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

Jana Drgonová, Tomás Drgon, Dong-Hyun Roh, and Enrico Cabib
National Institute of Diabetes and Digestive and Kidney Diseases, Laboratory of Biochemistry and Genetics, Bethesda, Maryland 20892

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 β(1→3)glucan synthase. Here we demonstrate a hitherto unknown function of Rho1p required for cell cycle progression and cell polarization. Cells of mutant rho1E45I 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, rho1V43T and rho1F44Y, showed a similar behavior, although some budding and actin polarization occurred at the nonpermissive temperature. This was also the case for rho1E45I 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 rho1E45I. 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 β(1→3)glucan synthase in rho1E45I, 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 P K C1. Reciprocally, when Pkc1p function was eliminated by the use of a temperature-sensitive mutation and β(1→3)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

Yeast 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 un budded cell, whereas DNA synthesis and nuclear division can continue, thus generating bi- and tetraneucleated 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 (H all, 1998). In budding yeast Rho1p, like Cdc42p, interacts with Bni1p and has been proposed as a regulator of actin reorganization (K ohno et al., 1996). However, a temperature-sensitive mutant of RH O1, rho1-104(D72N; C164Y), arrests at the nonpermissive temperature mainly as a cell with a small bud (Y amochi 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

Address correspondence to Enrico Cabib, National Institutes of Health, B Center Drive M SC 0851, Bethesda, M D 20892. Tel.: (301) 496-1008. Fax: (301) 496-9431. E-mail: enricoc@bdg10.niddk.nih.gov
regulate the activity of protein kinase C, hence of the corresponding MAP-kinase cascade (Nonaka et al., 1995; Kamaeda et al., 1996). It also regulates β(1→3)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 \textit{RHO1} are blocked at a cell cycle stage that precedes cell polarization. The defect does not appear to be related to β(1→3)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. \textit{Y}east cells were cultured either in minimal (2% glucose, 0.7% yeast nitrogen base without amino acids [Difco], plus requirements) or in \textit{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.

**Table I. \textit{S. cerevisiae} Strains Used in This Study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>OHNY1</td>
<td>MATa ura3 leu2 trpl his5 ade2</td>
<td>Y. Takai</td>
</tr>
<tr>
<td>DHNY10</td>
<td>MATa/MATa ura3/ura3 leu2/leu2 trpl1/trp1 his3/his3 ade2/ade2 rho1::HIS3/rho1::HIS3 pRS316(RHO1)[KpnI]</td>
<td>Y. Takai</td>
</tr>
<tr>
<td>HNY21</td>
<td>MATa ura3 leu2 trpl1 his3 ade2 rho1-104</td>
<td>Y. Takai</td>
</tr>
<tr>
<td>HNY93</td>
<td>MATa ura3 leu2 trpl1 his3 ade2 rho1^{E45I}</td>
<td>Y. Takai</td>
</tr>
<tr>
<td>HNY95</td>
<td>MATa ura3 leu2 trpl1 his3 ade2 rho1^{E45I}</td>
<td>Y. Takai</td>
</tr>
<tr>
<td>HNY97</td>
<td>MATa ura3 leu2 trpl1 his3 ade2 rho1^{E45I}</td>
<td>Y. Takai</td>
</tr>
<tr>
<td>JDY4</td>
<td>MATa ura3 leu2 trpl1 his3 ade2</td>
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<tr>
<td>JDY6-7A[pRS316(RHO1)]*</td>
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<td>R.S. Fuller</td>
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<td>This study</td>
</tr>
<tr>
<td>ECV44Δ[pRS316(RHO1)]</td>
<td>MATa/MATa ade2-1/ad2-1 his3-11 his3-11 leu2-3,112/trpl1-1/trpl1-1 ura3-1/[ura3-1] rho1::HIS3/RHO1 pRS316(RHO1)</td>
<td>This study</td>
</tr>
<tr>
<td>ECV44Δ[pRS316(rho1^{E45I})]</td>
<td>MATa/MATa ade2-1/ad2-1 his3-11 his3-11 leu2-3,112/trpl1-1/trpl1-1 ura3-1/[ura3-1] rho1::HIS3/RHO1 pRS316(rho1^{E45I})</td>
<td>This study</td>
</tr>
<tr>
<td>ECV44Δ[pRS316(rho1^{E45I})]-ID</td>
<td>MATa ade2-1 his3-11 leu2-3,112 trpl1-1 ura3-1 rho1::HIS3 pRS316(rho1^{E45I})</td>
<td>This study</td>
</tr>
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<td>D. Levin</td>
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<tr>
<td>DL503</td>
<td>MATa ade2-1 ura3-52 trpl1-1 his4 pkc1Δ::LEU2 YCP50[pkc1^{+}]</td>
<td>D. Levin</td>
</tr>
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</table>

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

†Resulting from cross between J DY6-7A[pRS316(rho1^{E45I})] and ECV44Δ[pRS316(rho1^{E45I})]-ID.
(RHO1 [KpnI]) (kindly furnished by T. Takai) was digested with SnaBI, 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'-ATGTCACAACCAGTTGGAAC-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 (Y amochi et al., 1994). To obtain a fragment containing only the open reading frame, genomic DNA was isolated, digested with BamHI and KpnI and ligated with pRS314 previously digested with MluI and HpaI. This 1,495-bp fragment, which contained promoter, coding region of RHO1, and the transcription terminator, was amplified by PCR with synthetic nucleotides containing engineered SacI and HindIII restriction sites, respectively, (underlined) 5'-AATGGGAGCTCACAATTGGAAC-3' and 5'-AAACTACATAAGCTTTACGCAGATCAAAAATAGGTGC-3' as primers. The amplified DNA was digested with SacI and HindIII and the resulting 1,495-bp fragment, which contained promoter, coding region of RHO1, and the transcription terminator, was ligated into pRS314. The resulting plasmid, pRS316 (Sikorski and Hieter, 1989), was then digested with SacI and XhoI. The excised fragment was isolated. Into this fragment were inserted the synthetic oligonucleotides 5'-CACTACGCGAGCCGCCACT-3' and 5'-AAATCAATGCTTACGCAGATCAAAAATAGGTGC-3'. This fragment was religated with pRS316 containing the KpnI site, inserted upstream of the open reading frame, 22 bp downstream of the ATG of RHO1, and 427 bp of the downstream region, was cloned into pRS316 (Sikorski and Hieter, 1989), previously digested with SacI and HindIII. The resulting plasmid, pRS316 (rho1^437), was digested with SnaBI and the vector-containing fragment was isolated. Into this fragment were cloned 594-bp pieces obtained by SnaBI digestion of either pRS316 (RHO1 [KpnI]), pRS316 (rho1F^44Y [KpnI]), or pRS316 (rho1V^43T [KpnI]). In the obtained plasmids, the region containing the mutation was sequenced with the synthetic oligonucleotide 5'-ATGTCACAACCAGTTGGAAC-3'. In addition, rho1 and rho1^491 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 pRS316 (Sikorski and Hieter, 1989) by digesting the latter with XbaI and XhoI and ligating a SacI-XhoI fragment from the corresponding pRS316 plasmid with the cut vector.

Strain JDY6-6A (pRS316 RHO1 [KpnI]) was a segregant dissected after sporulation of strain DHY10 (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 replaced (in DHY10, derived from W303-RHO1 [M adulea et al., 1987] by the multiplication of the promoter region that had been deleted). A 1,345-bp fragment containing the URA3 gene was excised from pYES2.0 (Invitrogen) by digestion with XmnI and inserted between the MluI site (located 382 bp upstream of RHO1) and the Hpal site (located 22 bp downstream of RHO1) of plasmid pRS316 (RHO1). Before ligation, the MluI digest was treated with T4 DNA polymerase to fill 5' overhangs. The resulting plasmid, pRS316 (rho1::URA3), was digested with XmaI and SacI; the excised rho1::URA3 fragment was isolated and used to transform JDY6-6A containing the pRS316 plasmid. The URA3-containing recombinant was used to replace the RHO1 genomic locus by PCR. The resulting plasmid, pRS316 (rho1::URA3), was digested with SacI and HindIII and the excised fragment was isolated. To obtain a fragment containing the entire RHO1 coding sequence, the PCR product was amplified from strain JDY6-7A (pRS316 RHO1::URA3) when a different marker was required. These were shuffled with the pRS316 plasmids (devoid of the KpnI site) when a different marker was required.

One of the above plasmids containing 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 the open reading frame of RHO1, genomic DNA was isolated, digested with SacI and HindIII, and the excised fragment was isolated. The resulting fragment, pEC14, was digested with SacI and XhoI. The excised 2.8-kb fragment containing the URA3 gene was isolated. This fragment was religated with pRS316 containing the KpnI site, inserted upstream of the open reading frame, 22 bp downstream of the ATG of RHO1, and 427 bp of the downstream region, was cloned into pRS316 (Sikorski and Hieter, 1989). DNA manipulations were performed according to standard protocols (Guthrie and Fink, 1991; A usubel et al., 1997).

Human p21 (p0112) 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 GI 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, resuspended 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 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 GI1 cells were cultured 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 (pXCl), cells were fixed with 5% formaldehyde before counting, to prevent lysing. Small buds were counted with Nomarski optics and the amino acids in yeast Rho1p corresponding to the p21 switch 1 domain were deduced from the alignment.

Cell viability was estimated by staining with methylene blue. Cells were pelleted and suspended in 2 µg/ml methylene blue in 0.05 M KHP04. 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% 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-coated slides, actin was stained with rhodamine-phalloidin (Molecular Probes) as described in A dams and Pringle (1991) and nuclei were stained with DAPI (1 µg/ml) for 5 min.

Col42p was stained with affinity-purified rabbit anti-Cl42 antibodies (kindly provided by D. Johnson) essentially as described by Ziman et al. (1993). For immunolocalization of Spa2p, the JDY6-7A strains were transformed with the pBu4 plasmid (pRS315 [3XHA-SPA2]) obtained from the laboratory of M. Snyder. After fixation as above, the cells were stained with 1 mg/ml of lyticase (Sigma Chemical Co.) for 30 min at 37°C, immobilized on polystyrene precoated slides, and permeobilized with 0.1% n-propanol.
Triton X-100 for 5 min. H A -Spa2p was immunostained as described by Pringle et al. (1995). Permeabilized protoplasts were incubated with anti-H A mouse monoclonal antibodies (12CA 5; Boehringer Mannheim) and fluorescing-conjugated goat anti-mouse IgG (Cappell) 1:400 in buffer F for 2.5 h at room temperature. In all cases, p-phénylendiamine-based mounting medium (Pringle et al., 1993) was used. The samples were observed in a Z eiss A xioscope and photographs were taken with an Olympus DM 4T camera.

Flow Cytometry

Cells were fixed with 70% ethanol and DNA was stained with propidium iodide as described in D en 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 F ast Systems.

Membrane Preparation and Measurement of β(1→3)Glucan Synthase Activity

Membranes were prepared from logarithmic phase cells as previously described (M ol et al., 1994), except that disruption with glass beads was carried out by vortexing. Glucan synthase was assayed as reported (M ol 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. U DP [14C]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 β(1→3)Glucan Synthesis by L-733,560

A n 80-ml culture of strain 1783 (wild-type) grown in minimal medium at 26°C and containing ~7 × 10^5 cells/ml was split in two. To one half L-733,560 (Douglas et al., 1994) was added to a final concentration of 1 μM. Thereafter, both cultures were treated identically. Flasks were transferred to a 37°C bath and 30 min later 40 μCi of U [14C]glucose (250 mCi/mmol) was added. A fter an additional 2 h at 37°C, cells were collected by centri- fugation and washed twice with water. A fter 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; B Braun Biotech), cells were disrupted by vortexing. T he extract was aspirated from the glass beads and the latter were washed with five 1-ml portions of buffer A. T he extract and washings were combined and centrifuged for 10 min at 4,000 g to sediment cell walls. T he 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 PM 5F-treated Z nyomyase 100,000 (K olár et al., 1997; Z nyomyase 100,000 was from SK 3AGAKA A merica). A fter a 12-h incubation at 37°C, insoluble material was sedimented by centrifugation and the supernatant was applied to a Sephadex G-100 column (1 × 85 cm), previously equilibrated with 0.1 M acetic acid. T he column was eluted with the equilibration sol- vent, 0.46-ml fractions were collected, and radioactivity was determined in the fractions.

Treatment of Cells with α-Factor

T o 5 ml of culture in minimal medium, containing ~10^7 cells/ml, two addi- tions of 80 μg of α-factor (B achem Bioscience, Inc.) were made 1 h apart. A fter a total incubation time of 2 h at 26°C, most cells carrying wild-type RHO1 showed a mating projection. A t 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 (A lpha Diag- nostic) against a purified maltose-binding protein (MBP)-Rho1p fusion protein obtained as follows. Antiserum against the 635-bp fragment containing the RHO1 coding se- quence was amplified by PCR from genomic DNA with an upstream primer containing the initiation codon (5′-AT GT CA CAA CAA C A G T T G G- TA CA A -3′) and a downstream primer containing an EcoRI restriction site (underlined) (5′-TG CCA CTA A G A T T G C A G T G A G A T C -3′). T he amplified fragment was digested with EcoRI and inserted into the plasmid <cml:pma-l2cexpression vector (New England Biolabs), previously digested

with X mnl and E coRI, to yield the in-frame MBP-Rho1 fusion protein. T he fusion protein was expressed in protease-deficient E scherichia coli BLR cells (Novagen), containing plasmid pCD 952 (kindly provided by J. R. Walker) that encodes r RNA 5 S to eliminate misincorporation of lysine in place of arginine (Calderone et al., 1996). Production of the fusion pro- tein was induced for 3 h at 30°C with 0.3 mM isopropylthio-β-D-galactoside. T he protein was purified by chromatography on amylose resin (New En gland Biolabs) according to manufacturer’s instructions, before injection.

For Western blot analysis, yeast cell lysates, obtained by glass bead dis-ruption and clarified by centrifugation, were separated by 5-10% polyacryl- amide electrophoresis (40 μg of protein/lane) in a 14% gel (Laemmli, 1970) and blotted onto a PVDF membrane (Novex) according to the manu- facturer’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. A ctin was stained with monoclonal mouse antiactin antibodies, clone C 4 (ICN) at 1:4,000, and HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) at 1:5,000. A n antibody-antigen complexes were detected with the ECL system (Pierce), following supplier’s instructions.

Preimmune serum did not produce any signal. T he antibody stained a band of the expected mobility (~26 K d) when wild-type cell lysates were used. T he band was stronger when RHO1 was overexpressed on a high- copy plasmid and much weaker with extracts from strain H N Y 23 (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 rho1F44Y, rho1E45V, and rho1E45I (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 func- tions of Rho1p were activation of Pkc1p and regulation of β(1→3)glucan synthase, we thought that rho1F44Y may be specifically impaired in glucan synthesis. However, we found that although glucan synthase was partially defec- tive in rho1E45V after growth at 37°C, the same defect was shown by the other two mutants. In the course of those ex- periments we observed that at the nonpermissive tempera- ture 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 (Y amochi 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, rho1E45I and, to some extent, rho1F44Y sporo- lated, whereas rho1E45V was found to contain two copies of the RHO1 gene in different parts of the genome (C abib, 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 (D HY 97, Table I) in which we placed the mutation rho1E45I into the RHO1 locus, in similar fashion to strain H N Y 97 from T akai’s laboratory, also appeared to have become aneuploid: the strain budded poorly and mated very poorly. S porulation of a cross with a wild-type strain gave rise to an abnormal distribution of markers (re- sults not shown). In a similar fashion, mutants in B EM 2, a gene that interacts genetically with RHO1, have been found to easily become aneuploid and to switch their mat- ing type (Peterson et al., 1994).

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Since uncertainty about ploidy would compromise the results, we constructed other strains harboring a disruption of \textit{RHO1} and a plasmid carrying either wild-type \textit{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 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 JDY 6-7A [pRS316(\textit{rho1E45I})], JDY 6-7A [pRS316(\textit{rho1V43T})], and JDY 6-7A [pRS314(\textit{rho1F44Y})], respectively. (E) Appearance of cells of JDY 6-7A [pRS316\textit{(RHO1})] as viewed with phase-contrast, after 6 h at 37°C. (F) Same as E, but with cells of JDY 6-7A [pRS316(\textit{rho1E45I})].
1953), started to decrease (Fig. 1 B). Similar results were obtained with the other two rho1 mutants, except that both rho1V43T and rho1F44Y 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 rho1F44Y; see Fig. 6 for rho1V43T), suggesting that they were unable to complete subsequent cell cycles. As will be shown below, when the mutation rho1E45I 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 rho1E45I (Fig. 2, A and C). With rho1V43T and rho1F44Y some polarization was found in those cells that formed buds (Fig. 2, E and G). To find out whether the lack of polarization in rho1E45I 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 (Ayrescough et al., 1997), Cdc42p and Spa2p. In both cases, the proportion of cells showing localization of these proteins was much lower in the rho1E45I than in wild-type (Fig. 3 and Table II).

In cdc42-1, a mutant with similar morphology to rho1E45I, 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 rho1E45I (Fig. 4). This experiment also confirms that the initial cell population was in G1.

Another difference between cdc42 and rho1E45I 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 rho1E45I cells do not (results not shown).

The rho1E45I Defect Results from Loss of a Previously Unknown Function

The defect in rho1E45I is recessive: neither strain

![Figure 2](image-url)

**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(rho1E45I)] (C), JDY6-7A[pRS316(rho1V43T)] (E), and JDY6-7A[pRS314(rho1F44Y)] (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.

![Figure 3](image-url)

**Figure 3.** Cdc42p and Spa2p fail to localize to the budding site in rho1E45I 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(rho1E45I)]; (C) Spa2p in JDY6-7A[pRS316(RHO1)]; (D) Spa2p in JDY6-7A[pRS316(rho1E45I)]. In C, the inset shows a cell undergoing cytokinesis with Spa2p at the septum formation site.
ECY44Δ(pRS316-RHO1) nor strain ECY44Δ(pRS316-rho1_E45) 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_E45), 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_E45 is due to loss of function and not to interference with some other pathway caused by abnormal targeting of the mutated protein.

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

<table>
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<th>26°C</th>
<th>37°C</th>
<th>26°C</th>
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<tbody>
<tr>
<td>Cdc42p</td>
<td>20%</td>
<td>17%</td>
<td>24%</td>
<td>3.6%</td>
</tr>
<tr>
<td>HA-Spa2p</td>
<td>15%</td>
<td>12%</td>
<td>11%</td>
<td>2%</td>
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In each case at least 100 unbudded cells were counted.

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...
here did not contain sorbitol. The activity of rho1E45I 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 rho1E45I, while it was lower in rho1-104. Therefore, it is unlikely that the defect of rho1E45I 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, pkc1R398P, suppresses the temperature sensitivity of strains HNY95 (rho1E45I) and HNY97 (rho1E45I) but not that of HNY93 (rho1V43T). 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(rho1V43T)] and JDY6-7A [pRS314 (rho1E45I)], 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 rho1V43T and the rho1E45I 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 pkc1R398P gene was carried by pRS315. A gain, 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, rho1V43T, rho1E45I, and rho1E45I 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(rho1V43T)] and JDY6-7A [pRS316(rho1E45I)] 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 rho1E45I 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 reasoned that cells containing a wild-type allele of RHO1 but in which Pkc1p activity and synthesis of β(1→3)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 Pkc1p, 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. When 1 μM L-733,560, that inhibits the formation of the polysaccharide in vivo and in vitro (Douglas et al., 1994), 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 A), whereas the oligo-
Figure 7. Suppression of Pkc1p function or β(1→3)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 [14C]glucose in the absence (A) or in the presence (B) of L-733,560. The peak at fractions 40–65 contains a mannoprotein-β(1→6)glucan complex, whereas the peak at fractions 140–160 (absent in B) corresponds to oligosaccharides resulting from the hydrolysis of β(1→3)glucan. For experimental details, see Materials and Methods. (C and D) Budding and viability in strain DL503 (pkc1ts) 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 β(1→3)glucan synthesis. Since in the original wall the mannoprotein-β(1→6)glucan complex is attached to β(1→3)glucan (Kapteyn et al., 1996; Kolár 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 particularly preexisting to prechains of β(1→3)glucan.

Having established the effectiveness of L-733,560, we proceeded to determine budding and viability (by methylene blue staining) of strain DL503 (pck1) under conditions where either Pck1p or β(1→3)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 α-factor (Fig. 7 E, arrows). At 37°C, when only Pck1p 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-H ebusch, 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 Pck1p and β(1→3)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^E45I and rho1^V43T 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 α-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 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^V43T and rho1^E45I, 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^E45I Mutation in Different Genetic Backgrounds

The observation that strain JDY 6-7A [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 DHNY 110 or introduced during our genetic manipulations, might contribute, together with rho1^E45I, to generate the observed phenotype. Whether a mutation had been introduced artificially was investigated by using strain DHY 1-5A (pGRT), which harbored a new deletion of the RHO1 gene (carried out in the diploid JDY 7, isogenic with OHNY 1) 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^E45I). The resulting strains behaved indistinguishably from the previously used strains JDY 6-7A[pRS316(RHO1)] and JDY 6-7A[pRS316(rho1^E45I)], 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 DHNY 110 and OHNY 1 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 ECY 44Δ[pRS316(rho1^E45I)] (Table I), a diploid obtained by mating strains CRY 1 and CRY 2 to yield ECY 44, followed by disruption of RHO1 and introduction of the...
plasmid carrying the rho1 mutation. Both CRY1 and CRY2, as well as ECY44 and ECY44Δ[pRS316(rho1E45I)], show robust growth at 37°C, clearly more vigorous than that of JDY6-7A[pRS316(RHO1)] or OHNY1 (results not shown). After sporulation of ECY44Δ[pRS316(rho1E45I)], six tetrads were analyzed. A II scored 2:2 for temperature sensitivity (results not shown), as expected, since all contain rho1E45I, 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 rho1E45I segregant it was only 0.6-fold.

It remained to be ascertained whether the difference in penetration of the rho1E45I mutation between the JDY6-7A or OHNY1 backgrounds on one hand and the ECY44Δ background on the other is due to a single mutation or to a more general genetic variation. To elucidate this point, we mated ECY44Δ-A1D (Fig. 10 A and B) to JDY6-7A[pRS316(rho1E45I)] 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. A II the progeny was temperature-sensitive (result not shown), as expected, since both mating partners carried a RHO1 deletion plus rho1E45I 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. A ctn 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 rho1E45I 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

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<tr>
<th>Spore</th>
<th>26°C</th>
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<tr>
<td>A</td>
<td>74</td>
<td>13</td>
</tr>
<tr>
<td>B</td>
<td>56</td>
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<td>C</td>
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<td>D</td>
<td>78</td>
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Approximately 200 cells were counted in each case.
rho1E45I 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, rho1V43T and rho1F44Y, 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 Rho1p 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 rho1E45I 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 comparison 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 rho1E45I, 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 rho1E45I 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; Miliburn et al., 1990). It is also called “putative effector recognition domain” (Marshall et al., 1991), since mutations of H-a-Ras in this region result in reduced in vitro affinity for p21-70G0. 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 β(1→3)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 (Dugan 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>E38K</sup> and rho1<sup>E41A</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. A nyway, none of the mutants was able to grow for more than one or a few generations, because they were all temperature-sensitive on plates.

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 Mpklp, a kinase controlled by Pkc1p, lysed while attempting to make a mating projection (Erede 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 bud site of 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 inhibitor p27<sup>Kip1</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>Kip1</sup>) for ubiquitination and proteolysis (Verma et al., 1997a,b). Therefore it is conceivable that Rho1p 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

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**Figure 11.** Alignment of the switch 1 domain of p21 (human H-a-Ras gene product) and 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).
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 (Cabb, E.-J., D. Drnovog, 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 sti4 (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” (Drnovog et al., 1996), is necessary for both processes to be accompanied.

We are deeply indebted to Y. Takai for strains and plasmids. We thank D.I. Johnson for an anti-Cdc42p antibody and for very useful advice on its use. We also thank A. Bender, J. Pringle, J.R. Walker, M. Snyder, and B. Santos for plasmids, D. Levin for strains, and M. Kurtz for a sample of L-733,560. We are grateful to L. Crotti, J.C. Ribas, A. Robbins, M. Schmidt, and R. Wickner for a critical reading of the manuscript.

Submitted: 29 July 1998
Revised: 11 June 1999
Acepted: 18 June 1999

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Downloaded from jcb.rupress.org on July 7, 2017


