Growth Site Localization of Rho1 Small GTP-binding Protein and Its Involvement in Bud Formation in Saccharomyces cerevisiae

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Abstract. The Rho small GTP-binding protein family regulates various actomyosin-dependent cell functions, such as cell morphology, locomotion, cytokinesis, membrane ruffling, and smooth muscle contraction. In the yeast Saccharomyces cerevisiae, there is a homologue of mammalian RhoA, RHO1, which is essential for vegetative growth of yeast cells. To explore the function of the RHO1 gene, we isolated a recessive temperature-sensitive mutation of RHO1, rhol-104. The rhol-104 mutation caused amino acid substitutions of Asp 72 to Asn and Cys 164 to Tyr of Rholp. Strains bearing the rhol-104 mutation accumulated tiny- or small-budded cells in which cortical actin patches were clustered to buds at the restrictive temperature. Cell lysis and cell death were also seen with the rhol-104 mutant. Indirect immunofluorescence microscopic study demonstrated that Rholp was concentrated to the periphery of the cells where cortical actin patches were clustered, including the site of bud emergence, the tip of the growing buds, and the mother-bud neck region of cells prior to cytokinesis. Indirect immunofluorescence study with cells overexpressing RHO1 suggested that the Rholp-binding site was saturable. A mutant Rhol with an amino acid substitution at the lipid modification site remained in the cytoplasm. These results suggest that Rhol small GTP-binding protein binds to a specific site at the growth region of cells, where Rhol exerts its function in controlling cell growth.

The yeast Saccharomyces cerevisiae grows by budding for cell division (Drubin, 1991; Nelson, 1992). This polarized cell growth is initiated by signals from the cell surface that result in realignment of the cytoskeleton and the biosynthetic machinery toward a targeting patch at the bud site. Membrane protein transport to the cell surface is mediated by vesicles, which become selectively targeted to the bud site. Bud site assembly and growth are also coupled to reorganization of the actin cytoskeleton. Disruption of the single actin gene in S. cerevisiae results in abnormal cell growth and intracellular accumulation of vesicles (Novick and Botstein, 1985). Moreover, there is a strong correlation between occurrence of active growth at the bud tip and clustering of cortical actin patches at the same tip. Cortical actin patches are concentrated at the site of bud emergence on unbudded cells and in small and medium size buds in budding cells, whereas actin fibers are generally oriented along the long axes of the mother–bud pairs (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Near the end of the cell cycle, cortical actin patches concentrate in the neck region connecting the mother cell to its bud. However, it is not clear how the actin cytoskeleton is linked to bud assembly at the membrane.

Genetic approaches have been exploited in S. cerevisiae to identify genes that are required for assembly and growth of a bud. Genes CDC24 and CDC42 have been identified to be necessary for bud site assembly (Sloat et al., 1981; Adams et al., 1990; Johnson and Pringle, 1990). Mutations in these genes do not result in bud formation, but in an overall increase of cell surface. In these mutants, cortical actin patches uniformly distribute over the entire cell surface. The CDC42 and CDC24 genes encode a small guanosine 5'-triphosphate (GTP)-binding protein (small G protein) (Johnson and Pringle, 1990) and a putative guanine nucleotide exchange protein for Cdc42p (Hart et al., 1991), respectively. Two other genes encoding small G proteins, RHO3 and RHO4.

Abbreviations used in this paper: 5-FOA, 5-fluoro-orotic acid; G protein, GTP-binding protein; GDI, GDP dissociation inhibitor; GDP, guanosine 5'-diphosphate; GDS, GDP dissociation stimulator.
have recently been shown to be involved in the control of bud growth (Matsui and Toh-e, 1992).

In the mammalian system, evidence is accumulating that small G proteins regulate various cell functions (Hall, 1990; Bourne et al., 1991; Takai et al., 1992). Of the many small G proteins, the Rho family, consisting of three members, A, B, and C, has been suggested to regulate the actomyosin system. This is based on the observations that Clostridium botulinum ADP-ribosyltransferase, Cε, which selectively ADP-ribosylates the Rho family (Aktories et al., 1988; Kikuchi et al., 1988; Narumiya et al., 1988), changes stress fibers (Paterson et al., 1990; Ridley and Hall, 1992). Moreover, it has recently been shown that RhoA plays an important role in various actomyosin-dependent cell functions, including cell morphology (Rubin et al., 1988; Paterson et al., 1990; Miura et al., 1993), smooth muscle contraction (Hirata et al., 1994), platelet aggregation (Morii et al., 1992), cell motility (Takashi et al., 1993, 1994), cytokinesis (Kishi et al., 1994), lymphocyte aggregation (Tomina et al., 1993), lymphocyte-mediated cytotoxicity (Lang et al., 1992), and membrane ruffling (Nishiyama et al., 1994).

Rho has guanosine 5'-diphosphate (GDP)-bound inactive and GTP-bound active forms which are interconvertible by GDP/GTP exchange and GTPase reactions. The conversion from the GDP-bound inactive form to the GTP-bound active form is regulated by GDP/GTP exchange proteins and the reverse conversion is regulated by GTPase-activating proteins. There are two types of GDP/GTP exchange proteins for Rho; one is a stimulatory type, named Rho GDP dissociation inhibitor (GDI) (Kuroda et al., 1992; Regazzi et al., 1993), and the other is an inhibitory type, named Rho GDP dissociation inhibitor (GDI) (Fukumoto et al., 1994). We have recently demonstrated that a proto-oncogene product, Dbl, acts as a GDS for Rho (Yaku et al., 1994). Recently, a homologue closely related to Rho GDI has been found and shown to be expressed in hematopoietic cells (Lelias et al., 1993). Rho undergoes three kinds of posttranslational modifications in the COOH-terminal region: geranylgeranylation of the cysteine residue, removal of the three COOH-terminal amino acids, and carboxymethyl esterification of the exposed cysteine residue (Katayama et al., 1991). Rho GDP and GDI are active on only the posttranslationally processed form of RhoA (Hori et al., 1991; Mizuno et al., 1991), whereas Dbl is active on both the posttranslationally processed and unprocessed form of RhoA, but is much more active on the former than on the latter form (Yaku et al., 1994).

In the cytosol of resting smooth muscle and insulinoma cells, Rho is present in the GDP-bound inactive form complexed with Rho GDI (Kuroda et al., 1992; Regazzi et al., 1992), and the inhibitory action of Rho GDI in the GDP/GTP exchange reaction is stronger than the stimulatory action of Smg GDS, Rho GDS, or Dbl if both are present (Kikuchi et al., 1992; Kuroda et al., 1992; Yaku et al., 1994). On the basis of these observations, we have tentatively proposed the following modes of activation and action of Rho. In resting cells, the posttranslationally processed form of Rho is present in the cytosol as the GDP-bound inactive form complexed with Rho GDI. Upon stimulation with some signals, the inhibitory action of Rho GDI is released in an unknown manner, the GDP-bound inactive form of Rho becomes sensitive to the action of Smg GDS, Rho GDS, or Dbl, and the GTP-bound active form is produced. By this activation, Rho interacts with its effector protein and exerts its biological function through this effector protein.

A homologue of mammalian RhoA gene, RHO1, has been identified in S. cerevisiae (Madaule et al., 1987). The amino acid sequence of Rhop has ~70% identity with that of RhoA, and Rhop like RhoA has been shown to be ADP-ribosylated by Cε (McCaffrey et al., 1991). A gene disruption experiment on RHO1 indicates that RHO1 is an essential gene, but the cellular function of RHO1 remains to be clarified. In this report, we have isolated a temperature-sensitive mutation of the RHO1 gene. The Rho1 mutation results in accumulation of tiny- or small-budded cells at nonpermissive temperature. Consistently, immunofluorescence study demonstrates that Rhop is localized at the periphery of the bud emergence sites or of the tips of growing buds. Cortical actin patches are overlappingly clustered to the Rhop-staining sites. We describe the function of RHO1 in the process of bud formation.

Materials and Methods

Strains, Media, and Cell Growth Conditions

Yeast strains used in this study are listed in Table I. Escherichia coli DH5α was used for the construction and propagation of plasmids. Yeast strains were grown on a rich medium, YPD, which contains 1% Bacto-yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto-peptone (Difco), 2% glucose, 0.04% adenine, and 0.02% uracil. Yeast transformations were performed by lithium acetate methods (Ito et al., 1983; Gietz et al., 1992), and other standard yeast genetic manipulations were performed as described by Sherman et al. (1986). Transformants were grown in SD medium which contained 2% glucose and 0.7% yeast nitrogen base without amino acid.
Plasmid Constructions

Standard molecular biological techniques were performed to construct plasmids (Sambrook et al., 1989). The plasmid pWT consists of the wild-type 1.4-kbp EcoRI-ClaI restriction fragment containing RHO1 cloned into the URA3-bearing high copy number vector YEp352 (Madaule et al., 1984). The 2.2-kbp Sau3A-BglII genomic fragment contained 1.2 kbp upstream and 414 bp downstream noncoding regions. This fragment was cloned into the URA3-bearing single-copy vector pRS316 (Sikorski and Hieter, 1989) to construct a plasmid pRS316-RHO1. A 6-bp untranslated sequence in pRS316-RHO1, AGAAAG, is located immediately upstream of the RHO1 open reading frame, was changed to the KpnI site, GGTTAC, by use of the in vitro mutagenesis technique to construct a plasmid pRS316-RHO1 (KpnI) (Higuchi, 1989). Synthetic oligonucleotides encoding the HA epitope, YPYDVPDYA, which is derived from the influenza hemagglutinin protein, was cloned into the KpnI site of pRS316-RHO1 (KpnI) to construct pRS316-HA-RHO1, which has two copies of the HA epitope (Wilson et al., 1984; Field et al., 1988; Kolodziej and Young, 1991). The 2.2-kbp DNA fragment of the HA-RHO1 gene was cloned into the URA3-bearing multicopy vector, YEp352 (Hill et al., 1986), and integration vector, pRS305 (Sikorski and Hieter, 1989), to construct YEp5352-HA-RHO1 and pRS306-HA-RHO1, respectively. A plasmid pRS316-HA-RHO1 (C2000) was constructed from pRS306-HA-RHO1 (C2000) by changing Ura7 to C in the RHO1 open reading frame by use of the in vitro mutagenesis technique, which caused an amino acid substitution of cysteine 206 of Rho1p to serine. Expected changes of DNA sequence induced by in vitro mutagenesis or by the insertion of oligonucleotides were confirmed by DNA sequencing. The EcoRI-Sall PCR fragments containing the RHO1 and RhoA open reading frames were cloned into YCP-LEU2-GALI, which carried the LEU2 marker and the GALI promoter on a single copy plasmid, to construct YCP-LEU2-GALI-RHO1 and YCP-LEU2-GALI-RhoA, respectively.

Hydroxyamine Mutagenesis

The plasmid pWT was mutagenized in vitro by hydroxyamine as described (Rose and Fink, 1987). Briefly, 10 μg of pWT DNA was added to 500 μl of a fresh hydroxyamine solution (0.09 g NaOH and 0.35 g hydroxyamine·HCl in 5 ml ice-cold water), and the reaction mixture was incubated at 37°C for 20 h. Plasmid DNA was recovered by precipitation at -80°C with ethanol, and was transformed into a yeast strain TM2-1A. The rhol::HIS3 disruption mutation in TM2-1A was previously made by replacing the 460-bp EcoRI-ClaI fragment of RHO1 with the HIS3 DNA fragment (Madaule et al., 1987).

Construction of the rhol-104 Mutant

The genomic rhol::HIS3 disrupted gene was replaced by the rhol-104 gene by pop-in/pop-out replacement (Rotondo, 1991). The KpnI-Sall DNA fragment containing the rhol-104 mutant gene was cut out from pRS316-rho1-104 and was cloned into the KpnI-Sall site of an integration vector pRS306 (Sikorski and Hieter, 1989). The resulting plasmid pRS306-rho1-104 was cut at the Hpal site located 22 bp downstream of the RHO1 open reading frame and was subsequently transformed into TM2-1A. A transformant of TM2-1A in which pRS306-rho1-104 was targeted at the RHO1 locus was selected by Southern hybridization analysis. Since the recombination occurred downstream of the HIS3 insertion site, a second recombination upstream of HIS3 would give rise to a strain in which the rhol::HIS3 gene is replaced by the rhol-104 gene. The resulting strain would show a Ura-, His+, and temperature-sensitive growth phenotype. Cells of the resulting transformant were spread out on plates containing 5-FOA to select clones as described (Boeke et al., 1984). Among 5-FOA-resistant clones His+ clones were searched for and one of such clones, HNY21, was isolated. Southern hybridization analysis confirmed that the rhol::HIS3 gene was replaced by the rhol-104 gene in HNY21. The temperature-sensitive growth phenotype of HNY21 was suppressed by pRS316-RHO1.

Western Blot Analysis of Rho1p

HA-Rho1p proteins expressed in yeast cells were detected by Western blot analysis with 12CA5 mAb (W'dson et al., 1984; Field et al., 1988). Total yeast protein was extracted from exponentially growing cells by the method described previously (Kuchler et al., 1993). About 1 × 10⁶ cells grown in YPD medium were harvested by brief centrifugation, washed once in a lysis buffer (0.01 M Tris·HCl and 1 mM EDTA, pH 7.5, containing 2% 2-mercaptoethanol and 100 μM p-amidinophenyl methanesulfonfluoride·hydrochloride), and resuspended in 500 μl of the same cold lysis buffer. All subsequent steps were carried out at 4°C. About 1 g of prechilled glass beads (0.45-mm diam) were added to the cell suspension and lysis was achieved by vigorous vortex mixing for five 2-min intervals with 1 min of cooling in between. The resulting homogenates were collected and unbroken cells, glass beads, and large debris were removed twice by centrifugation for 5 min at 450 g. Membranes were collected from the clarified lysate by sedimentation for 1 h at 100,000 g. The particulate fraction was rinsed with the lysis buffer and resuspended in the same buffer. 60 μg of each protein sample from the homogenates and the cytosolic and particulate fractions was subjected to SDS-PAGE and separated proteins were electrophoretically transferred to a nitrocellulose membrane sheet (BA85, pore size: 0.45 μm; Schleicher & Schuell Inc., Keene, NH). The sheet was probed to detect HA-Rho1p with 10 μg/ml of affinity-purified 12CA5 mAb as a primary antibody by using the ECL detection kit (Amersham Corp., Arlington Heights, IL).

Cytological Techniques

For staining of actin and DNA in rhol mutants, cells cultured under the permissive or restrictive conditions were fixed with 4% of formaldehyde at 25°C for 30 min. After washing once with PBS, cells were stained with rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) at a final concentration of 0.5 μM for 30 min (Adams and Pringle, 1991). After washing three times with PBS, cells were costained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co.) at a final concentration of 0.1 μg/ml for 15 min. After washing twice with PBS, cells were observed and photographed on Neopan Super Presto film (Fuji Photo Film, Tokyo, Japan) using a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

To visualize the intracellular localization of HA-Rho1p, yeast cells expressing HA-Rho1p were processed for immunofluorescence microscopy by the methods of Pringle et al. (1991). Cells were fixed with 4% of formaldehyde at 25°C for 2.5 h. Affinity-purified 12CA5 mAb was used as a primary antibody at a concentration of 25 μg/ml and the FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, Malvern, PA) was used as a secondary antibody at a concentration of 10 μg/ml. After washing with PBS, cells were stained with rhodamine phalloidin. Stained cells were observed and photographed as described above.

Results

Isolation of a Temperature-sensitive Mutation in RHO1

The RHO1 gene has been demonstrated to be essential for vegetative growth of cells in S. cerevisiae (Madaule et al., 1987). To explore the function of the RHO1 gene, a conditional mutation of RHO1, which is a temperature-sensitive mutation, was isolated. An episomal plasmid bearing the RHO1 and URA3 genes, pWT-RHO1, was mutagenized with hydroxyamine as described in Materials and Methods. This mutagenized plasmid DNA was directly transformed into the yeast strain TM2-1A, which carried the rhol mutation disrupted with HIS3 and a plasmid, YCP-LEU2-GALI-RHO1, bearing the GALI-RHO1 gene and the LEU2 gene. TM2-1A grows on a galactose-containing medium but not on a glucose-containing medium since the GALI promoter is repressed by a glucose. Transformants were selected on SD-
uridine plate media at 24°C, and colonies were replica-plated onto SD-ura plate media which were subsequently incubated at 37°C for 2 d. From ~10,000 clones screened, candidate clones which showed temperature-sensitive growth phenotype were isolated, and the plasmids were recovered through *E. coli* transformation. One plasmid which conferred a temperature-sensitive growth phenotype on TM2-1A was identified and used for further study. The 1.4-kbp EcoRI-EcoRI restriction fragment carrying the mutant *rho1* gene was cloned into a single copy vector pRS316 (Sikorski and Hieter, 1989). This plasmid again conferred a temperature-sensitive growth phenotype on TM2-1A. The plasmid carrying the temperature-sensitive mutation of *RHO1*, *rho1-104*, did not retard growth of wild-type cells, indicating that the mutation was recessive.

The mutation site in *rho1-104* responsible for the temperature-sensitive trait was mapped to a 476-bp *Nhel-HpaI* restriction fragment in the *RHO1* open reading frame. DNA sequencing of this fragment of the mutant gene resulted in the identification of two transition mutations: both G214 and G491 in the *RHO1* open reading frame were changed to A. These mutations were consistent with the specificity of base changes which could be induced by the hydroxylamine mutagenesis as described (Sikorski and Boeke, 1991). The first mutation changed Asp 72 to Asn (D72N) and the second mutation changed Cys 164 to Tyr (C164Y). The D72N substitution occurred five amino acids downstream of one of the two conserved phosphate/Mg²⁺-binding loops, Asp-Thr-Ala-Gly-Gln, while the C164Y substitution occurred at X in Glu-X-Ser-Ala, which is one of the guanine base-binding loops (Valencia et al., 1991). We have not determined which substitution is responsible for the temperature-sensitive trait of the *rho1-104* mutation.

The genomic *rho1* gene was replaced with the *rho1-104* mutant gene by the pop-in/pop-out method as described in Materials and Methods. This strain, HNY21, showed a temperature-sensitive growth phenotype which could be suppressed by a plasmid bearing the wild-type *RHO1* gene. HNY21 or its derivatives were used for further experiments.

**Characterization of the rho1-104 Mutant**

Examination of time courses of cell growth of the wild type and the *rho1-104* mutant cells (HNY30) indicated that the growth of the *rho1-104* cells was retarded even at 24°C and was inhibited at 37°C (Fig. 1, A and B). Neither the cell number nor optical density of the culture significantly increased at 37°C in the *rho1-104* mutant culture, suggesting that the *rho1-104* cells had halted cell growth as well as cell division. To examine whether the arrested cells lose viability or not, viable cells were counted after temperature up-shift. As shown in Fig. 1 C, the *rho1-104* mutant cells lost viability rapidly when incubated at 37°C, suggesting that the deficiency of cell surface growth of the arrested *rho1-104* cells were mainly due to their loss of viability. Microscopic examination of the *rho1-104* cells growing at 24°C revealed that cells with a tiny or small bud accumulated compared with the wild-type cells: 48% of the *rho1-104* cells versus 38% of the wild-type cells (Fig. 2). In contrast, in the *rho1-104* cells arrested at 37°C, the population of tiny- or small-budded cells increased up to 80%, which presented a clear contrast to 34% in the wild-type control culture (Fig. 2). To confirm that cell surface growth was inhibited at 37°C, length of the long axes of tiny- or small-budded cells was measured in more than 500 cells of the *rho1-104* mutant and wild-type cells. The average cell length of the *rho1-104* cells grown at 24°C was not longer than that of the wild-type cells grown at 24°C and did not change after incubation at 37°C for 6 h (data not shown).

Cells of the *rho1-104* mutant and the *RHO1*-depleted mutant grown under the permissive conditions or arrested under nonpermissive conditions were stained with rhodamine phalloidin and DAPI to visualize actin and DNA, respectively, by fluorescence microscopy (Fig. 3). In both mutant cells arrested as tiny- or small-budded cells, cortical actin patches were concentrated to budding sites or in buds (Fig. 3, A and B, *Actin*) as described in tiny- or small-budded cells of the wild-type strain (Adams and Pringle, 1984; Kilmartin and Adams, 1984). Tiny-budded cells were more frequently...
seen in the rho1-104 cells (~70% of tiny-budded cells and 30% of small-budded cells) than in the RHO1-depleted cells (~30% of tiny-budded cells and 70% of small-budded cells) (data not shown). Staining with DAPI showed that the rho1-104 cells arrested at 37°C had a single nucleus (Fig. 3, A and B, DNA). To examine the state of nuclear DNA further, flow cytometric analysis was conducted to determine the DNA content of the arrested cells of the rho1-104 mutant (Fig. 4). Cells of the wild-type strain RAY3A-D or rho1-104 mutant strain HNY30 (rho1-104/rho1-104) growing exponentially at 24°C were further incubated at 24, 33, or 37°C for 5 h, 65, 45, and 40% of the wild-type cells showed 4-n peaks when incubated at 24, 33, and 37°C, respectively (Fig. 4 A). In contrast, 70, 95, and 65% of the rho1-104 mutant cells showed 4-n peaks when incubated at 24, 33, and 37°C, respectively. These results indicate that the nuclear cycle of the rho1-104 cells was not arrested in a cell cycle-specific manner at 37°C, but was arrested at the G2/M phase at 33°C.

Cell cycle arrest at the small-budded stage has been observed with a mutant in PKCl which is a yeast homologue of mammalian protein kinase C of an αβγ type (Levin and Bartlett-Heubusch, 1992). Since cell lysis phenotype has also been found in the pclk1 mutant, cell lysis of the rho1-104 or RHO1-depleted cells was examined by assaying alkaline phosphatase activity which was normally localized to vacuoles (Fig. 5). This method was used to detect the cell lysis phenotype of the pclk1 mutant (Paravicini et al., 1992). Substantial activity of alkaline phosphatase was detected in the RHO1-depleted cells incubated in YPD medium for 16 h (Fig. 5 B) and in the rho1-104 cells incubated at 37°C for 1 h (Fig. 5, C and D). Although cell lysis was not microscopically evident in the rho1 mutant cultures, the temperature-sensitive pclk1 mutant was also found to release intracellular materials without apparent cell lysis at the restrictive temperature (Levin and Bartlett-Heubusch, 1992). The lethality caused by the cell lysis phenotype of the pclk1 mutant has been found to be suppressed by an osmotic stabilizer such as 1 M sorbitol (Levin and Bartlett-Heubusch, 1992). In contrast, the lethality of the rho1 disruption mutant was not suppressed by the presence of 1 M sorbitol in the medium, although the rho1-104 temperature-sensitive mutant cells grew at 37°C in the presence of 1 M sorbitol (Fig. 6).

**Suppression of the Growth Deficiency of the rho1 Mutant by Expression of Mammalian RhoA**

Among the mammalian small G proteins, the mammalian RhoA protein is the most similar to the yeast Rho1p. For this purpose, human RhoA cDNA was cloned into a yeast single copy expression vector, YCp-LEU2-GAL1. The resulting plasmid, YCp-LEU2-GAL1-RhoA, expressed RhoA under control of the GAL1 promoter. A strain KTI10-1A carried the rho1 gene disrupted with HIS3 and the resulting lethality was suppressed by the presence of the plasmid pMM105, a URA3-bearing single copy plasmid containing the GAL1::RHO1 gene. KTI10-1A was transformed with YCp-LEU2-GAL1-RHO1, YCp-LEU2-GAL1-RhoA, or a vector plasmid, YCp-LEU2-GAL1. Transformants which grew on the SG-ura-leu medium at 30°C were streaked on the SG-ura-leu medium containing uracil and 5-FOA. The plate was subsequently incubated at 24°C for 4 d. Since 5-FOA inhibits the growth of Ura+ cells, only cells that had lost pMM105 should grow on the 5-FOA-containing medium. As shown in Fig. 7, cell growth on the 5-FOA-containing medium was seen with cells bearing YCp-LEU2-GAL1-RhoA as well as YCp-LEU2-GAL1-RHO1. Therefore, expression of RhoA suppressed the growth deficiency of the rho1 disruption mutant, although the suppression was not seen when incubated at 37°C (data not shown). Suppression of growth deficiency of the rho1 disruption mutant was also seen with a single copy plasmid carrying the RhoA gene under the control of the RHO1 promoter (data not shown). These results suggest that mammalian RhoA is functionally homologous to yeast Rho protein, in addition to the structural homology.

**Intracellular Localization of Rho1p**

Phenotypic analysis of the rho1-104 temperature-sensitive mutant and the RHO1-depleted mutants suggested that RHO1 is involved in the process of bud growth. Indirect immunofluorescence method was used to reveal the intracellular localization of Rho1p. For this purpose, oligonucleotides encoding the HA epitope of influenza hemagglutinin were added to the 5' end of the RHO1 gene. This HA-Rho1p tagged with the HA epitope was cloned into a single copy vector, pRS316, and an integration vector, pRS306, to construct plasmids pRS316-HA-RHO1 and pRS306-HA-RHO1, respectively. Plasmid pRS316-HA-RHO1 suppressed the temperature-sensitive growth phenotype of HNY30 (rho1-104/rho1-104) as well as the lethality of TM2-1A-D (rho1::HIS3/rho1::HIS3). The lethality of TM2-1A-D was also suppressed by integrating pRS306-HA-RHO1 at the rho1::HIS3 locus. These rho1 mutant cells expressing HA-RHO1 were morphologically indistinguishable from the wild-type cells. These results indicate that HA-Rhop functions as wild-type Rho1p.

Cells of three strains described above carrying the HA-RHO1 gene were grown at 30°C and processed for indirect immunofluorescence microscopic analysis. To visualize the HA-Rho1p protein, cells were stained with 12CA5 mAb and subsequently with the FITC-conjugated goat anti-mouse IgG antibody. The same preparation was doubly stained with rhodamine phalloidin to visualize actin. The results are shown in Fig. 8. Distribution of cortical actin patches at specific stages of cell cycle is well established in S. cerevisiae (Adams and Pringle, 1984; Kilmartin and Adams, 1984). In the small unbudded cells, cortical actin patches distributed randomly on the cell surface (Fig. 8, A, B, and C, 1, Actin) and the staining with 12CA5 mAb was seen in the cytosol (Fig. 8, A, B, and C, 1, HA-Rho1p). This is consistent with the result that the substantial portion of HA-Rho1p was present in the cytosolic fraction as estimated by Western blot analysis (see below). However, at least a part of the staining with 12CA5 was due to nonspecific staining, since 12CA5 mAb similarly stained the cytoplasm of cells which did not express the HA epitope (Fig. 8, A, B, and C, 1, Control). In the large unbudded cells, cortical actin patches were concentrated at the site where budding would occur (Fig. 8, A, B, and C, 2, Actin). In these cells, HA-Rho1p was found to be colocalized with cortical actin patches, although HA-Rho1p was stained uniformly at the periphery of cells (Fig.
Figure 3. Morphology of the rho1-104 (A) and the RHO1-depleted cells (B). Asynchronous culture of a diploid strain HNY30 (rho1-104/rho1-104) grown at 24°C was further incubated at 24°C or at 37°C for 3 h. Asynchronous culture of a diploid strain TM2-1A-D (rho1::His3/rho1::His3) grown at 30°C in YPGal medium was further incubated in YPGal or in YPD medium for 15 h. Fixed cells were then stained with rhodamine phalloidin (Actin) and with DAPI (DNA), and the stained cells were observed under a light (Phase contrast) or fluorescence microscope. Tiny buds of the rho1-104 cells arrested at 37°C are shown with arrowheads.
Figure 4. Flow cytometric analysis of the rhol mutant. Cells of the wild-type strain RAY-3A-D (A) or rhol-104 mutant strain HNY30 (B) growing at 24°C were further incubated at 24, 33, or 37°C for 5 h. Flow cytometric analysis of DNA contents was subsequently conducted on these cells.

8, A, B, and C, 2, HA-Rholp). In cells forming small buds, cortical actin patches were localized into the buds as well as to the budding sites (Fig. 8, A, B, and C, 3, Actin). In these cells, HA-Rholp was localized to the periphery covering the entire buds (Fig. 8, A, B, and C, 3, HA-Rholp). In cells with larger buds in which cortical actin patches were concentrated towards the bud tips (Fig. 8, A, B, and C, 4, Actin), staining of HA-Rholp was also dense around the bud tips (Fig. 8, A, B, and C, 3, HA-Rholp). It is known that cortical actin patches randomly redistribute throughout mother and daughter cells after the G2 phase, but before cytokinesis (Adams and Pringle, 1984; Kilmartin and Adams, 1984) (Fig. 8, A, B, and C, 5, Actin). Staining of HA-Rholp was hardly seen in these cells (Fig. 8, A, B, and C, 5, HA-Rholp). Following mitosis, cortical actin patches became clustered at the mother–bud neck region before cytokinesis (Fig. 8, A, B, and C, 6, Actin). HA-Rholp was localized again at the periphery of cells where cortical actin patches clustered in these cells (Fig. 8, A, B, and C, 6, HA-Rholp). The staining of HA-Rholp at the cytokinesis site raised a possibility that unbudded cells with HA-Rholp colocalized with cortical actin patches were in the postcytokinetic stage. To answer this question, the appearance of HA-Rholp at the presumptive budding site was observed on cells emerging from stationary phase. Cells of HNY30 carrying pRS316-HA-RHO1 were grown to stationary phase in YPD medium and released into fresh YPD medium for 1 h. Cells with HA-Rholp colocalized with actin patches at the presumptive budding site significantly increased after the 1-h incubation (data not shown). Therefore, it seems that HA-Rholp colocalized with actin patches in cells at the budding stage.

Among ~500 cells observed in each HA-Rholp–expressing strain, more than 70% of cells in large unbudded, small-budded, medium-budded, or cytokinetic cell cycle stage were stained at the growth site as shown in Fig. 8, while no such staining was observed in more than 500 observed cells of each strain carrying the RHO1 gene in place of the HA-RHO1 gene. Representatives of these control cells are shown (Fig. 8, A, B, and C, 1–6, Control).

Figure 5. Cell lysis phenotype of the rhol mutants. Wild-type strain RAY-3A-D (A), rhol disruption mutant TM2-1A-D (B), and rhol-104 temperature-sensitive mutants of a haploid strain HNY21 (C) and of a diploid strain HNY30 (D) were streaked on YPD plate, incubated at 24°C for 15 h, and then incubated further at 37°C for 1 h. The patches were stained with a chromogenic substrate, BCIP, of alkaline phosphatase for 15 h at 25°C.

Figure 6. Suppression of the temperature-sensitive growth phenotype of the rhol-104 cells by osmotic stabilizer. The wild-type strain RAY-3A-D (WT), rhol disruption mutant TM2-1A-D (rhol::HIS3), and rhol-104 temperature-sensitive mutant HNY30 (rhol⁰) were streaked on YPD and YPD+1 M sorbitol media and incubated at 37°C for 3 d. Residual growth of TM2-1A-D seems to be due to a time lag for the repression of the GAL1 promoter.

Fractionation of Rholp into Both Cytosolic and Particulate Fractions

Results shown in Fig. 8 suggested that at least a part of HA-Rholp might be localized to the plasma membrane. To exam-
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Requirement of Posttranslational Modifications for the Function and Localization of Rholp

Most small G proteins including Rholp undergo postranslational modifications including the attachment of lipid moieties to cysteine residues at the carboxyl terminus (Katayama et al., 1991; Qadota et al., 1992). To examine whether these modifications are important for the function and localization of Rholp, the cysteine residue at amino acid position 206 was changed to serine by use of the in vitro mutagenesis technique. The HA-rhol (C206S) gene was cloned into a single copy plasmid pRS316 and the resulting plasmid, pRS316-HA-rhol (C206S), was transformed into the strain HNY30 (rho1-104/rho1-104). The transformants did not grow at 37°C, indicating that HA-rhol (C206S)p was not functional. Cells of a transformant were grown in a rich medium at 24°C and were stained with rhodamine phalloidin and with 12CA5 mAb. As shown in Fig. 10 (rho1 [C206S]p, HA), no particular staining with 12CA5 was observed except that the cytoplasm was stained to a certain extent, suggesting that HA-rhol (C206S)p remained in the cytoplasm. Western blot analysis indicated that most of HA-rhol(C206S)p was present in the cytosolic fraction (Fig. 9 C, lanes 2 and 3). These results indicate that the postranslational modifications of Rholp are essential for Rhopl to localize to the site of cell growth, where, most probably, Rhopl performs its function.

A Saturable Binding Site for Rholp at the Growth Site

The results described above suggest that there may be a specific binding site for Rhopl at the plasma membrane in the sites of cell growth. To examine whether the binding site is saturable, we transformed HNY30 with a multicopy plasmid YEp352-HA-Rho1, which overexpressed HA-Rho1. Although very high expression of RHO1 by a strong promoter was detrimental to the growth of cells, overexpression with the RHO1 promoter on a multicopy plasmid affected neither the rate of cell growth nor the cell morphology (data not shown). Western blot analysis demonstrated that HA-Rho1 was overexpressed about three- to fivefold both in the cytosolic and particulate fractions (Fig. 9 D, lanes 2 and 3). The increase of the overexpressed HA-Rho1 in the cytosolic fraction might not simply be due to the incomplete postranslational modifications of HA-Rho1, because HA-Rho1 fully underwent the postranslational modifications in the HA-RHO1 overexpressing strain, as estimated by the SDSPAGE method described previously (Mizuno et al., 1991), in which the postranslationally modified RhoA migrates faster than the postranslationally unmodified RhoA. Transformants were processed for indirect immunofluorescence microscopy with 12CA5 mAb and for staining with rhodamine phalloidin. As shown in Fig. 10 (Rholp, overproduced), overexpression of HA-RHO1 did not significantly affect the distribution of cortical actin patches (Actin), while the staining of HA-Rho1 was seen at the almost entire periphery of cells as well as in the cytoplasm irrespective of cell cycle stage (HA). This result suggests that the overexpressed Rhopl which was recovered as the particulate fraction nonspecifically bound to the plasma membrane region. Therefore, the Rhopl-binding site at the growth site seems to be saturable and there may be a specific protein which binds to Rhopl at the sites of cell growth.

Discussion

Isolation of a temperature-sensitive mutation of the RHO1 gene has revealed that RHO1 is required for the budding process. The rho1-104 cells were arrested in the cell cycle as tiny- or small-budded cells and no cell surface growth occurred after arrest. Since the phenotype was seen in the RHO1-
Figure 8.

The Journal of Cell Biology, Volume 125, 1994

A

Actin   HA-Rho1p   Control

1

2

3

4

5

6

Figure 8.
Figure 8.
Although the thesis, but not nuclear division, as the restrictive temperature (Hartwell, 1971; Pringle and Hartwell, 1981). The morphological phenotype of rho1-104 was different from those of previously described cdc mutants: many of the cdc mutants that have entered the cell division cycle continue cell growth after division has been arrested (Hartwell et al., 1974; Pringle and Hartwell, 1981). Flow cytometric analysis revealed that the nuclear cycle of the rho1-104 mutant was not arrested in a cell cycle-specific manner at 37°C, but was arrested at the G2/M phase at 33°C. We favor a hypothesis that the rho1-104 mutation indirectly caused the arrest of the nuclear cycle, because Rholp was localized at the sites of cell growth, not around the nuclei. Arrest of the nuclear cycle and cell growth in the rho1-104 cells may be caused by the rapid loss of viability at the restrictive temperature.

We have noted that the phenotypes of the rho1 mutant are similar to those of the two previously described mutants, cdcl and pkcl. The cdcl mutant arrests its cell division with un budded or tiny-budded cells and loses cell viability rapidly at the restrictive temperature (Hartwell, 1971; Pringle and Hartwell, 1981). The cdcl mutant could complete DNA synthesis, but not nuclear division, as the rho1-104 mutant arrested at 33°C (Hartwell, 1971; Pringle and Hartwell, 1981). Although the RHO1 gene did not complement the cdcl mutation even on a multicopy plasmid (data not shown), CDC1 might encode a protein which functions in the RHO1-mediated signalling pathway. The PKCl gene encodes a yeast homologue of mammalian protein kinase C of αβγ type (Levin and Bartlett-Heubusch, 1992). Cells of the galactose-dependent pkcl mutant arrests as small-budded cells which are not detectably enlarged. It has been also shown that temperature-sensitive pkcl mutant cells lose their viability at the restrictive temperature as rapidly as the rho1-104 mutant cells did. This rapid loss of viability in the pkcl mutant has been shown to be caused by cell lysis which could be suppressed by osmotic stabilizer (Levin and Bartlett-Heubusch, 1992). We have noted that the rho1-104 and RHO1-depleted mutants also show the cell lysis phenotype under the restrictive conditions. Recently, we have isolated a gene which suppresses the temperature-sensitive growth phenotype of the rho1 mutant at 30°C on a multicopy vector. DNA sequencing of this gene has revealed that it is identical to PPZ2, encoding a type 1 protein phosphatase (T. Musha, K. Tanaka, H. Nonaka, and Y. Takai, manuscript in preparation). The PPZ2 gene has been also isolated as a multicopy suppressor of the pkcl mutant (Lee et al., 1993), indicating that RHO1 is functionally relevant to PKC1. One major phenotypic difference between rho1 and pkcl mutants is that cell growth of a gene disruptant of PKC1, but not of RHO1, is supported by osmotic stabilizer such as 1 M sorbitol. RHO1 plays an essential role in the bud growth and a part of the RHO1 function may be related to the osmotic integrity which is controlled by the PKC1 gene.

Epitope tagging method enabled us to demonstrate the intracellular localization of Rholp. Rholp was stained uniformly at the sites of cell growth where cortical actin patches clustered. Consistently, Rholp was found to be localized at the tip of shmoo-like region in the cdc28-13 mutant cells incubated at 37°C (data not shown). In contrast, the rho1-104(C206S) mutant protein seemed to remain in the cytoplasm. The rho1(C206S) protein has an amino acid substitution at the cysteine residue that receives posttranslational modifications which are important for membrane binding of GTP-binding proteins. Therefore, Rholp appears to bind to the plasma membrane or vesicles near the plasma membrane.

We have recently shown that the posttranslational modifications are also required for a small G protein to activate its target protein. The posttranslational modifications are essential for Rac to activate the superoxide generation (Ando et al., 1992) and for Ras to activate adenylyl cyclase in S. cerevisiae (Horiuchi et al., 1992) and MAP kinase in Xenopus laevis (Itoh et al., 1993). Therefore, there seems to be a target protein for Rholp at the sites of cell growth. It has previously been reported by immunofluorescence microscopy that Rholp is detected as a punctate pattern, with the signal concentrated toward the cell periphery and in the buds (McCaffrey et al., 1991). These stained regions have been
Figure 10. Intracellular localization of HA-rhol (C206S) mutant protein and overexpressed HA-Rholp. HNY30 (rho1-104/rho1-104) cells bearing the HA-rhol (C206S) gene on a single copy plasmid (rhol(C206S)p) or the HA-RHOI gene on a multicopy plasmid (Rholp, overproduced) were fixed and costained with rhodamine phalloidin (Actin) and with 12CA5 mAb (HA) as described in Materials and Methods. Cells at various cell cycle stages (1–6) as in Fig. 8 are shown.

We do not know the reason for this discrepancy, the punctate staining pattern of Rholp observed by McCaffrey et al. (1991) might be due to cross-reaction of the polyclonal anti-Rholp antibody with Rholp-like protein, such as Cdc42p, Rho2p, Rho3p, or Rho4p. In mammalian cells, it has been proposed to be post-Golgi vesicles by subcellular fractionation experiments (McCaffrey et al., 1991). HA-Rholp was also stained at the peripheral regions of buds in our immunofluorescence microscopic study. However, we did not see any vesicle-like staining pattern of HA-Rholp. Although...
demonstrated that RhoA is present in the both cytosolic and particulate fractions as in yeast cells, and immunofluorescence microscopy has revealed that RhoA is present in the cytoplasm and that no particular intracellular regions have been stained (Adamson et al., 1992). Further detailed studies are required to know whether RhoA is present in any particular intracellular space(s).

Western blot analysis demonstrated that ~70 and 30% of HA-Rho1ps were present in the 100,000 g cytosolic fraction and in the particulate fraction, respectively. In mammalian cells, the cytosolic RhoA has been found to be complexed with Rho GDI, which is a GDI for GTP-binding proteins of the Rho family including RhoA (Kuroda et al., 1992; Regazzi et al., 1992). Thus, we presume that the cytosolic Rho1p may be complexed with a counterpart of Rho GDI in yeast. In fact, we have recently found that there is a substantial Rho GDI-like activity in the cytosolic fraction of yeast cells (T. Masuda, K. Tanaka, H. Nonaka, W. Yamochi, A. Maeda, and Y. Takai, manuscript in preparation). If the cytosolic Rho1p is complexed with Rho GDI, it might be in the GDP-bound form, since Rho GDI preferentially interacts with the GDP-bound form of Rho proteins (Hori et al., 1991; Mizuno et al., 1991; Takai et al., 1992). On the contrary, Rho1p localized at the sites of cell growth should be in the GTP-bound form, since it is well established that small G proteins are active in the GTP-bound form and are inactive in the GDP-bound form. It may be noted that McCaffrey et al. (1991) has reported that ~20 and 80% of Rho1ps are present in the 100,000 g cytosolic fraction and in the particulate fraction, respectively. This result is inconsistent with our result that HA-Rho1ps was mostly present in the cytosolic fraction. The exact reason for the discrepancy between their and our results is not known at present, but our result is more likely because of the following two reasons: (a) we have previously shown that the substantial amount of Rho1ps is present in the cytosolic fraction in a complex with Rho GDI in bovine aortic smooth muscle (Kawahara et al., 1990; Kuroda et al., 1992); and (b) McCaffrey et al. (1991) have quantitated Rho1p by measuring the ADP-ribosylation of Rho1p. We have previously shown that the ADP-ribosylation of Rho1ps is inhibited by Rho GDI, which is present in the cytosolic fraction in a complex with Rho GDI (Kikuchi et al., 1992). Therefore, McCaffrey et al. (1991) might underestimate the amount of cytosolic Rho1p.

It has not been clarified to which protein Rho1p is bound at the sites of cell growth. This protein, which might be a target protein of Rho1p, was clearly limited in amount since overexpressed Rho1p was nonspecifically localized to the periphery of cells. How is Rho1p localized to the sites of cell growth? We presume that Cdc42p may play a crucial role in this process. A temperature-sensitive mutant defective in the target protein of Rho1p, was clearly limited in amount since unbudded cells that often contain two or more nuclei. Conversely, staining of cdc42 mutant cells with rhodamine phalloidin results in an uniform distribution of cortical actin patches (Adamson et al., 1990). We stained the cdc42 mutant cells expressing HA-Rho1p with 12CA5 mAb and found that HA-Rho1p was not localized to any specific site of arrested cells (data not shown). Therefore, Cdc42p appears to regulate the localization of Rho1p or the Rho1p-binding protein.

Based on the results of immunofluorescence microscopy of Rho1p and of phenotypic analysis of the rhol-104 mutant, we presume that Rho1p plays an important role, directly or indirectly, in the process of localized cell growth, including vesicle fusion to the plasma membrane or cell wall growth at the bud tips. Since cell polarity was established in the arrested rhol mutant cells, Rho1p seemed to function downstream of Cdc42p. Another phenotypic difference between cdc42 and rhol mutants was that rhol mutants halted cell growth under the restrictive conditions, probably due to cell death. A possible cause for cell death may be lysis of the rhol mutant cells, whose molecular mechanism remains to be elucidated. Rho1p was also stained in cells prior to or during cytokinesis. In these cells, staining was observed at the plasma membrane region at the mother–bud neck, where cortical actin patches were also concentrated. This result may suggest that Rho1p plays a role in localized cell growth accompanying cytokinesis. In accordance with this, we have recently shown that the microinjection of C3 or Rho GDI inhibits the cytokinesis in Xenopus embryo (Kishi et al., 1993). However, the rhol-104 cells arrested at nonpermissive temperature did not accumulate cells with large buds. Further investigations are needed with respect to the role of Rho1p in cytokinesis.

Suppression of the rhol-104 mutation by the expression of Rho1s suggests that the function of Rha1s is conserved in lower eukaryotes such as yeast. Although the precise mechanism has not been established yet, Rha1s has been proposed to be involved in the reorganization of actin stress fibers in mammalian cells (Ridley and Hall, 1992). The GTP-bound form of RhoA rapidly stimulates the stress fiber and focal adhesion formation when microinjected into serum-starved Swiss 3T3 cells. Colocalization of Rho1p with cortical actin patches raises a possibility that the function of Rho1p is also somehow concerned with the actin cytoskeleton system in yeast. Although cortical actin patches were concentrated to tiny or small buds in the rhol-104 cells arrested at nonpermissive temperatures, it remains possible that Rho1p is involved in the reorganization of the actin system after the cell polarity has been established. Identification of genes functionally interacting with RHO1 may reveal a relationship between RHO1 and the actin cytoskeleton system.

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