a regulator of actin reorganization (Kohno et al., 1996). However, a temperature-sensitive mutant of *RHO1*, *rho1-104(D72N; C164Y)*, arrests at the nonpermissive temperature mainly as a cell with a small bud (Yamochi et al., 1994), a stage subsequent to actin reorganization. Mammalian Rho has also been implicated in cell cycle progression (Olson et al., 1995). One of the difficulties in determining a specific role for Rho1p is that this protein appears to have several functions. It has been shown to

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regulate the activity of protein kinase C, hence of the corresponding MAP-kinase cascade (Nonaka et al., 1995; Kamada et al., 1996). It also regulates  $\beta(1\rightarrow3)$ glucan synthase and is required for its activity (Drgonová et al., 1996; Qadota et al., 1996).

Here we show that certain temperature-sensitive mutants of *RHO1* are blocked at a cell cycle stage that precedes cell polarization. The defect does not appear to be related to  $\beta(1\rightarrow3)$ glucan synthase or Pkc1p activity. The mutants are also defective in cell polarization before conjugation.

## Materials and Methods

### Yeast Strains and Yeast Growth

The strains used in this study are listed in Table I. Yeast cells were cultured either in minimal (2% glucose, 0.7% yeast nitrogen base without amino acids [Difco], plus requirements) or in YEPD medium (1% yeast extract [Difco], 2% peptone [Difco], and 2% glucose) to which adenine was added to a final concentration of 40  $\mu$ g/ml. Solid media contained 2% agar.

Strain JDY4 was derived from OHNY1 (Table I) by switching its mating type with HO recombinase as described by Herskowitz and Jensen (1991). JDY4 and OHNY1 were then mated to yield the diploid JDY7.

Yeast transformation was carried out either by the lithium acetate method (Ito et al., 1983) or by electroporation with an Eppendorf electroporator 2510, according to the manufacturer's directions. Genetic manipulations were done as described (Rose et al., 1990).

### Plasmid and Strain Construction and Manipulation

Yeast genomic DNA was prepared as described in Ausubel et al. (1997) except that 10-ml glass test tubes were substituted for plastic tubes. Preparative PCR was performed with Pfu DNA polymerase (Stratagene). For analytical purposes TaKaRa DNA polymerase (PanVera) was used following manufacturer's directions.

To construct plasmids containing genomic mutations of *RHO1*, 720-bp fragments of *rho1*<sup>V43T</sup>, *rho1*<sup>F44Y</sup>, and *rho1*<sup>E45I</sup> were obtained by PCR from genomic DNA of strains HNY93, HNY95, and HNY97, respectively, with synthetic oligonucleotides (5'-ATGTCACAACAAGTTGGTAACA-3' for the 5' end [ATG in bold in this and subsequent oligonucleotides] and 5'-AAAGGCATACGTACATACAATGAGAAA-3' for the 3' end [SnaBI site underlined]). PCR fragments were digested with SnaBI, which cuts at a site located 118 bp downstream from the RHO1 ATG and at another site (included in the oligonucleotide), 86 bp downstream from the stop codon, and purified by agarose gel electrophoresis. Plasmid pRS316

Table I. *S. cerevisiae* Strains Used in This Study

Strain	Genotype	Source
OHNY1	<i>MATa ura3 leu2 trp1 his3 ade2</i>	Y. Takai (Yamochi et al., 1994)
DHNY110	<i>MATa/MAT<math>\alpha</math> ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 rho1::HIS3/rho1::HIS3</i> pRS316( <i>RHO1</i> [KpnI])	Y. Takai
HNY21	<i>MATa ura3 leu2 trp1 his3 ade2 rho1-104</i>	Y. Takai (Yamochi et al., 1994)
HNY93	<i>MATa ura3 leu2 trp1 his3 ade2 rho1</i> <sup>V43T</sup>	Y. Takai (Nonaka et al., 1995)
HNY95	<i>MATa ura3 leu2 trp1 his3 ade2 rho1</i> <sup>F44Y</sup>	Y. Takai (Nonaka et al., 1995)
HNY97	<i>MATa ura3 leu2 trp1 his3 ade2 rho1</i> <sup>E45I</sup>	Y. Takai (Nonaka et al., 1995)
JDY4	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2</i>	This study
JDY6-7A[pRS316( <i>RHO1</i> )]*	<i>MATa ura3 leu2 trp1 his3 ade2 rho1::HIS3</i> pRS316( <i>RHO1</i> )	This study
JDY7	<i>MATa/MAT<math>\alpha</math> ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2</i>	This study
JDY8	<i>MATa/MAT<math>\alpha</math> ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 rho1::HIS3/rho1::HIS3</i> pRS316( <i>rho1</i> <sup>E45I</sup> )	This study <sup>‡</sup>
DHY5D	<i>MATa/MAT<math>\alpha</math> ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 rho1::URA3/RHO1</i>	This study
DHY1-5A (pGRT)	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2 rho1::URA3</i> pGRT	This study
DHY-W	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2</i>	This study
DHY93	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2 rho1</i> <sup>V43T</sup>	This study
DHY95	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2 rho1</i> <sup>F44Y</sup>	This study
DHY97	<i>MAT<math>\alpha</math> ura3 leu2 trp1 his3 ade2 rho1</i> <sup>E45I</sup>	This study
CRY1	<i>MATa ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1</i>	R.S. Fuller
CRY2	<i>MAT<math>\alpha</math> ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1</i>	R.S. Fuller
ECY44	<i>MATa/MAT<math>\alpha</math> ade2-1/ade2-1 his3-11/his3-11</i> <i>leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1</i>	This study
ECY44A	<i>MATa/MAT<math>\alpha</math> ade2-1/ade2-1 his3-11/his3-11</i> <i>leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 rho1::HIS3/RHO1</i>	This study
ECY44A[pRS316( <i>RHO1</i> )]	<i>MATa/MAT<math>\alpha</math> ade2-1/ade2-1 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1</i> <i>ura3-1/ura3-1 rho1::HIS3/RHO1</i> pRS316( <i>RHO1</i> )	This study
ECY44A[pRS316( <i>rho1</i> <sup>E45I</sup> )]	<i>MATa/MAT<math>\alpha</math> ade2-1/ade2-1 his3-11/his3-11 leu2-3,112/leu2-3,112 trp1-1/trp1-1</i> <i>ura3-1/ura3-1 rho1::HIS3/RHO1</i> pRS316( <i>rho1</i> <sup>E45I</sup> )	This study
ECY44A[pRS316( <i>rho1</i> <sup>E45I</sup> )]-1D	<i>MAT<math>\alpha</math> ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 rho1::HIS3</i> pRS316( <i>rho1</i> <sup>E45I</sup> )	This study
1783	<i>MATa leu2,3-112 ura3-52 trp1-1 his4</i>	D. Levin
DL503	<i>MATa leu2,3-112 ura3-52 trp1-1 his4 pkc1<math>\Delta</math>::LEU2</i> YCP50[ <i>pkc1</i> <sup>ts</sup> ]	D. Levin

\* Because of the *RHO1* disruption, this strain requires a *RHO1*-carrying plasmid for survival. In different experiments, the resident plasmid was one of the following: vector pRS316, in which either *RHO1*, *rho1*<sup>V43T</sup>, *rho1*<sup>F44Y</sup>, or *rho1*<sup>E45I</sup> were cloned, or vector pRS314 with one of the same three alleles cloned into it.

<sup>‡</sup> Resulting from cross between JDY6-7A[pRS316(*rho1*<sup>E45I</sup>)] and ECY44A[pRS316(*rho1*<sup>E45I</sup>)]-1D.

(*RHO1* [KpnI]) (kindly furnished by Y. Takai) was digested with *Sna*BI, dephosphorylated, and ligated with the fragments prepared above. The presence of the respective mutation was confirmed by sequencing the plasmid DNA with a synthetic oligonucleotide (5'-ATGTCACAACAAGTTGGTAACA-3') as a primer.

All of the above plasmids contained a KpnI site, inserted upstream of the open reading frame of *RHO1* during construction of the original *RHO1* plasmid (Yamochi et al., 1994). To obtain a fragment containing only genomic sequences, genomic DNA from strain HNY93 (*rho1*<sup>V43T</sup>) was amplified by PCR with synthetic nucleotides containing engineered *Sac*I and *Hind*III restriction sites, respectively, (underlined) 5'-AATGGGGAGCTCGACGACATATTCGAGGTTGAC-3' and 5'-AAACTACATAAGCTTTACGCAGATCAAAAATAGGTGC-3' as primers. The amplified DNA was digested with *Sac*I and *Hind*III and the resulting 2,042-bp fragment, which contained promoter, coding region of *rho1*<sup>V43T</sup>, and 427 bp of the downstream region, was cloned into pRS316 (Sikorski and Hieter, 1989), previously digested with *Sac*I and *Hind*III. The resulting plasmid, pRS316(*rho1*<sup>V43T</sup>), was digested with *Sna*BI and the vector-containing fragment was isolated. Into this fragment were cloned 594-bp pieces obtained by *Sna*BI digestion of either pRS316 (*RHO1*[KpnI]), pRS316(*rho1*<sup>E44Y</sup>[KpnI]), or pRS316(*rho1*<sup>E45I</sup>[KpnI]). In the obtained plasmids, the region containing the mutation was sequenced with the synthetic oligonucleotide 5'-ATGTCACAACAAGTTGGTAACA-3'. In addition, *RHO1* and *rho1*<sup>E45I</sup> were completely sequenced in both directions with appropriate oligonucleotides. In this way, a complete set of plasmids containing *RHO1* or each of its three mutant versions without the KpnI site was obtained. Each gene was also recloned into vector pRS314 (Sikorski and Hieter, 1989), by digesting the latter with *Sac*I and *Xho*I and ligating a *Sac*I-*Xho*I fragment from the corresponding pRS316 plasmid with the cut vector.

Strain JDY6-7A (pRS316(*RHO1*[KpnI])) was a segregant dissected after sporulation of strain DHNY110 (Table I). The resident plasmid was exchanged with any of those from the pRS314 series by shuffling. In turn, these were shuffled with the pRS316 plasmids (devoid of the KpnI site) when a different marker was required.

To reintroduce the mutated *RHO1* into its chromosomal locus, the entire *RHO1* coding sequence was deleted (in DHNY110, derived from W303-RHO1 [Madaule et al., 1987] about half of the 3' end of the reading frame is still present, although the promoter region had been deleted). A 1,345-bp fragment containing the *URA3* gene was excised from pYES2.0 (Invitrogen) by digestion with *Xmn*I and inserted between the *Mlu*I site (located 382 bp upstream of *RHO1*) and the *Hpa*I site (located 22 bp downstream of *RHO1*) of plasmid pRS316(*RHO1*). Before ligation, the *Mlu*I digest was treated with T4 DNA polymerase to fill 5' overhangs. The resulting plasmid, pRS316(*rho1::URA3*), was digested with *Xma*I and *Sac*I; the excised *rho1::URA3* fragment was isolated and used to transform JDY7. Genomic DNA was isolated from transformants and correct replacement of the *RHO1* genomic locus was verified by PCR analysis. The *RHO1* null mutant used in further work is DHY5D.

The centromeric plasmid pGRT, containing the *GAL1-RHO1* and the *TRP1* gene, was constructed as follows: a synthetic oligonucleotide containing a *Hind*III restriction site (underlined) and the 5' end of the *RHO1* reading frame, 5'-AAAATTAAGCTTGAAGATGTCACAACAAG-3', was used as upstream primer and one bearing an *Eco*RI restriction site (underlined) and sequence 36 bp downstream of the *RHO1* stop codon, 5'-TGCCACTAAGAATTTCGACTGAGAGATC-3', as downstream primer in a PCR reaction with OHNY1 genomic DNA as template. The amplified product was digested with *Hind*III and *Eco*RI and ligated with pYES2.0, previously digested with the same restriction enzymes to yield plasmid pYES-*RHO1*. To transfer the *GAL1-RHO1* fusion to a centromeric plasmid, a primer bearing a *Bam*HI restriction site, 5'-CGGGATC-CAGTACGGATTAGAAGCCG-3', and one with a KpnI site, 5'-GAG-CTACCGGGCCGCAAATTAAGCC-3', were used to amplify a 1,495-bp fragment of pYES-*RHO1* containing the *GAL1* promoter, the ORF of *RHO1*, and the transcription terminator. The PCR product was digested with *Bam*HI and KpnI and ligated with pRS314 previously digested with the same enzymes to yield plasmid pGRT. This plasmid was found to support growth of *rho1Δ* mutants in galactose medium.

Strain DHY5D was transformed with pGRT and sporulated; tetrads were dissected on minimal medium containing 2% galactose and 0.2% sucrose in place of glucose. A Ura<sup>+</sup> and Trp<sup>+</sup> segregant, DHY1-5A (pGRT), was transformed with DNA fragments containing either *RHO1*, *rho1*<sup>V43T</sup>, *rho1*<sup>E44Y</sup>, or *rho1*<sup>E45I</sup>, obtained by digesting the respective pRS316 plasmids with *Sac*I and *Xho*I. Ura<sup>-</sup> colonies were selected on glucose plates containing 5-fluoroorotic acid (Boeke et al., 1984) and the pGRT plasmid was eliminated by growth in tryptophan-containing medium. The correct

reintroduction of each mutated gene was confirmed by sequencing a PCR-amplified fragment containing the corresponding mutation. The final strains were DHY-W (*RHO1*), DHY93 (*rho1*<sup>V43T</sup>), DHY95 (*rho1*<sup>E44Y</sup>), and DHY97 (*rho1*<sup>E45I</sup>).

To place the *rho1* mutation in a different genetic background, strain ECY44 was obtained by mating CRY1 and CRY2 (Table I). A deletion of *RHO1* was carried out by digesting pRS316(*RHO1*) with *Mlu*I and *Hpa*I (as above), followed by blunting and treating with alkaline phosphatase; the excised fragment was replaced with *HIS3*, obtained by digestion of pJJ217 (Jones and Prakash, 1990) with *Eco*RI and *Xba*I and also blunted before ligation, to yield plasmid pEC14. A 2.8-kb fragment containing *HIS3* and *RHO1*-flanking regions was amplified from pEC14 by PCR, with oligonucleotides 5'-CACTACGCCGAGCCGCACT-3' and 5'-AAACTACATAAGCTTTACGCAGATCAAAAATAGGTGC-3'. This fragment was transformed into ECY44 and disruption of *RHO1* in the transformants (ECY44Δ) was verified by PCR. ECY44Δ was transformed with pRS316(*RHO1*) or with pRS316(*rho1*<sup>E45I</sup>); the transformed strains were sporulated and segregants harboring the *RHO1* disruption and the respective plasmid (His<sup>+</sup> Ura<sup>+</sup>) were isolated after tetrad dissection.

The plasmid YCp50 (*PKC1*<sup>R398F</sup>), carrying a constitutively active allele of *PKC1*, was provided by Y. Takai. To overcome marker limitations in some host strains, the mutated gene was excised from the plasmid with *Pst*I and recloned into the *Pst*I site of the centromeric vector pRS315.

DNA manipulations were performed according to standard protocols (Guthrie and Fink, 1991; Ausubel et al., 1997).

Human p21 (p01112) and yeast Rho1p (p06780) sequences were aligned with program PILEUP and LINEUP of the GCG package (Wisconsin Package Version 9.1; Genetics Computer Group) and the amino acids in yeast Rho1p corresponding to the p21 switch 1 domain were deduced from the alignment.

## Isolation of G1 Cells and Determination of Budding

Yeast cells were grown in 160 ml of minimal medium to early log phase (0.3 g cells, wet weight/100 ml culture). The cells were harvested by centrifugation, suspended in 1 ml of 0.75 M methylmannoside, and sonicated briefly to break up clumps. Portions of the suspension (720 μl each) were applied on top of two 12-ml linear sucrose gradients (15–40%) and centrifuged at 400 *g* for 10 min. The cells formed a wide band in the gradient. Three 0.5-ml fractions from the upper part of the band were collected with a J-shaped needle with the help of a peristaltic pump and checked microscopically. Those fractions that contained <5% of budding cells were pooled, washed with distilled water, and used to inoculate 5 ml of minimal medium. The G1 cells were cultivated at 26°C or 37°C and every 2 h cells were counted to determine percentage of budding. More than 300 cells were counted in each sample.

In the experiments with strain DL503 (*pkc1<sup>Δ</sup>*), cells were fixed with 5% (final concentration) formaldehyde before counting, to prevent lysis. Small buds were counted with Nomarski optics under oil immersion. For photographic purposes, the tiny buds were best visualized in unfixed cells with phase-contrast under oil immersion.

Cell viability was estimated by staining with methylene blue. Cells were pelleted and suspended in 2 μg/ml methylene blue in 0.05 M KH<sub>2</sub>PO<sub>4</sub>. 5 min later, cells were observed in the microscope with Nomarski optics without removing the excess dye; the percentage of blue cells was determined. The Nomarski optics did not interfere with color observation and facilitated visualization of the unstained cells for counting.

## Fluorescence Microscopy

For all experiments, cells were fixed with 5% formaldehyde at 4°C overnight as described by Pringle et al. (1991). For visualization of actin, the cells were protoplasted with 5.5% Glusulase (Endo Laboratories) and 1% of mercaptoethanol for 30 min at 37°C and permeabilized with 0.1% Triton X-100 for 3 min. After immobilization of the cells on Con A-precoated slides, actin was stained with rhodamine-phalloidin (Molecular Probes) as described in Adams and Pringle (1991) and nuclei were stained with DAPI (1 μg/ml) for 5 min.

Cdc42p was stained with affinity-purified rabbit anti-Cdc42p antibodies (kindly provided by D.I. Johnson) essentially as described by Ziman et al. (1993). For immunolocalization of Spa2p, the JDY6-7A strains were transformed with the pBU4 plasmid [pRS315(3×*HA-SPA2*)] obtained from the laboratory of M. Snyder. After fixation as above, the cells were digested with 1 mg/ml of lyticase (Sigma Chemical Co.) for 30 min at 37°C, immobilized on polylysine precoated slides, and permeabilized with 0.1%

Triton X-100 for 5 min. HA-Spa2p was immunostained as described by Pringle et al. (1991). Permeabilized protoplasts were incubated with anti-HA mouse monoclonal antibodies (12CA5; Boehringer Mannheim) 1:1,000 in buffer F (0.01%  $\text{KH}_2\text{PO}_4$ , pH 7.4, 0.85% NaCl, 0.1% BSA, 0.1% sodium azide) for 1.5 h at room temperature followed by fluorescein-conjugated goat anti-mouse IgG (Cappel) 1:400 in buffer F for 2.5 h at room temperature. In all cases, *p*-phenylenediamine-based mounting medium (Pringle et al., 1991) was used. The samples were observed in a Zeiss Axioscope and photographs were taken with an Olympus OM4T camera.

### Flow Cytometry

Cells were fixed with 70% ethanol and DNA was stained with propidium iodide as described in Dien et al. (1994) except that before the analysis the propidium iodide solution was not removed from the cell suspension. Flow cytometric analysis was performed by Fast Systems.

### Membrane Preparation and Measurement of $\beta(1\rightarrow3)$ Glucan Synthase Activity

Membranes were prepared from logarithmic phase cells as previously described (Mol et al., 1994), except that disruption with glass beads was carried out by vortexing. Glucan synthase was assayed as reported (Mol et al., 1994). When the activity was measured at different temperatures (see Fig. 5 B) the membranes were preincubated for 15 min at the respective temperature in the presence of all components except substrate. UDP[ $^{14}\text{C}$ ]glucose was added to start the reaction, followed by the standard 20-min incubation. Protein was measured according to Lowry et al. (1951).

### Inhibition of $\beta(1\rightarrow3)$ Glucan Synthesis by L-733,560

An 80-ml culture of strain 1783 (wild-type) grown in minimal medium at 26°C and containing  $\sim 7 \times 10^6$  cells/ml was split in two. To one half L-733,560 (Douglas et al., 1994) was added to a final concentration of 1  $\mu\text{M}$ . Thereafter, both cultures were treated identically. Flasks were transferred to a 37°C bath and 30 min later 40  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]glucose (250 mCi/mmol) was added. After an additional 2 h at 37°C, cells were collected by centrifugation and washed twice with water. After addition of 0.8 ml of 50 mM Tris-chloride, pH 7.5 (buffer A), and 1.5 g of glass beads (0.4 mm diameter; Braun Biotech), cells were disrupted by vortexing. The extract was aspirated from the glass beads and the latter were washed with five 1-ml portions of buffer A. The extract and washings were combined and centrifuged for 10 min at 4,000 *g* to sediment cell walls. The walls were washed once with buffer A, twice with 1% SDS, and twice with water. Portions of each cell wall suspension containing 50,000 cpm were centrifuged and each pellet was suspended in 0.8 ml of buffer A, followed by 0.4 ml of PMSF-treated Zymolyase 100,000 (Kollár et al., 1997; Zymolyase 100,000 was from Seikagaku America). After a 16-h incubation at 37°C, insoluble material was sedimented by centrifugation and the supernatant was applied to a Sephadex G-100 column (1  $\times$  85 cm), previously equilibrated with 0.1 M acetic acid. The column was eluted with the equilibration solvent, 0.46-ml fractions were collected, and radioactivity was determined in the fractions.

### Treatment of Cells with $\alpha$ -Factor

To 5 ml of culture in minimal medium, containing  $\sim 10^7$  cells/ml, two additions of 80  $\mu\text{g}$  of  $\alpha$ -factor (Bachem Bioscience, Inc.) were made 1 h apart. After a total incubation time of 2 h at 26°C, most cells carrying wild-type *RHO1* showed a mating projection. At this point, cells were photographed under phase-contrast or fixed for subsequent actin visualization.

### Production of an Antibody against Rho1p and Western Blot Analysis

Polyclonal anti-Rho1p antiserum was raised in two rabbits (Alpha Diagnostic) against a purified maltose-binding protein (MBP)-Rho1p fusion obtained as follows. A 655-bp fragment containing the *RHO1* coding sequence was amplified by PCR from genomic DNA with an upstream primer containing the initiation codon (5'-ATGTCACAACAAGTTGGTAACA-3') and a downstream primer containing an EcoRI restriction site (underlined) (5'-TGCCACTAAGAATTCGACTGAGAGATC-3'). The amplified fragment was digested with EcoRI and inserted into the pMAL-c2 expression vector (New England Biolabs), previously digested

with XmnI and EcoRI, to yield the in-frame MBP-Rho1 fusion protein. The fusion protein was expressed in protease-deficient *Escherichia coli* BLR cells (Novagen), containing plasmid pDC952 (kindly provided by J.R. Walker) that encodes tRNA $_{\text{UCC}}^{\text{Arg}}$  to eliminate misincorporation of lysine in place of arginine (Calderone et al., 1996). Production of the fusion protein was induced for 3 h at 30°C with 0.3 mM isopropylthio- $\beta$ -D-galactoside. The protein was purified by chromatography on amylose resin (New England Biolabs) according to manufacturer's instructions, before injection.

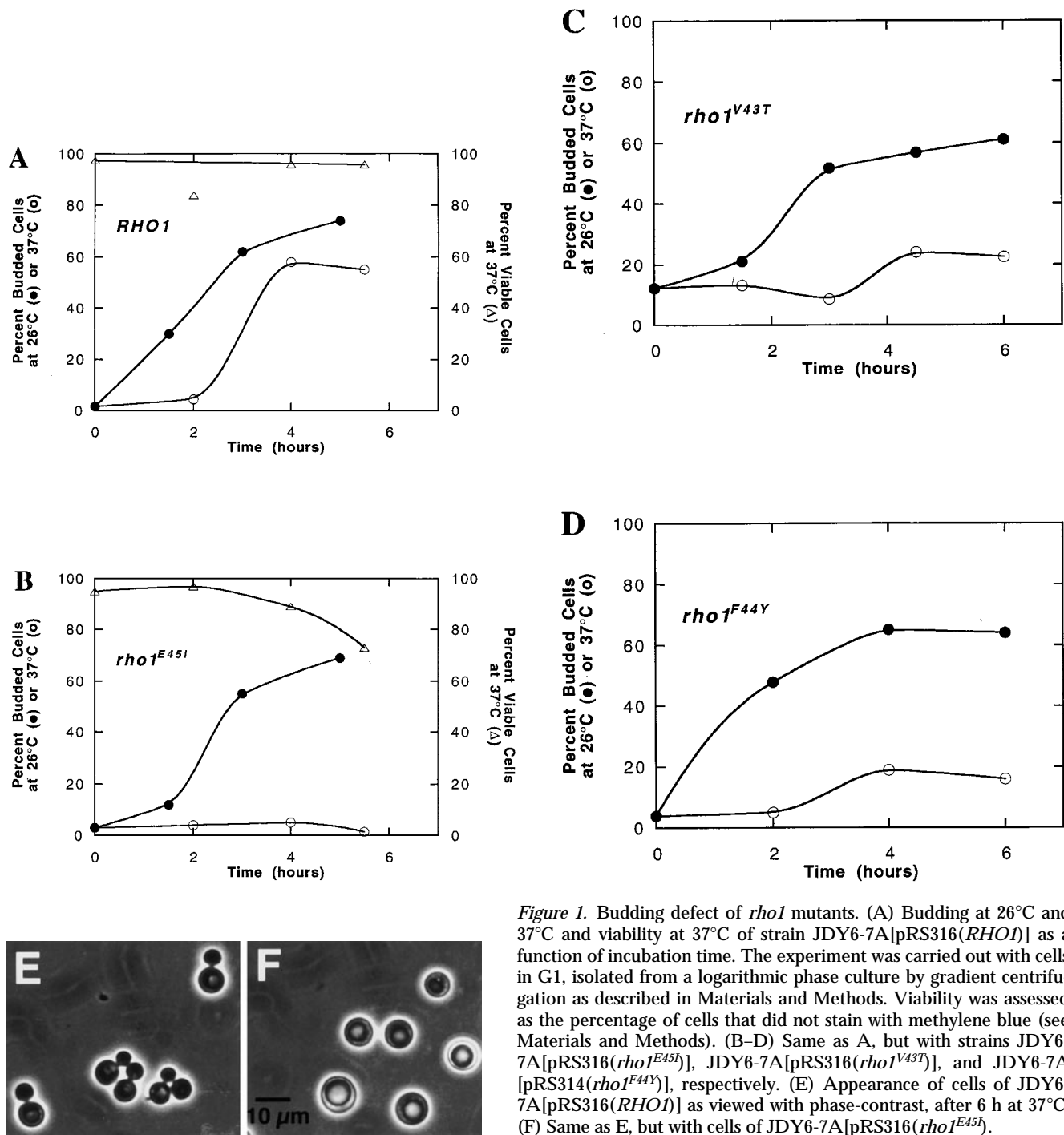
For Western blot analysis, yeast cell lysates, obtained by glass bead disruption and clarified by centrifugation, were separated by SDS-polyacrylamide electrophoresis (40  $\mu\text{g}$  of protein/lane) in a 14% gel (Laemmli, 1970) and blotted onto a PVDF membrane (Novex) according to the manufacturer's directions. Rho1p was detected with anti-Rho1p antiserum at 1:4,000 and HRP-conjugated goat anti-rabbit IgG (Pierce) at 1:5,000. Actin was stained with monoclonal mouse antiactin antibodies, clone C4 (ICN) at 1:4,000, and HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) at 1:5,000. Antibody-antigen complexes were detected with the ECL system (Pierce), following supplier's instructions.

Preimmune serum did not produce any signal. The antibody stained a band of the expected mobility ( $\sim 26$  kD) when wild-type cell lysates were used. The band was stronger when *RHO1* was overexpressed on a high-copy plasmid and much weaker with extracts from strain HNY21 (*rho1-104*), which was found (Drgonová et al., 1996) to contain low levels of the mutant protein (results not shown).

## Results

### Certain Temperature-sensitive *rho1* Mutants Are Blocked before Budding

Our initial interest in the *RHO1* mutants *rho1*<sup>V43T</sup>, *rho1*<sup>F44Y</sup>, and *rho1*<sup>E45I</sup> (kindly provided by Y. Takai) stemmed from the report (Nonaka et al., 1995) that the temperature sensitivity of the last two could be suppressed by a dominant positive allele of *PKC1*, whereas that of the first could not. Since at the time the only two known functions of Rho1p were activation of Pkc1p and regulation of  $\beta(1\rightarrow3)$ glucan synthase, we thought that *rho1*<sup>V43T</sup> may be specifically impaired in glucan synthesis. However, we found that although glucan synthase was partially defective in *rho1*<sup>V43T</sup> after growth at 37°C, the same defect was shown by the other two mutants. In the course of those experiments we observed that at the nonpermissive temperature large unbudded cells predominated in the population, indicating a budding defect in all three strains. In contrast, under the same conditions, mutant *rho1-104* cells mainly arrest with a very small bud (Yamochi et al., 1994). Initial studies of the budding defect were carried out with the three above-mentioned mutants, but it was later realized that, although constructed as haploids (Nonaka et al., 1995), they had become aneuploids. In fact, *rho1*<sup>E45I</sup> and, to some extent, *rho*<sup>F44Y</sup> sporulated, whereas *rho1*<sup>V43T</sup> was found to contain two copies of the *RHO1* gene in different parts of the genome (Cabib, E., J. Drgonová, and T. Drgon, unpublished experiments; the development of aneuploidy in *rho1* mutants will be the subject of a separate study). A strain (DHY97, Table I) in which we placed the mutation *rho1*<sup>E45I</sup> into the *RHO1* locus, in similar fashion to strain HNY97 from Takai's laboratory, also appeared to have become aneuploid: the strain budded polarly and mated very poorly. Sporulation of a cross with a wild-type strain gave rise to an abnormal distribution of markers (results not shown). In a similar fashion, mutants in *BEM2*, a gene that interacts genetically with *RHO1*, have been found to easily become aneuploid and to switch their mating type (Peterson et al., 1994).



**Figure 1.** Budding defect of *rho1* mutants. (A) Budding at 26°C and 37°C and viability at 37°C of strain JDY6-7A[pRS316(*RHO1*)] as a function of incubation time. The experiment was carried out with cells in G1, isolated from a logarithmic phase culture by gradient centrifugation as described in Materials and Methods. Viability was assessed as the percentage of cells that did not stain with methylene blue (see Materials and Methods). (B–D) Same as A, but with strains JDY6-7A[pRS316(*rho1<sup>E45I</sup>*)], JDY6-7A[pRS316(*rho1<sup>V43T</sup>*)], and JDY6-7A[pRS314(*rho1<sup>F44Y</sup>*)], respectively. (E) Appearance of cells of JDY6-7A[pRS316(*RHO1*)] as viewed with phase-contrast, after 6 h at 37°C. (F) Same as E, but with cells of JDY6-7A[pRS316(*rho1<sup>E45I</sup>*)].

Since uncertainty about ploidy would compromise the results, we constructed other strains harboring a disruption of *RHO1* and a plasmid carrying either wild-type *RHO1* or an appropriate mutation thereof (see Materials and Methods and Table I). These strains remained haploid under usual laboratory conditions, although some aneuploids arose if the cells were left on plates for very long periods (Cabib, E., J. Drgonová, and T. Drgon, unpublished experiments).

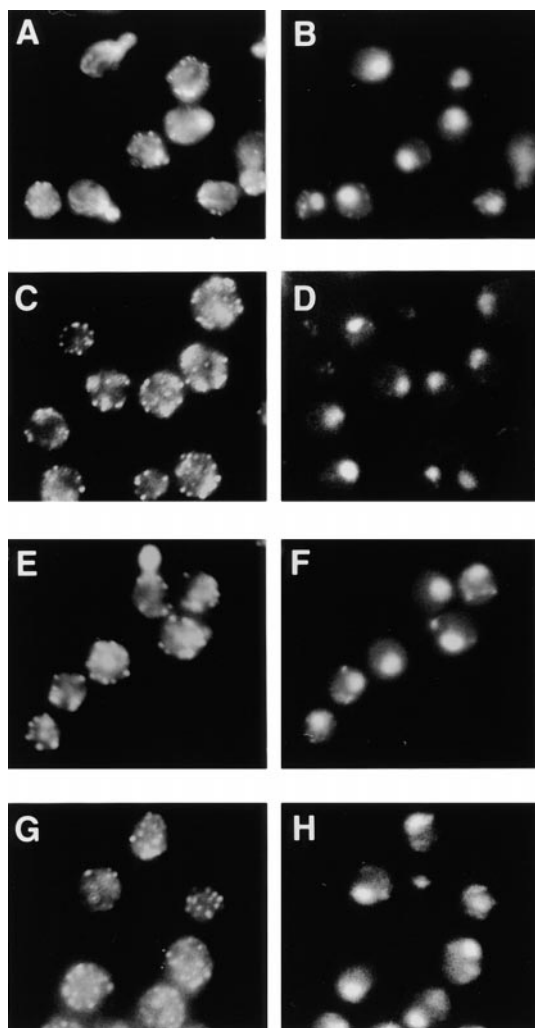
Because Rho1p has different functions (see above) which may be executed at different stages of the cell cycle,

it seemed desirable to study the presumed budding defect with a uniform cell population in the G1 stage of the cycle. Such a population can be obtained by gradient centrifugation (Mitchison and Vincent, 1965). The upper layers of the gradient contain mainly small daughter cells that still must grow in size before budding. When such cells were incubated at 26°C or 37°C, the wild-type budded at both temperatures, but *rho1<sup>E45I</sup>* only did so at 26°C (Fig. 1, A and B). At 37°C, cells of the mutant rounded up and enlarged (Fig. 1, E and F). After ~2 h, viability, as measured by staining with methylene blue (Pepler and Rudert,

1953), started to decrease (Fig. 1 B). Similar results were obtained with the other two *rho1* mutants, except that both *rho1*<sup>V43T</sup> and *rho1*<sup>F44Y</sup> underwent a partial round of budding (Fig. 1, C and D). However, the two latter strains did not grow at 37°C on plates (not shown for *rho1*<sup>F44Y</sup>; see Fig. 6 for *rho1*<sup>V43T</sup>), suggesting that they were unable to complete subsequent cell cycles. As will be shown below, when the mutation *rho1*<sup>E45I</sup> was placed in a different genetic background, some leakiness was also observed.

### Cells of *rho1* Mutants Fail to Polarize

Cell polarization can be estimated by the distribution of actin (Kilmartin and Adams, 1984). Fluorescence microscopy with rhodamine-phalloidin showed that at 37°C actin localized to the presumptive bud site and to the emerging bud in wild-type cells but remained depolarized in *rho1*<sup>E45I</sup>



**Figure 2.** In cells of *rho1* mutants actin fails to become polarized and nuclei do not divide at 37°C. Actin distribution as observed by fluorescence microscopy with rhodamine-phalloidin of JDY6-7A[pRS316-*(RHO1)*] (A), JDY6-7A[pRS316(*rho1*<sup>E45I</sup>)] (C), JDY6-7A[pRS316(*rho1*<sup>V43T</sup>)] (E), and JDY6-7A[pRS314(*rho1*<sup>F44Y</sup>)] (G), after 4 h of incubation at 37°C. The cultures were started with cells in G1, as in Fig. 1. Notice, in E, a budded cell in which the actin was polarized. (B, D, F, and H) DAPI staining of nuclei.

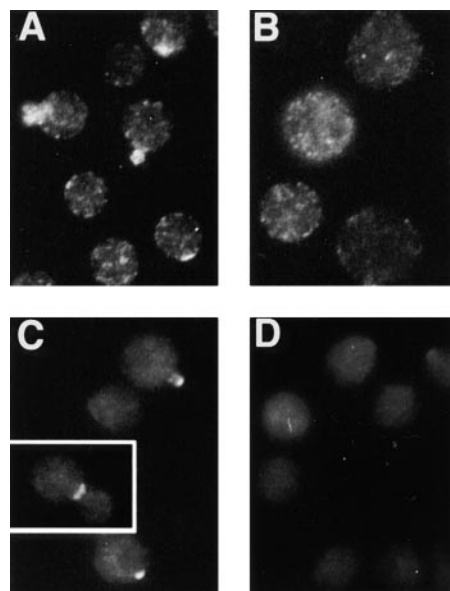
(Fig. 2, A and C). With *rho1*<sup>V43T</sup> and *rho1*<sup>F44Y</sup> some polarization was found in those cells that formed buds (Fig. 2, E and G). To find out whether the lack of polarization in *rho1*<sup>E45I</sup> was limited to actin or was a general effect, we investigated the distribution of two proteins that do not depend on actin for their localization at the bud site (Ayscough et al., 1997), Cdc42p and Spa2p. In both cases, the proportion of cells showing localization of these proteins was much lower in the *rho1*<sup>E45I</sup> than in wild-type (Fig. 3 and Table II).

In *cdc42-1*, a mutant with similar morphology to *rho1*<sup>E45I</sup>, DNA synthesis and nuclear division continue after the 37°C block. In contrast, DAPI staining showed a single nucleus per cell in all three *rho1* mutants (Fig. 2, B, D, F, and H). However, determination of DNA content per cell by fluorescence-activated cell sorting showed that DNA is slowly duplicated in the *rho1*<sup>E45I</sup> (Fig. 4). This experiment also confirms that the initial cell population was in G1.

Another difference between *cdc42* and *rho1*<sup>E45I</sup> is the randomized production of chitin at the cell surface as assessed by Calcofluor white staining. After incubation at 37°C, *cdc42* cells stain very strongly with Calcofluor (Adams et al., 1990), but *rho1*<sup>E45I</sup> cells do not (results not shown).

### The *rho1*<sup>E45I</sup> Defect Results from Loss of a Previously Unknown Function

The defect in *rho1*<sup>E45I</sup> is recessive: neither strain



**Figure 3.** Cdc42p and Spa2p fail to localize to the budding site in *rho1*<sup>E45I</sup> cells at 37°C. The localization of Cdc42p and Spa2p was determined by indirect immunofluorescence after incubation of G1 cells for 4 h at 37°C, as described under Materials and Methods. (A) Cdc42p in JDY6-7A[pRS316(*RHO1*)]; (B) Cdc42p in JDY6-7A[pRS316(*rho1*<sup>E45I</sup>)]; (C) Spa2p in JDY6-7A[pRS316(*RHO1*); pRS315(3×*HA-SPA2*)]; (D) Spa2p in JDY6-7A[pRS316(*rho1*<sup>E45I</sup>); pRS315(3×*HA-SPA2*)]. In C, the inset shows a cell undergoing cytokinesis with Spa2p at the septum formation site.

Table II. Percentage of Unbudded Cells with Polarized Location of Cdc42p and HA-Spa2p

	<i>RHO1</i>		<i>rho1<sup>E45I</sup></i>	
	26°C	37°C	26°C	37°C
Cdc42p	20%	17%	24%	3.6%
HA-Spa2p	15%	12%	11%	2%

In each case at least 100 unbudded cells were counted.

ECY44Δ(pRS316-*RHO1*) nor strain ECY44Δ(pRS316-*rho1<sup>E45I</sup>*) was temperature sensitive (result not shown). Both strains are diploids containing one chromosomal copy and one deletion of *RHO1*, plus a plasmid carrying either a wild-type or a mutated allele of the same gene, as indicated. However, segregants from sporulation of ECY44Δ(pRS316-*rho1<sup>E45I</sup>*), that harbored the *RHO1* deletion and the plasmid with the mutation, were temperature sensitive, as expected (data not shown). These results show that the defect in *rho1<sup>E45I</sup>* is due to loss of function and not to interference with some other pathway caused by abnormal targeting of the mutated protein.

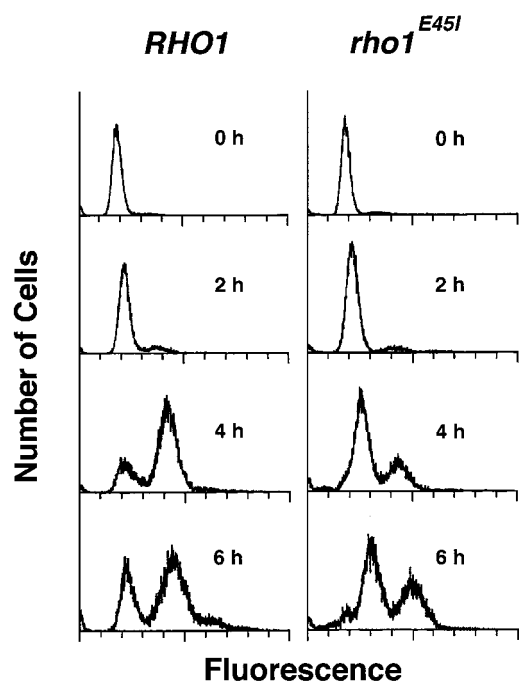


Figure 4. Cell cycle analysis of JDY6-7A[pRS316(*RHO1*)] and JDY6-7A[pRS316(*rho1<sup>E45I</sup>*)] cells grown at 37°C. The culture was started with G1 cells isolated by gradient centrifugation. In each graph, the left- and right-hand peaks represent cells containing 1 N and 2 N DNA, respectively. At the nonpermissive temperature, DNA replication in the mutant cells is much delayed: after 6 h only 35% of the cells had duplicated their DNA content. The shift observed at late times in the 1 N and 2 N fluorescence values of *rho1<sup>E45I</sup>*, relative to wild-type, may be due, at least in part, to an unspecific increase in fluorescence caused by growth in size of the mutant cells (Dien et al., 1994). At 26°C, duplication of the DNA occurred at about the same time in both strains (results not shown).

It was important to separate the function of Rho1p in cell polarization from those already known in β(1→3)glucan synthesis and Pkc1p activation. Therefore, we studied the effect of temperature on glucan synthase activity in two different ways: in one of them the enzyme was measured at 30°C with membrane preparations obtained from cells grown at 26°C or from cells shifted to 37°C for 2 h; in the other, membranes from cells grown at 26°C were assayed both at 26°C and 37°C. The first condition assesses the irreversible inactivation of Rho1p in vivo at 37°C, whereas the second one takes into account the possibility that the inactivation at 37°C may be reversible upon cooling. Membranes from a wild-type strain and from mutant *rho1-104* were included for comparison (Fig. 5). Results for the latter two strains under the first condition were similar to those already reported (Drgonová et al., 1996), except for some decline in activity in the wild-type strain exposed to 37°C (Fig. 5 A). This may be due to the fact that the growth medium used in the experiment shown

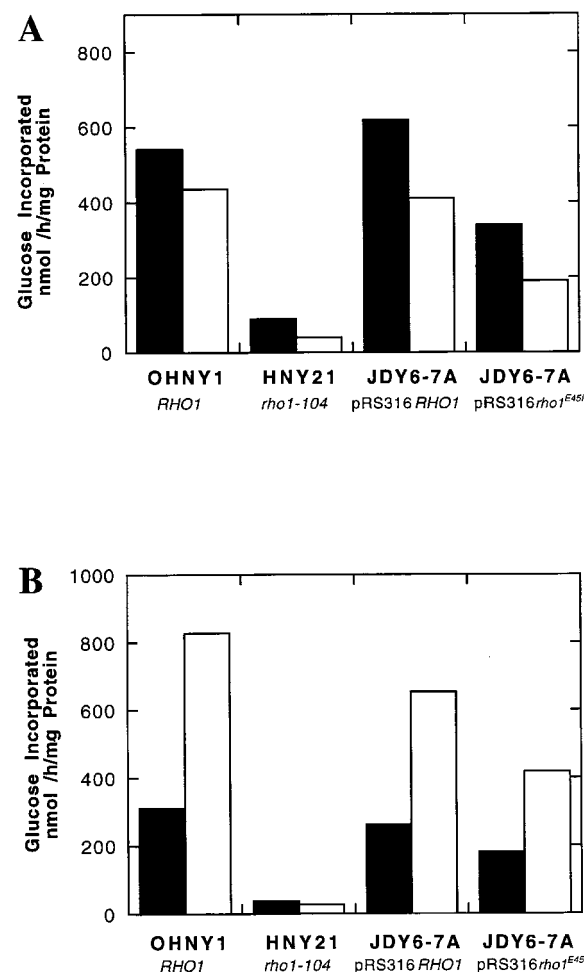


Figure 5. β(1→3)glucan synthase activity in membrane preparations of different strains. (A) Membranes prepared after growth of cells in YEPD at 26°C (solid bars) or after shift to 37°C for 2 h (empty bars). The enzyme was assayed at 30°C. (B) Membranes prepared from cells grown at 26°C. The enzyme was assayed at 26°C (solid bars) or at 37°C (empty bars). For experimental details see Materials and Methods.

here did not contain sorbitol. The activity of *rho1*<sup>E45I</sup> was lower than that of wild-type at both temperatures and under both conditions (Fig. 5, A and B). However, the activity at 37°C was much greater, especially under the second condition (Fig. 5 B), than that of *rho1-104* at 26°C, a temperature at which the latter strain grows normally. Notice also that under the second condition the enzymatic activity was much higher at 37°C than at 26°C in three of the strains, including *rho1*<sup>E45I</sup>, while it was lower in *rho1-104*. Therefore, it is unlikely that the defect of *rho1*<sup>E45I</sup> is due to low activity of glucan synthase.

As for Pkc1p, Nonaka et al. (1995) showed that a constitutively active allele of the *PKC1* gene, *pkc1*<sup>R398P</sup>, suppresses the temperature sensitivity of strains HNY95 (*rho1*<sup>F44Y</sup>) and HNY97 (*rho1*<sup>E45I</sup>) but not that of HNY93 (*rho1*<sup>V43T</sup>). We confirmed that result with the original strains under the conditions used by Nonaka et al., i.e., YEPD as medium and 35°C as the nonpermissive temperature. At that temperature, our reconstructed strains, JDY6-7A[pRS314(*rho1*<sup>V43T</sup>)] and JDY6-7A[pRS314(*rho1*<sup>E45I</sup>)], grew rather abundantly, preventing the use of 35°C for the test. At 37°C, the temperature used in the experiment of Fig. 1, both the *rho1*<sup>V43T</sup> and the *rho1*<sup>E45I</sup> mutant did not grow either in the absence or in the presence of a centromeric plasmid carrying the *PKC1* constitutively active allele (Fig. 6). Similar results were obtained in YEPD or in minimal medium, although a slight growth was observed at 37°C in YEPD in both mutants (Fig. 6). In other experiments, the wild-type and mutated *RHO1* genes were carried by vector pRS316, whereas the *pkc1*<sup>R398P</sup> gene was carried by pRS315. Again, no growth was observed at 37°C in the cells harboring both plasmids (data not shown). Finally, to exclude the possibility that the discrepancy between our results and those of Nonaka et al. (1995) was due to the chromosomal or plasmid localization of *RHO1*, a new, more extensive disruption of *RHO1* with *URA3* was carried out that completely eliminated the coding sequence (see Materials and Methods). *RHO1*, *rho1*<sup>V43T</sup>, *rho1*<sup>F44Y</sup>, and *rho1*<sup>E45I</sup> were integrated into the disruption and the resulting strains (DHY-W, DHY93, DHY95, and DHY97, respectively) were transformed with a plasmid carrying the constitutively active *PKC1* allele. The latter was unable to suppress the temperature sensitivity of any of the mutant strains (results not shown). We conclude that under the conditions of our experiments the constitutively active allele of *PKC1* does not suppress the growth defect. Transformation of strains JDY6-7A [pRS316(*rho1*<sup>V43T</sup>)] and JDY6-7A [pRS316(*rho1*<sup>E45I</sup>)] with a multicopy plasmid carrying the wild-type allele of *PKC1* also did not affect the temperature sensitivity of the strains (data not shown). In contrast, *PKC1* on a multicopy plasmid did suppress the temperature sensitivity of a *swi4* mutant that also arrests before budding, which suggests that in this case Pkc1p functions in a different pathway (Gray et al., 1997).

These results indicate that the cell cycle block in mutant *rho1*<sup>E45I</sup> is not due to defects in glucan synthase or protein kinase C, although the latter may well be inactive in the mutant.

To confirm these findings and to obtain evidence on glucan synthesis independent of in vitro measurements of enzymatic activity, we used a different approach. It was rea-

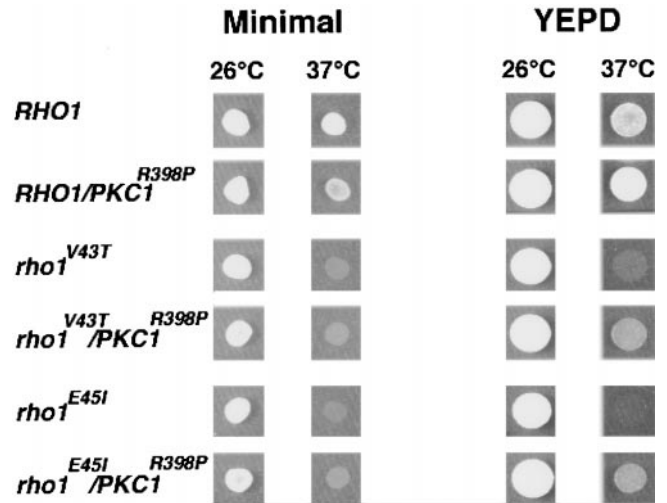
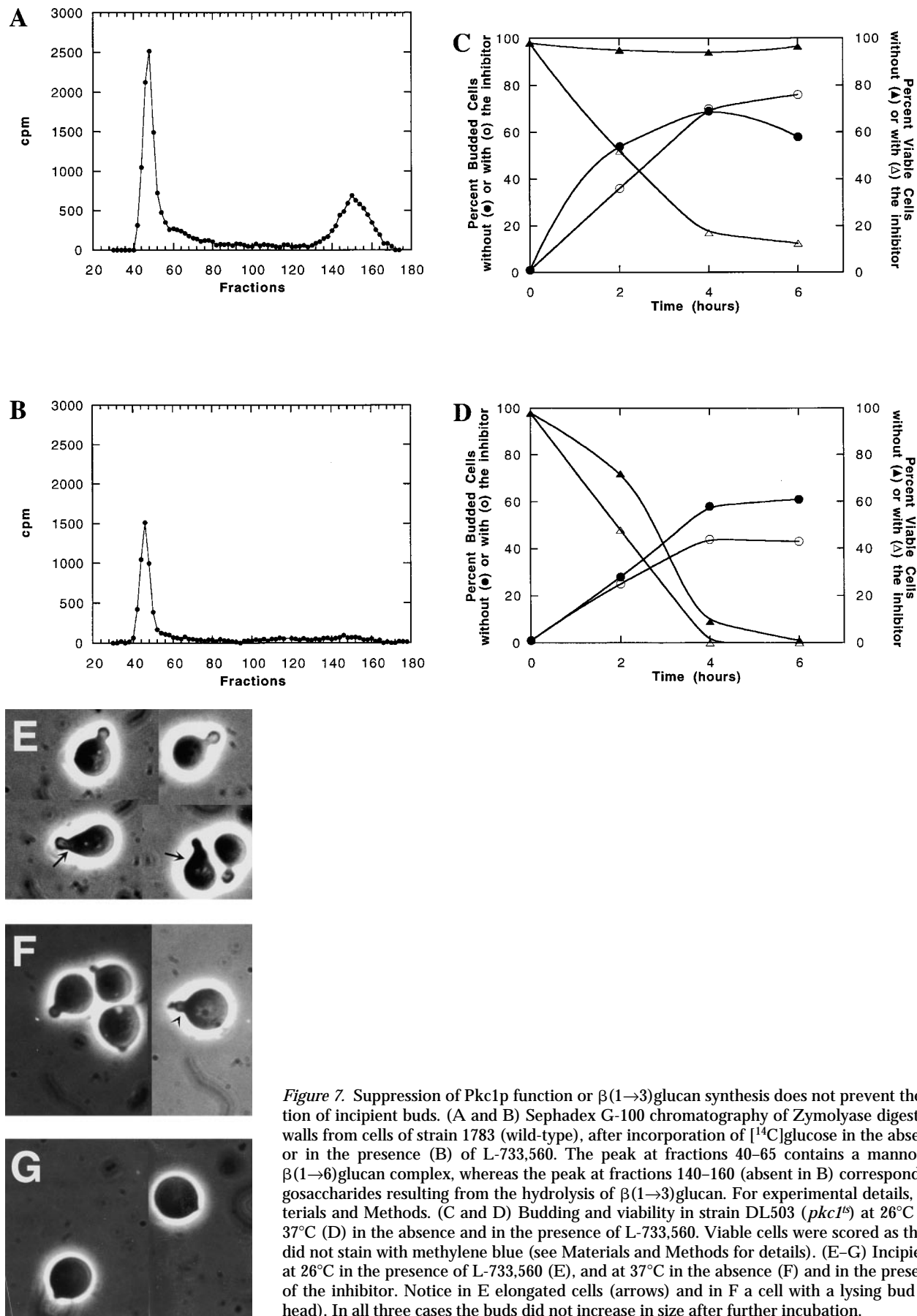


Figure 6. The constitutively active allele of *PKC1*, *pkc1*<sup>R398P</sup>, does not suppress the temperature sensitivity of *rho1* mutants. Notice that in YEPD some growth at 37°C takes place in cells of *rho1* mutants carrying the *pkc1*<sup>R398P</sup> plasmid; however, this growth is slight compared with that of wild-type and it occurs in both *rho1*<sup>E45I</sup> and *rho1*<sup>V43T</sup>. All strains are in a JDY6-7A background. Only the name of the genes carried by the resident plasmids is shown in the figure, as follows: *RHO1*, *rho1*<sup>V43T</sup>, and *rho1*<sup>E45I</sup> were in the pRS314 vector, whereas *pkc1*<sup>R398P</sup> was in the YCp50 vector.

soned that cells containing a wild-type allele of *RHO1* but in which Pkc1 activity and synthesis of  $\beta(1\rightarrow3)$ glucan had been turned off should be able to form at least incipient buds, if the two latter functions were not required for cell polarization. To inactivate Pkc1, we used a temperature-sensitive *pkc1* mutant. The terminal phenotype of such a mutant in asynchronous cultures at the nonpermissive temperature is that of a mother cell with a small bud lysing at the tip (Levin and Bartlett-Heubusch, 1992; Levin et al., 1994). To eliminate  $\beta(1\rightarrow3)$ glucan synthesis, we employed the semisynthetic echinocandin L-733,560, that inhibits the formation of the polysaccharide in vivo and in vitro (Douglas et al., 1994). When 1  $\mu$ M L-733,560 was added to asynchronous cultures of strain 1783 (wild-type) growing at 26°C or 37°C or DL503 (*pkc1*) at 26°C, the optical density of the culture doubled and then stopped abruptly (results not shown). Most cells ended up with a small bud and seemed to be lysing. To assess the extent of inhibition of  $\beta(1\rightarrow3)$ glucan synthesis, cells growing in the absence or in the presence of L-733,560 were labeled with <sup>14</sup>C-glucose and the cell walls were digested with Zymolyase, an endo- $\beta(1\rightarrow3)$ glucanase preparation. Incubation with this enzyme leads to solubilization of most of the cell wall components, resulting in a mixture of short  $\beta(1\rightarrow3)$ -linked glucose oligosaccharides and large mannoproteins attached to  $\beta(1\rightarrow6)$ glucan (Kapteyn et al., 1996; Kollár et al., 1997). These two groups of substances can be easily separated on a Sephadex G-100 column (Fig. 7 A). When the inhibitor was added to the culture, the total incorporation of radioactivity in the cell wall was 80% inhibited. Upon chromatography, the remaining radioactivity was found to be in the mannoprotein peak (Fig. 7 B), whereas the oli-





**Figure 7.** Suppression of Pkc1p function or  $\beta(1\rightarrow3)$ glucan synthesis does not prevent the formation of incipient buds. (A and B) Sephadex G-100 chromatography of Zymolyase digests of cell walls from cells of strain 1783 (wild-type), after incorporation of  $[^{14}\text{C}]$ glucose in the absence (A) or in the presence (B) of L-733,560. The peak at fractions 40–65 contains a mannoprotein- $\beta(1\rightarrow6)$ glucan complex, whereas the peak at fractions 140–160 (absent in B) corresponds to oligosaccharides resulting from the hydrolysis of  $\beta(1\rightarrow3)$ glucan. For experimental details, see Materials and Methods. (C and D) Budding and viability in strain DL503 (*pkc1<sup>ts</sup>*) at 26°C (C) and 37°C (D) in the absence and in the presence of L-733,560. Viable cells were scored as those that did not stain with methylene blue (see Materials and Methods for details). (E–G) Incipient buds at 26°C in the presence of L-733,560 (E), and at 37°C in the absence (F) and in the presence (G) of the inhibitor. Notice in E elongated cells (arrows) and in F a cell with a lysing bud (arrowhead). In all three cases the buds did not increase in size after further incubation.

gosaccharide peak was completely absent. These results show that L-733,560 caused total inhibition of  $\beta(1\rightarrow3)$ glucan synthesis. Since in the original wall the mannoprotein- $\beta(1\rightarrow6)$ glucan complex is attached to  $\beta(1\rightarrow3)$ glucan (Kapteyn et al., 1996; Kollár et al., 1997), it is easily understandable why inhibition of the formation of the latter polysaccharide leads to a large decrease in incorporation of the other components. The remaining mannoproteins (Fig. 7 B) were probably attached to preexisting chains of  $\beta(1\rightarrow3)$ glucan.

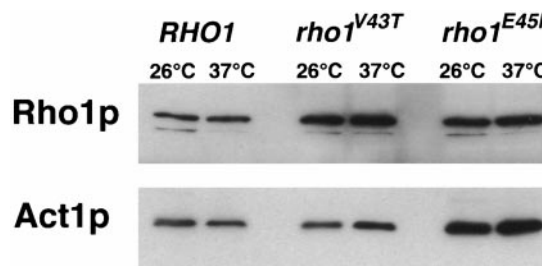
Having established the effectiveness of L-733,560, we proceeded to determine budding and viability (by methylene blue staining) of strain DL503 (*pkc1*) under conditions where either Pkc1p or  $\beta(1\rightarrow3)$ glucan synthase or both were not functional. At 26°C, in the presence of the inhibitor, cells gave rise to buds almost as efficiently as in its absence, but died rapidly (Fig. 7 C) with a small bud (Fig. 7 E). Many cells acquired an elongated shape, somewhat akin to that of the shmoos formed in the presence of  $\alpha$ -factor (Fig. 7 E, arrows). At 37°C, when only Pkc1p was inactivated, the cells also lost viability (Fig. 7 D) and ended up with small buds (Fig. 7 F), as previously found with unsynchronized cultures (Levin and Bartlett-Heubusch, 1992; Levin et al., 1994). Finally, when both functions were abolished by adding L-733,560 at 37°C, the cells behaved essentially in the same way (Fig. 7 D), but in this case the buds were extremely small (Fig. 7 G). Since it was only possible to count the buds visible around the circumference of the cell, there is little doubt that in this case the number of buds was underestimated (in fact, some buds became visible when an occasional cell turned while being observed). This also applies to some extent to the cells incubated at 37°C in the absence of inhibitor, because many buds were very small there too (Fig. 7 F). Therefore, the production of buds at 37°C was probably not very inferior to that observed at 26°C.

We conclude that a functional Rho1p is sufficient for cell polarization, even when Pkc1p and  $\beta(1\rightarrow3)$ glucan synthesis have been inactivated.

We previously found (Drgonová et al., 1996; Cabib, E., J. Drgonová, and D.-H. Roh, unpublished results) that in mutant *rho1-104* the amount of Rho1p is severely reduced at permissive temperature and even more at 37°C. In contrast, Western blot analysis of *rho1<sup>E45I</sup>* and *rho1<sup>V43T</sup>* showed that in these mutants the protein is conserved both at 26°C and 37°C (Fig. 8), in consonance with a partial rather than complete loss of function.

### Polarization of Cells before Conjugation Is Defective in the *rho1* Mutants

The polarization defect in the *rho1* mutants is not limited to the budding cycle. Most cells in the mutant failed to produce a mating projection when incubated with the sexual pheromone  $\alpha$ -factor at the permissive temperature (26°C). After a 2-h incubation with the pheromone, the percentage of cells with a visible mating projection was 74 and 75 in two determinations with wild-type and 18 and 21 in the mutant. In the latter, most cells remained roundish with a large vacuole (Fig. 9, A and B). Correspondingly, in the wild-type shmoos actin was recruited to the mating projection (Fig. 9 C), whereas in the mutant cells there was



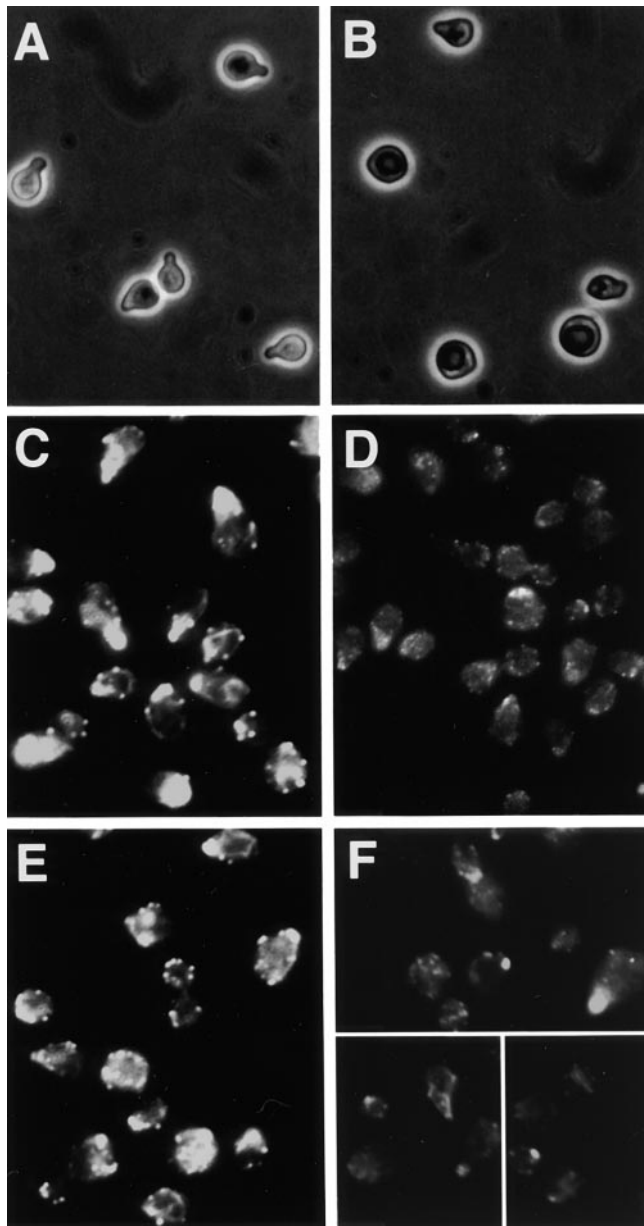
**Figure 8.** Mutant forms of Rho1p are present in cells at normal levels both at 26°C and 37°C. Rho1 proteins were detected by Western blot analysis (see Materials and Methods). Actin was used as a control. 40  $\mu$ g of protein was applied to each lane. Each of the indicated genes was in vector pRS316 in a JDY6-7A background (see Table I).

some localization of actin only where a mating projection appeared (Fig. 9 D). However, the block in mating projection was leaky enough to permit mating with the opposite mating type (results not shown). A similar defect in actin polarization was observed with the other two *rho1* mutants (Fig. 9, E and F). In an experiment in which wild-type yielded 55% cells with a mating projection, the values were 21 and 23% for *rho1<sup>V43T</sup>* and *rho1<sup>F44Y</sup>*, respectively. Thus the defect was somewhat decreased, relative to wild-type, in these two strains, in accordance with their leakiness in budding (Fig. 1).

### Variability in Penetration of the *rho1<sup>E45I</sup>* Mutation in Different Genetic Backgrounds

The observation that strain JDY6-7A[pRS316(*RHO1*)] grows on plates somewhat slowly at 37°C suggested the possibility that a temperature-sensitive mutation in another gene, either preexisting in the mother strain DHNY110 or introduced during our genetic manipulations, might contribute, together with *rho1<sup>E45I</sup>*, to generate the observed phenotype. Whether a mutation had been introduced artificially was investigated by using strain DHY1-5A(pGRT), which harbored a new deletion of the *RHO1* gene (carried out in the diploid JDY7, isogenic with OHNY1) with a different marker (*URA3*) from that used previously (*HIS3*). Plasmid pGRT present in that strain was substituted by either pRS314(*RHO1*) or pRS314(*rho1<sup>E45I</sup>*). The resulting strains behaved undistinguishably from the previously used strains JDY6-7A[pRS316(*RHO1*)] and JDY6-7A[pRS316(*rho1<sup>E45I</sup>*)], i.e., at 37°C G1 cells carrying *RHO1* budded and those carrying the mutation did not (results not shown). Since the probability of having introduced the same mutation with completely independent manipulations on different strains is vanishingly small, these results effectively eliminate that possibility.

It was still conceivable that both DHNY110 and OHNY1 originally harbored a temperature-sensitive mutation in another gene that was required for a stringent block of the cell cycle. To address this point, we used strain ECY44Δ[pRS316(*rho1<sup>E45I</sup>*)] (Table I), a diploid obtained by mating strains CRY1 and CRY2 to yield ECY44, followed by disruption of *RHO1* and introduction of the



**Figure 9.** *rho1* mutants are defective in mating projection formation and actin polarization in the presence of  $\alpha$ -factor. (A and C) JDY6-7A[pRS316(*RHO1*)]; (B and D) JDY6-7A[pRS316(*rho1*<sup>E45I</sup>)]; (E) JDY6-7A[pRS316(*rho1*<sup>V43T</sup>)]; (F) JDY6-7A(*rho1*<sup>E44Y</sup>). (A and B) Phase-contrast; (C-F) distribution of actin as observed by fluorescence microscopy with rhodamine-phalloidin. In all cases cells shown were incubated for 2 h with  $\alpha$ -factor (see Materials and Methods).

plasmid carrying the *rho1* mutation. Both CRY1 and CRY2, as well as ECY44 and ECY44 $\Delta$ [pRS316(*rho1*<sup>E45I</sup>)], show robust growth at 37°C, clearly more vigorous than that of JDY6-7A[pRS316(*RHO1*)] or OHNY1 (results not shown). After sporulation of ECY44 $\Delta$ [pRS316(*rho1*<sup>E45I</sup>)], six tetrads were analyzed. All scored 2:2 for temperature sensitivity (results not shown), as expected, since all contain *rho1*<sup>E45I</sup>, whereas half of them carry *RHO1* and the other half *rho1::HIS3*. These results also confirm that the

*rho1* mutation is recessive. One of the tetrads was monitored for budding of G1 cells at 37°C (Fig. 10 A), under the same conditions of the experiment of Fig. 1. Although the temperature-sensitive segregants (A and D) budded much less than their temperature-resistant counterparts (B and C), there was still 15–20% residual budding in A and D. Thus, there is some leakiness in this genetic background. To obtain a more precise estimate of the leakiness in terms of cells that had completed one or more cell cycles, we repeated the experiment with segregants C and D, but counted the total cell number, where a bud was counted as an independent cell (Fig. 10 B). The increase in cell number in the wild-type segregant over a 6-h period was 5-fold with fairly synchronous growth, whereas in the *rho1*<sup>E45I</sup> segregant it was only 0.6-fold.

It remained to be ascertained whether the difference in penetration of the *rho1*<sup>E45I</sup> mutation between the JDY6-7A or OHNY1 backgrounds on one hand and the ECY44 $\Delta$  background on the other is due to a single mutation or to a more general genetic variation. To elucidate this point, we mated ECY44 $\Delta$ -1D (Fig. 10, A and B) to JDY6-7A[pRS316(*rho1*<sup>E45I</sup>)] and sporulated the diploid. It was reasoned that, if the difference between the two strains was due to a single allele, the leakiness at 37°C should segregate 2:2, whereas if many genes were involved in the effect, a more randomized distribution would be found. All the progeny was temperature-sensitive (result not shown), as expected, since both mating partners carried a *RHO1* deletion plus *rho1*<sup>E45I</sup> on a plasmid. Three tetrads were further analyzed by obtaining G1 cells from all segregants and incubating them at 26°C or 37°C. The number of cells was monitored as in the experiment of Fig. 10 B. The increase in cell number in the different segregants over a 6-h period was variable, ranging between 0.2- and 2-fold, with one strain reaching 3.5-fold (Fig. 10 C). There was no clear 2:2 segregation of the increase in cell number. Actin distribution was determined by fluorescence microscopy in all components of tetrad 6 of Fig. 10 C, after incubation at 26°C or 37°C (Table III). The results basically confirmed those observed by measuring cell number, since the great majority of cells did not show actin polarization at 37°C. In conclusion, these results support the notion that the penetration of mutation *rho1*<sup>E45I</sup> is determined by the genetic background rather than by a specific gene. The reasons for the variability will be discussed below.

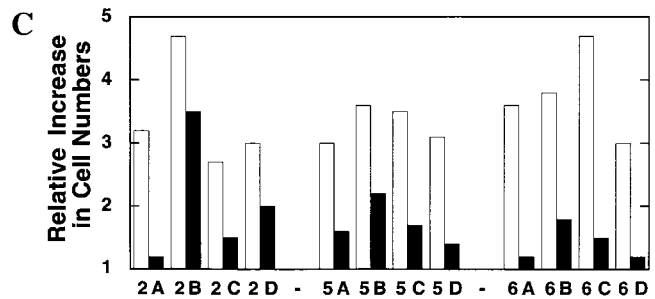
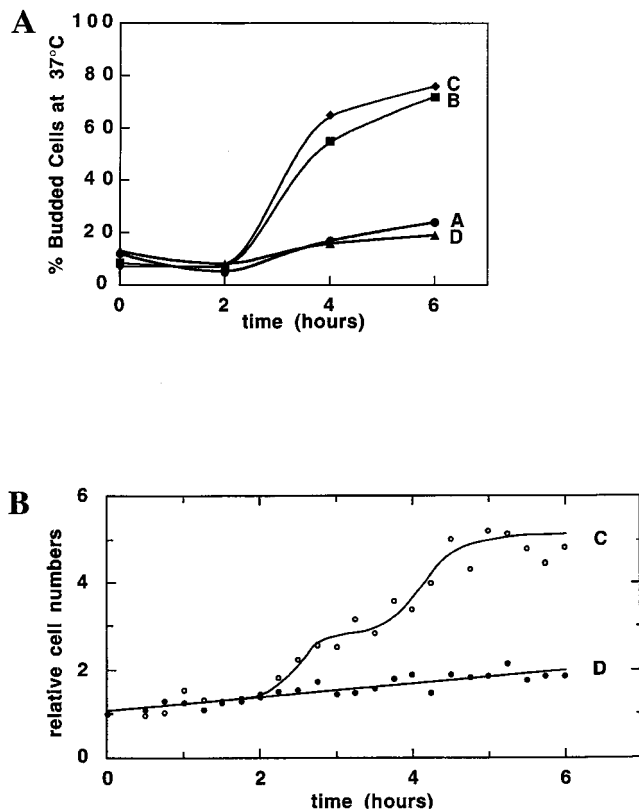
## Discussion

The experiments described above show that mutant

**Table III.** Budding and Polarization of Actin in Tetrad 6 after a 3 h Incubation at 26°C or 37°C

Spore	% budding		% cells with polarized actin	
	26°C	37°C	26°C	37°C
A	74	13	62	5.6
B	56	11	33	3.2
C	77	13	62	10
D	78	10	63	11

Approximately 200 cells were counted in each case.



**Figure 10.** Penetration of the *rho1*<sup>E45I</sup> mutation in different genetic backgrounds. (A) Budding at 37°C of G1 cells of tetrad 1 segregants from sporulation of strain ECY44Δ[pRS316 (*rho1*<sup>E45I</sup>)]. Segregants A and D were temperature-sensitive on plate. (B) Same as in A, but only segregants C and D were used and total cell number was determined (a bud counted as a cell). (C) Relative increase in cell number at 26°C (open bars) and 37°C (solid bars) in 6 h. of G1 cell cultures from three tetrads (2, 5, and 6) resulting from sporulation of strain JDY8.

*rho1*<sup>E45I</sup> is defective in cell polarization: at 37°C cells do not bud; they enlarge and become round; actin is not reorganized and recruited to the presumptive bud site; certain proteins, such as Cdc42p and Spa2p, also usually found at the budding site, do not localize. The blocked cells show a single nucleus, although DNA duplication proceeds at 37°C, albeit at a greatly reduced rate. The mutant also shows a defective response to pheromone, both in the formation of a mating projection and in concentrating actin at the projection. Two *rho1* mutants in adjacent amino acids, *rho1*<sup>V43T</sup> and *rho1*<sup>F44Y</sup>, also were defective in cell polarization at the nonpermissive temperature, but they showed some leakiness (Fig. 1, C and D), consistent with a partial function of the mutated Rho1p.

The phenotype of the *rho1* mutants analyzed in this study differs from that of mutant *rho1-104*, which arrests at the nonpermissive temperature with a preponderance of cells bearing a small bud (Yamochi et al., 1994), as found when either Pkc1p function or glucan synthesis is impaired (Fig. 7). Our interpretation of this difference is that in *rho1-104*, in which the Rho1 protein gradually disappears at 37°C (Cabib, E., J. Drgonová, and D.-H. Roh, unpublished results), the Pkc1p function and/or glucan synthesis become defective before the budding function, thus giving rise to the observed phenotype.

The recessive character of the *rho1*<sup>E45I</sup> mutation indicates that the phenotype is a consequence of loss of function. That loss does not appear to be in one of the already known roles of Rho1p: the *in vitro* glucan synthase activity of the mutant, although reduced at 37°C, should be sufficient for normal *in vivo* β(1→3)glucan synthesis, by com-

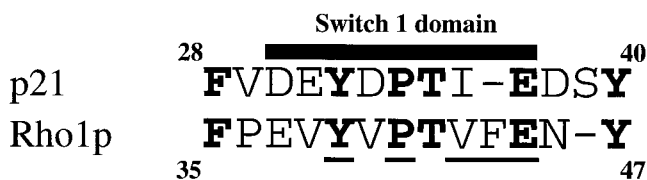
parison with that of mutant *rho1-104* grown at 26°C (Fig. 5). As for Pkc1p, transformation of our reconstructed strains with a constitutively active allele of the corresponding gene failed to suppress their temperature sensitivity at 37°C. In contrast, Nonaka et al. (1995) detected such a suppression with the original *rho1*<sup>E45I</sup>, using rich medium and 35°C as nonpermissive temperature. We were able to reproduce their results using their original strains and conditions; however, the finding that those strains were aneuploid, at least after we received them, prevents a final clarification of the discrepancy.

Some uncertainty lingered because of this disagreement and of the difficulty in extrapolating from measurements of glucan synthase activity *in vitro* to its performance *in vivo*. Therefore, it was desirable to use a different approach to find out whether Pkc1p and synthase activity are required for cell polarization and budding. This was achieved by using a *pkc1* temperature-sensitive mutant and an inhibitor that totally abolished β(1→3)glucan synthesis, in cells containing a wild-type *RHO1* allele. Turning off either Pkc1p activity or glucan synthesis or both did not abolish budding, an event subsequent to cell polarization, although at 37°C and in the presence of inhibitor the buds were extremely small. This reduction in size is not surprising, because each one of the two defects alone results in the production of small buds, followed by cell death. The rapid loss in cell viability stands in contrast with the very slow decrease in viable cells observed in *rho1*<sup>E45I</sup> at 37°C (Fig. 1 B), another indication that the defect in the mutant does not reside in a lack of Pkc1p or glucan synthase function. These results provide a counterpart

to those obtained with the *rho1* mutants. Taken together, the two groups of findings indicate that Rho1p is endowed with a hitherto unknown function, necessary for polarization of the yeast cell.

It has not yet been established whether there is a relationship between specific regions of Rho1p and each of the functions performed by the protein. The mutations we examined are located in the “switch 1” domain of Ras-related proteins (Fig. 11), one of the two regions that change their conformation in response to GTP/GDP exchange (Sigal et al., 1986; Pai et al., 1989; Milburn et al., 1990). It is also called “putative effector recognition domain” (Marshall et al., 1991), since mutations of Ha-Ras in this region result in reduced in vitro affinity for p21-GAP. Our results indicate that the switch 1 domain is very important for the cell polarization function of Rho1p but less so for the regulation of  $\beta(1\rightarrow3)$ glucan synthase. On the other hand, no conclusion can be made about protein kinase C activation. Constitutively active Pkc1p failed to suppress the temperature sensitivity of the mutants; therefore, an activation of the kinase by the mutated Rho1p would not have been detected in our experiments. In mammalian cells, however, a Ras1 mutation equivalent to *rho1*<sup>E45I</sup> resulted in impaired activation of the protein kinase Raf1, which, like Pkc1p, regulates a MAP kinase cascade (Drugan et al., 1996). To further investigate the switch 1 domain of Rho1p we obtained mutations in two more amino acids in this region by directed mutagenesis. However, the corresponding mutants, *rho1*<sup>Y39A</sup> and *rho1*<sup>P41A</sup>, did not show a detectable phenotype (results not shown).

As shown above, the penetration of the *rho1*<sup>E45I</sup> defect was influenced by the genetic background. This is not surprising, if one considers that Rho1p has several essential functions and that it may therefore be very difficult to obtain a mutant in which one of those functions has been completely abolished while maintaining enough of the others to survive at a permissive temperature. Thus, it seems probable that *rho1*<sup>E45I</sup> still maintains at 37°C some residual function for cell cycle progression that enables it to cross the block under favorable conditions. These may entail, for instance, certain levels of expression of other interacting proteins that may vary with the genetic background. This explanation also accounts for the finding that some segregants of cross JDY8 were more leaky than either of the two parents, depending on the gene mix they inherited. Anyway, none of the mutants was able to grow for more than one or a few generations, because they were all temperature-sensitive on plates.



**Figure 11.** Alignment of the switch 1 domain of p21 (human Ha-Ras gene product) with corresponding part of yeast Rho1p sequence. Identical amino acids are shown in bold letters. The position of the switch 1 domain in p21 is indicated by black bar. Underlined amino acids were mutated in this study and in that of Nonaka et al. (1995).

What is the nature of the Rho1p function required for cell polarization? One aspect of the cell cycle block we examined is the lack of actin reorganization and it is certainly possible that Rho1p has a direct role in that process. As mentioned above, Rho has been shown to participate in actin organization in animal cells. Furthermore, Takai and associates have found interactions between Rho1p and Bni1p, a protein that binds to profilin, which in turn stimulates actin polymerization (Imamura et al., 1997). However, that pathway does not seem essential for actin organization, because a deletion of *BNI1* does not alter growth. Perhaps Bni1p could participate in the effect of the *rho1* mutation on polarization caused by pheromone, since *bni1* mutants were found to be defective in that process (Evangelista et al., 1997). In *Schizosaccharomyces pombe*, overexpression of certain *rho1* mutants, some corresponding to the mutants used in this study, or *rho1p* depletion resulted in loss of actin organization (Arellano et al., 1997). However, the mechanism of this effect has not been studied. A role for a glucan synthase or Pkc1p defect in the shmooing impairment cannot be discarded outright but is very unlikely, because of the relatively high level of the synthase, especially at the permissive temperature, and because mutants in Mpk1p, a kinase controlled by Pkc1p, lysed while attempting to make a mating projection (Errede et al., 1995).

There are some indications that the Rho1p function discussed here involves more than actin organization: Cdc42p and Spa2p, two proteins that do not depend on actin for their localization, were not found in the presumptive budding area in *rho1*<sup>E45I</sup>. This result could be interpreted to mean that these proteins are unable to reach their destination or that they reach it but are unable to maintain localization in the presence of a defective Rho1p. However, this finding, together with the observation that the mutant does not undergo nuclear division and duplicates its DNA slowly, suggests that the execution point of Rho1p might precede that of Cdc42p. This is also in agreement with the lack of randomized deposition of chitin, which may require more than one round of DNA replication (Shaw et al., 1991). Furthermore, the already mentioned finding that overexpression of *PKC1* suppresses the budding defect of *swi4*, but not that of our *rho1* mutants, may indicate that Rho1p function precedes Swi4p function in the cell cycle. Thus, the function of Rho1p affected by the mutation may be connected to cell cycle control rather than specifically to cell polarization. Involvement of RhoA, the mammalian counterpart of Rho1p, in cell cycle progression and Ras-dependent cell transformation has been well documented (Olson et al., 1995; Qiu et al., 1995; Hirai et al., 1997). Recently, Hu et al. (1999) showed that RhoA controls ubiquitin-directed degradation of the CDK kinase inhibitor p27<sup>Kip</sup> through regulation of cyclin E/CDK2 activity. In yeast, a similar G1 cyclin-dependent kinase activity (Clnp/Cdc28p) is also required for targeting of its inhibitor Sic1p (yeast counterpart of p27<sup>Kip</sup>) for ubiquitination and proteolysis (Verma et al., 1997a,b). Therefore it is conceivable that Rho1 could have a role in this pathway.

We sought information about the Rho1p effectors by looking for suppressors of the temperature sensitivity of *rho1*<sup>E45I</sup>. No effect was found by transformation with *CDC42* on a high-copy plasmid (results not shown). This

does not exclude the possibility that Cdc42p is a direct or indirect target of Rho1p, because mere increase in expression may be ineffective if an activation step is involved in the pathway and the *rho1* mutant is unable to provide it. Sorbitol, at 1 M concentration, was able to suppress the temperature sensitivity (result not shown). This effect, however, may be due to protection of the mutated Rho1p protein by glycerol accumulated intracellularly in response to the external osmolyte, as we recently found for *rho1-104* (Cabib, E., J. Drgonová, and D.-H. Roh, manuscript in preparation).

A genetic screen for high-copy suppressors yielded in seven cases *RHO1* and in one the *SSD1* gene (results not shown). The latter encodes a cytoplasmic RNA-binding protein (Uesono et al., 1997) which is able to suppress a wide range of mutations including deletion of *sit4* (Sutton et al., 1991), *cln1* and *cln2* mutations (Cvrckova and Nasmyth, 1993), *rpc31* (Stettler et al., 1993), *pde2* and *bcy1* (Sutton et al., 1991; Wilson et al., 1991), and *mpk1* (Lee et al., 1993). The mechanism of the suppressions is not understood, therefore at present this finding does not provide useful information about the Rho1p targets.

In conclusion, although clarification of the mechanism by which Rho1p acts in G1 must await further experimentation, our results clearly show that this yeast protein, "at the interface between cell polarization and morphogenesis" (Drgonová et al., 1996), is necessary for both processes to take place.

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## References

Adams, A.E.M., and J.R. Pringle. 1991. Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol.* 194:729-731.

Adams, A.E.M., D.I. Johnson, R.M. Longnecker, B.F. Sloat, and J.R. Pringle. 1990. Budding and establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* 111:131-142.

Arellano, M., A. Duran, and P. Perez. 1997. Localization of the *Schizosaccharomyces pombe* rho1p GTPase and its involvement in the organization of the actin cytoskeleton. *J. Cell Sci.* 110:2547-2555.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1997. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Ayscough, K.R., J. Stryker, N. Pokala, M. Sanders, P. Crews, and D.G. Drubin. 1997. High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor Latrunculin-A. *J. Cell Biol.* 137:399-416.

Boeke, J.D., F. LaCroute, and G.R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoroorotic acid resistance. *Mol. Gen. Genet.* 197:345-346.

Cabib, E., J. Drgonová, and T. Drgon. 1998. Role of small G proteins in yeast cell polarization and wall biosynthesis. *Annu. Rev. Biochem.* 67:307-333.

Calderone, T.L., R.D. Stevens, and T.G. Oas. 1996. High-level misincorporation of lysine for arginine at AGA codons in a fusion protein expressed in *Escherichia coli*. *J. Mol. Biol.* 262:407-412.

Chant, J. 1996. Generation of cell polarity in yeast. *Curr. Opin. Cell Biol.* 8:557-565.

Cvrckova, F., and K. Nasmyth. 1993. Yeast G1 cyclins CLN1 and CLN2 and a GAP-like protein have a role in bud formation. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:5277-5286.

Dien, B.S., M.S. Peterson, and F. Sreenc. 1994. Cell cycle analysis of *Saccharomyces cerevisiae*. *Methods Cell. Biol.* 42:457-475.

Douglas, C.M., J.A. Marrinan, W. Li, and M.B. Kurtz. 1994. A *Saccharomyces cerevisiae* mutant with echinocandin-resistant 1,3- $\beta$ -D-glucan synthase. *J. Bacteriol.* 176:5686-5696.

Drgonová, J., T. Drgon, K. Tanaka, R. Kollar, G.C. Chen, R.A. Ford, C.S. Chan, Y. Takai, and E. Cabib. 1996. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science.* 272:277-279.

Drubin, D.G., and J. Nelson. 1996. Origins of cell polarity. *Cell.* 84:335-344.

Drugan, J.K., R. Khosravi-Far, M.A. White, C.J. Der, Y.-J. Sung, Y.-W. Hwang, and S.L. Campbell. 1996. Ras interaction with two distinct binding domains in Raf-1 may be required for Ras transformation. *J. Biol. Chem.* 271:233-237.

Errede, B., R.M. Cade, B.M. Yashar, Y. Kamada, D.E. Levin, K. Irie, and K. Matsumoto. 1995. Dynamics and organization of MAP kinase signal pathways. *Mol. Reprod. Dev.* 42:477-485.

Evangelista, M., K. Blundell, M.S. Longtine, C.J. Chow, N. Adams, J.R. Pringle, M. Peter, and C. Boone. 1997. Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science.* 276:118-122.

Gray, J.V., J.P. Ogas, Y. Kamada, M. Stone, D.E. Levin, and I. Herskowitz. 1997. A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:4924-4937.

Guthrie, C., and G.R. Fink. 1991. Guide to Yeast Genetics and Molecular Biology. Academic Press, London.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279:509-514.

Herskowitz, I., and R.E. Jensen. 1991. Putting the HO gene to work: practical uses for mating type switching. *Methods Enzymol.* 194:132-145.

Hirai, A., S. Nakamura, Y. Noguchi, T. Yasuda, M. Kitagawa, I. Tatsuno, T. Oeda, K. Tahara, T. Terano, S. Narumiya, et al. 1997. Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27<sup>Kip1</sup> and facilitate the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells. *J. Biol. Chem.* 272:13-16.

Hu, W., C.J. Bellone, and J.J. Baldassare. 1999. RhoA stimulates p27<sup>Kip</sup> degradation through its regulation of cyclin E/CDK2 activity. *J. Biol. Chem.* 274:3396-3401.

Imamura, H., K. Tanaka, T. Hihara, M. Umikawa, T. Kamei, K. Takahashi, T. Sasaki, and Y. Takai. 1997. Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:2745-2755.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153:163-168.

Jones, J.S., and L. Prakash. 1990. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast.* 6:363-366.

Kamada, Y., H. Qadota, C.P. Python, Y. Anraku, Y. Ohya, and D.E. Levin. 1996. Activation of yeast protein kinase C by Rho1 GTPase. *J. Biol. Chem.* 271:9193-9196.

Kapteyn, J.C., R.C. Montijn, E. Vink, J. De La Cruz, A. Llobell, J.E. Douwes, H. Shimoi, P.N. Lipke, and F.M. Klis. 1996. Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked  $\beta$ 1,3- $\beta$ 1,6-glucan heteropolymer. *Glycobiology.* 6:337-345.

Kilmartin, J.V., and A.E.M. Adams. 1984. Structural rearrangements of tubulin and actin during the cycle of the yeast *Saccharomyces*. *J. Cell Biol.* 98:922-933.

Kohno, H., K. Tanaka, A. Mino, M. Umikawa, H. Imamura, T. Fujiwara, Y. Fujita, K. Hotta, H. Qadota, T. Watanabe, Y. Ohya, and Y. Takai. 1996. Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6060-6068.

Kollár, R., B.B. Reinhold, E. Petraková, H.J.C. Yeh, G. Ashwell, J. Drgonová, J.C. Kapteyn, F.M. Klis, and E. Cabib. 1997. Architecture of the yeast cell wall.  $\beta$ (1 $\rightarrow$ 6)glucan interconnects mannoprotein,  $\beta$ (1 $\rightarrow$ 3)glucan, and chitin. *J. Biol. Chem.* 272:17762-17775.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature.* 227:680-685.

Leberer, E., D.Y. Thomas, and M. Whiteway. 1997. Pheromone signalling and polarized morphogenesis in yeast. *Curr. Opin. Genet. Dev.* 7:59-66.

Lee, K.S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto, and D.E. Levin. 1993. A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. *Mol. Cell. Biol.* 13:3067-3075.

Levin, D.E., and E. Bartlett-Heubusch. 1992. Mutants in the *S. cerevisiae* PKC1 gene display a cell cycle-specific osmotic stability defect. *J. Cell Biol.* 116:1221-1229.

Levin, D.E., B. Bowers, C.Y. Chen, Y. Kamada, and M. Watanabe. 1994. Dissecting the protein kinase C/MAP kinase signalling pathway of *Saccharomyces cerevisiae*. *Cell. Mol. Biol. Res.* 40:229-239.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.

Madaule, P., R. Axel, and A.M. Myers. 1987. Characterization of two members of the rho gene family from the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 84:779-783.

Marshall, M.S., L.J. Davis, R.D. Keys, S.D. Mosser, W.S. Hill, E.M. Scolnick, and J.B. Gibbs. 1991. Identification of amino acid residues required for Ras p21 target activation. *Mol. Cell. Biol.* 11:3997-4004.

Milburn, M.V., L. Tong, A.M. deVos, A. Brunger, Z. Yamaizumi, S. Nishimura, and S.H. Kim. 1990. Molecular switch for signal transduction: struc-

- tural differences between active and inactive forms of protooncogenic ras proteins. *Science*. 247:939-945.
- Mitchison, J.M., and W.S. Vincent. 1965. Preparation of synchronous cell cultures by sedimentation. *Nature*. 205:987-989.
- Mol, P.C., H.-M. Park, J.T. Mullins, and E. Cabib. 1994. A GTP-binding protein regulates the activity of (1→3)-β-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis. *J. Biol. Chem.* 269:31267-31274.
- Nonaka, H., K. Tanaka, H. Hirano, T. Fujiwara, H. Kohno, M. Umikawa, A. Mino, and Y. Takai. 1995. A downstream target of *RHO1* a small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:5931-5938.
- Olson, M.F., A. Ashworth, and A. Hall. 1995. An essential role for Rho, Rac and Cdc42 GTPases in cell cycle progression through G1. *Science*. 269:1270-1272.
- Pai, E.F., W. Kabsch, U. Krengel, K.C. Holmes, J. John, and A. Wittinghofer. 1989. Structure of the guanine-nucleotide-binding domain of the Ha-Ras oncogene product p21 in the triphosphate conformation. *Nature*. 341:209-214.
- Peppler, H.J., and F.J. Rudert. 1953. Comparative evaluation of some methods for estimation of the quality of active dry yeast. *Cereal. Chem.* 30:146-152.
- Peterson, J., Y. Zheng, L. Bender, A. Myers, R. Cerione, and A. Bender. 1994. Interactions between the bud emergence proteins Bem1p and Bem2p and Rho-type GTPases in yeast. *J. Cell Biol.* 127:1395-1406.
- Pringle, J.R., A.E.M. Adams, D.G. Drubin, and B.K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565-601.
- Qadota, H., C.P. Python, S.B. Inoue, M. Arisawa, Y. Anraku, Y. Zheng, T. Watanabe, D.E. Levin, and Y. Ohya. 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. *Science*. 272:279-281.
- Qiu, R.G., J. Chen, F. McCormick, and M. Symons. 1995. A role for Rho in Ras transformation. *Proc. Natl. Acad. Sci. USA*. 92:11781-11785.
- Rose, M.D., F. Winston, and P. Hieter. 1990. *Methods in Yeast Genetics. A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shaw, J.A., P.C. Mol, B. Bowers, S.J. Silverman, M.H. Valdivieso, A. Durán, and E. Cabib. 1991. The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 114:111-123.
- Sigal, I.S., J.B. Gibbs, J.S. D'Alonzo, and E.M. Scolnick. 1986. Identification of effector residues and a neutralizing epitope of Ha-Ras-encoded p21. *Proc. Natl. Acad. Sci. USA*. 83:4725-4729.
- Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 122:19-27.
- Stettler, S., N. Chiannikulchai, S.H. Denmat, D. Lalo, F. Lacroute, A. Sentenac, and P. Thuriaux. 1993. A general suppressor of RNA polymerase I, II and III mutations in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 239:169-176.
- Sutton, A., F. Lin, and K.T. Arndt. 1991. The *SIT4* protein phosphatase is required in late G1 for progression into S phase. *Cold Spring Harb. Symp. Quant. Biol.* 56:75-81.
- Uesono, Y., A. Toh-e, and Y. Kikuchi. 1997. Ssd1p of *Saccharomyces cerevisiae* associates with RNA. *J. Biol. Chem.* 272:16103-16109.
- Verma, R., R.M.R. Feldman, and R.J. Deshaies. 1997a. SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34 and cyclin/CDK activities. *Mol. Biol. Cell.* 8:1427-1437.
- Verma, R., R.S. Anan, M.J. Huddleston, S.A. Carr, G. Reynard, and R.J. Deshaies. 1997b. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science*. 278:455-460.
- Wilson, R.B., A.A. Brenner, T.B. White, M.J. Engler, J.P. Gaughran, and K. Tatchell. 1991. The *Saccharomyces cerevisiae* *SRK1* gene, a suppressor of *bcy1* and *ins1*, may be involved in protein phosphatase function. *Mol. Cell Biol.* 11:3369-3373.
- Yamochi, W., K. Tanaka, H. Nonaka, A. Maeda, T. Musha, and Y. Takai. 1994. Growth site localization of Rho1 small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 125:1077-1093.
- Ziman, M., D. Preuss, J. Mulholland, J.M. O'Brien, D. Botstein, and D.I. Johnson. 1993. Subcellular localization of Cdc42p, a *Saccharomyces cerevisiae* GTP-binding protein involved in the control of cell polarity. *Mol. Biol. Cell.* 4:1307-1316.