Genetic Analysis of the Saccharomyces cerevisiae RHO3 Gene, Encoding a Rho-Type Small GTPase, Provides Evidence for a Role in Bud Formation

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ABSTRACT

RHO3 encodes a Rho-type small GTPase of the yeast Saccharomyces cerevisiae. We isolated temperature-sensitive alleles and a dominant active allele of RHO3. Ts rho3 cells lost cell polarity during bud formation and grew more isotropically than wild-type cells at nonpermissive temperatures. In contrast, cells carrying a dominant active mutant RHO3 displayed cold sensitivity, and the cells became elongated and bent, often at the position where actin patches were concentrated. These phenotypes of the rho3 mutants strongly suggest that RHO3 is involved in directing the growing points during bud formation. In addition, we found that SRO6, previously isolated as a multicopy suppressor of rho3, is the same as SEC4. The sec4-2 mutation was synthetic lethal with temperature-sensitive rho3 mutations and suppressed the cold sensitivity caused by a dominant active mutant RHO3. The genetic interactions between RHO3 and SEC4, taken together with the fact that the Rab-type GTPase Sec4p is required to fuse secretory vesicles together with plasma membrane for exocytosis, suggest a model in which the Rho3p pathway modulates morphogenesis during bud growth via directing organization of the actin cytoskeleton and the position of the secretory machinery for exocytosis.

SMALL GTPases of the Ras superfamily act as molecular switches through their conformational change between the GTP-bound active form and GDP-bound inactive form of the proteins (Barral 1987; Bourne et al. 1991; Boguski and McCormick 1993). In the yeast Saccharomyces cerevisiae, three Rho-type GTPases of the Ras superfamily, Cdc42p, Rho3p, and Rho4p, participate in the morphogenetic event of bud formation (Adams et al. 1990; Johnson and Pringle 1990; Matsui and Toh-e 1992a,b). In bud formation, cell polarity is established and the cytoskeleton is reorganized. Patches of actin filaments become concentrated at the bud site, to which transport of secretory vesicles is directed for the construction of the daughter cell surface (Tkačz and Lampen 1973; Field and Schekman 1980; Pringle and Hartwell 1981; Cabib et al. 1982; Adams and Pringle 1984; Kilmartin and Adams 1984; Novick and Botstein 1985; Pringle et al. 1986; Drubin 1991; Johnston et al. 1991). Loss of Cdc42p function disrupts the asymmetric localization of actin filaments and causes cells to become unbudded, large, and round (Adams et al. 1990; Johnson and Pringle 1990; Ziman and Johnson 1994). Cells carrying a dominant active CDC42 mutation (e.g., CDC42<sup>A151T</sup>) initiate bud emergence independent of the cell cycle and cells with multiple buds are accumulated (Ziman et al. 1991). These facts suggest that Cdc42p is required for cell polarity establishment at the initiation of bud emergence.

Cell deleted for RHO3 grow very slowly, and simultaneous disruption of RHO4 enhances the growth defect, whereas disruption of RHO4 alone does not inhibit cell growth. In addition, RHO4 is a multicopy suppressor of rho3 (Matsui and Toh-e 1992a), suggesting that Rho4p possesses a Rho3p-related function. Δrho3 Δrho4 cells, where both Rho3p and Rho4p are depleted using a conditionally expressed promoter, undergo lysis with small buds (Matsui and Toh-e 1992b). These facts suggest that Rho3p and Rho4p are required for bud growth. The genes SRO1 ~ SRO9 were previously isolated as multicopy suppressors of rho3. SRO2 is the same as CDC42 and SRO1 is identical to BEM1 (Matsui and Toh-e 1992b). BEM1 encodes a SH3 domain-bearing protein required for cell polarization both during bud formation and during the formation of mating projections (Bender and Pringle 1991; Chant et al. 1991; Cheever et al. 1992). The genetic interactions of RHO3 with BEM1 and CDC42 suggest that RHO3 is involved in development or maintenance of cell polarization.

During bud formation, the function of the exocytic machinery is restricted to the site of cell-surface growth (Tkačz and Lampen 1972; Field and Schekman 1980). SEC4 encodes a Rab-type GTPase of the Ras superfamily and is essential for exocytosis at the step of fusion of secretory vesicles with the plasma membrane (Novick and Schekman 1979; Salminen and Novick 1987). Not surprisingly, Sec4p is concentrated in the growing bud (Salminen and Novick 1989), and it may play a pivotal role in exocytosis by controlling a plasma membrane-associated complex including other essential components for exocytosis, such as Sec8p and Sec15p (Salminen and Novick 1989; Bower and Novick 1991).

In this study, we identified and characterized Ts mutants and a dose-dependent dominant active mutant of RHO3. In addition, we show genetic interaction between RHO3 and SEC4, which establishes a connection...
between components necessary for cell polarization and components of the exocytic apparatus.

**MATERIALS AND METHODS**

**Microbiological techniques:** Yeast transformations were performed by the method of ITO *et al.* (1985). Rich medium containing glucose (YPD) and synthetic minimal medium (SD) were as described (SHERMAN *et al.* 1986). SC contains 0.5% casamino acid (Difco) and 100 mg/l each of uracil, adenine sulfate, and tryptophan in SD. YGal and SCGal are YPD and SC, respectively, except that 2% glucose is replaced with 5% galactose and 0.3% sucrose. SC-U and SCGal-U are SC and SCGal, respectively, lacking uracil. SC-T is SC lacking tryptophan.

**Strains, plasmids, and replacement of RHO3:** The yeast strains used are listed in Table 1. Plasmids pRS314-RHO3 and pRS316-RHO3 carry the 1.8-kb *KpnI-XhoI* fragment (Figure 1) of *RHO3* in centromeric plasmids pRS314 and pRS16 (SIKORSKI and HIER (1989), respectively. Plasmid KS' *RHO3* carries the 1.8-kb *KpnI-XhoI* fragment of *RHO3* in pBluescript KS' (Stratagene). Plasmids pSR06, pSR06-1, pSR06-2, and pSR06-3, carrying *SRO6*, were isolated previously (MATSUI and TOH-E 1992a) from a yeast genomic library based on the multiple-copy-plasmid YEp24 (CARLSON and BOTSTEIN 1982). pSR06 was digested with *SmaI* and *BstXI* and ligated after blunting the *BstXI* overhang to construct pSR06ΔB. pSR06-3 was digested with *PvuII* and ligated to construct pSR06ΔA. The 3.5-kb *SacI-SalI* fragment from pSR06-3 was inserted into pBluescript KS', digested with *HindIII*, and ligated after blunting the *HindIII* overhang to construct pKSSRO6AH. To construct pKSSRO6AH, the 3.5-kb *SacI-SalI* fragment from pKSSRO6AH was inserted into pYO326 (OHTA *et al.* 1991), a high-copy-number plasmid that harbors the 2μm plasmid ori-

### Table 1

**Yeast strains used in this study**

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| YJR3-5A       | MATa rho3::rho4 2pm plasmid carry the l.8kb *KpnI-XhoI* fragment (Figure 1) of *RHO3* in centromeric plasmids pRS314 and pRS16 (SIKORSKI and HIER (1989), respectively. Plasmid KS' *RHO3* carries the 1.8-kb *KpnI-XhoI* fragment of *RHO3* in pBluescript KS' (Stratagene). Plasmids pSR06, pSR06-1, pSR06-2, and pSR06-3, carrying *SRO6*, were isolated previously (MATSUI and TOH-E 1992a) from a yeast genomic library based on the multiple-copy-plasmid YEp24 (CARLSON and BOTSTEIN 1982). pSR06 was digested with *SmaI* and *BstXI* and ligated after blunting the *BstXI* overhang to construct pSR06ΔB. pSR06-3 was digested with *PvuII* and ligated to construct pSR06ΔA. The 3.5-kb *SacI-SalI* fragment from pSR06-3 was inserted into pBluescript KS', digested with *HindIII*, and ligated after blunting the *HindIII* overhang to construct pKSSRO6AH. To construct pKSSRO6AH, the 3.5-kb *SacI-SalI* fragment from pKSSRO6AH was inserted into pYO326 (OHTA *et al.* 1991), a high-copy-number plasmid that harbors the 2μm plasmid ori-

All strains listed above, except for ANS4-8C, YJS-2A, and YJR34 strains, are isogenic to YPH499 except for the indicated genotype. YJS-2A and YJR34 strains are congenic to YPH499 except for the indicated genotype.
Yeast rho3 Mutants

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Figure 1.—Mutations in H-ras and RH03 (above) and the restriction map of RH03 (below). The coding regions are represented by boxes. GTPase domains and CAAX motif are represented by black boxes. The KpnI site in the multicloning site of the original RH03 clone (Matsui and Toh-e 1992a) is shown. B, BamHI; C, BclI; E, EcoR1III; H, HindIII; K, KpnI; V, EcoRV; and X, XhoI.

The mutant rho3 alleles, rho3<sup>Val-25</sup>, rho3<sup>Val-30</sup>, rho3<sup>Val-47</sup>, and rho3<sup>Val-131</sup> were constructed with the oligonucleotide-directed in vitro mutagenesis technique (Amersham) using pRS314-RH03 as a template and the following primers: 5'-TTGGGCGAC-CAATGCTGGTGTG (rho3<sup>Val-25</sup>), 5'-GGCTCGTCTTTGTTAAGAT (rho3<sup>Val-30</sup>), 5'-GGGCTTGGCGAAGAAT (rho3<sup>Val-47</sup>) and 5'-GGGCTGTTGGCGAACCA (rho3<sup>Val-131</sup>). The EcoR1 site in the coding region (3'-noncoding region of rho3) and the EcoR1 site (3'-noncoding region of rho3) were replaced with the corresponding sequence of rho3<sup>Val-25</sup>, rho3<sup>Val-30</sup>, rho3<sup>Val-47</sup> and rho3<sup>Val-131</sup>, respectively, rho3<sup>Val-30</sup>, Ala<sup>Val-30</sup> and rho3<sup>Val-47</sup>, respectively, were inserted into the sequence downstream of the HindIII site (Figure 1) of pRS306 (Sikorski and Hieter 1988).

The mutant rho3 alleles were constructed 1.4-kb KpnI-EcoRI fragments carrying rho3<sup>Val-25</sup> and rho3<sup>Val-131</sup> into pY0324 (for overexpression) and pY13 (for replacement).

The RH03 alleles under the control of the GAL7 promoter (pGAL7::rho3) were constructed as follows. The complete RH03 coding region was amplified from each of the mutated rho3 genes by PCR using the two convergent primers, 5'-CCGAGATCTCAATCAGCGCCTTTAGTT-3' and 5'-CCGAGATCTCAATCAGCGCCTTTAGTT-3' of the coding region and noncoding region of RH03 and respectively.

For the construction of multicopy plasmids carrying RH03 and mutated rho3 alleles, the 1.8-kb KpnI-XhoI fragment (Figure 1), carrying RH03 or one of the mutated rho3 alleles, was inserted between KpnI and XhoI sites of pY0324 (Ohya et al. 1991), a high-copy-number plasmid that harbors the 2-microm, but grows well on galactose-containing medium, but grows poorly on glucose-containing medium by the method of Sanger et al. (1977).

Suppression of rho3: A rho3 disruptant (strain YMR505), which carries pGAL7::RHO4 (RHO4 under the control of the GAL7 promoter), grows poorly on glucose-containing medium, but grows well on galactose-containing medium because rho4 can serve as a multicopy suppressor of rho3 (Matsui and Toh-e 1992a,b). YMR505 with a multicopy plasmid based on Yep24 (Botstein et al. 1979) or pY0326 (Ohya et al. 1991), growing on Sgal-U, was streaked on SC-U or YPD and incubated for 3 days at 30°.

Isolation of a temperature-sensitive allele of RH03: pRS314-RH03 was mutagenized by treatment with hydroxylamine as described by Hashimoto and Sekiguchi (1976). Two micrograms of DNA were incubated with 3.6 ml hydroxylamine hydrochloride at 37°, and fractions were recovered during the incubation of 24–48 hr. The treated DNA was introduced into Escherichia coli to amplify the DNA. The amplified DNA was introduced into Δrho3 Δrho4 pGAL7::RHO4 cells (strain YMR510). The resulting transformants were streaked on two YPD plates, and one plate was incubated at 25° and the other at 37°. Transformants that grew at 25° but not at 37° on YPD were selected and plasmids were recovered from the transformants. The recovered plasmids were reintroduced into YMR510 to test whether the plasmid conferred the Ts phenotype on YPD plates. RH03 in the wild-type strain YPH501 and the Δrho4 strain YMR402 was replaced with rho3 from each of the candidate plasmids using pY13 (see above) by replacement transformation. When the Trp<sup>+</sup> segregants from the transformants were streaked on YPD plates, the Δrho4 phenotype, we judged that the rho3 allele used in the transformation was a temperature-sensitive allele of RH03.

Anti-Rho3p antiserum: EcoRI-EcoRI fragment from pOPR3
(Matsui and Toh-e 1992a), carrying the RHO3 coding region, was inserted into the EcoRI site of pGEXKG vector for production of GST-fused Rho3p in E. coli. Purification of the GST-fused protein was performed as described previously (Shirama et al. 1995). The purified GST-fused Rho3p was used as an antigen to raise anti-Rho3p antibodies in rabbits.

**Morphological observations:** Cells were stained with rhodamine-phalloidin (to reveal actin filaments), and 4′,6-diamidino-2-phenylindole (DAPI) (to reveal DNA) as described (Pringle et al. 1989). Samples were observed with an epifluorophotomicroscope (Olympus BH-2).

**Assay for the secretion of invertase:** The assay method was described by Goldstein and Lampen (1975). rho3 cells, sec4-2 cells (for the control of a secretion-deficient strain), and wild-type cells were cultured in YPD with 5% glucose, harvested, and inoculated into YPD that contained 0.2% glucose instead of 2% and incubated at nonpermissive temperatures. Aliquots of the culture were fractionated after 0, 1, 2, 3, and 4 hr from the inoculation and activities of invertase were measured.

## RESULTS

**Identification of temperature-sensitive rho3 mutants:** Δrho3 cells grow very slowly at 25, 30, and 37°C, whereas Δrho3 Δrho4 cells do not grow at 30°C or above (Matsui and Toh-e 1992a; data not shown). Therefore we screened temperature-sensitive alleles of RHO3 in a Δrho3 Δrho4 background. As a result, a temperature-sensitive allele of RHO3, designated rho3-1, which conferred temperature-sensitive growth on Δrho3 Δrho4 cells when introduced, was isolated by random mutagenesis with hydroxylamine. rho3-1 Δrho4 cells (strain YMR3732-5D) did not grow at 37°C and, unexpectedly, rho3-1 RHO4 cells (strain YMR3732-2B) also did not grow at 37°C (Figure 2A; see DISCUSSION). Disruption of RHO4 enhanced the Ts+ phenotype of rho3-1 cells; the rho3-1 Δrho4 strain YM3732-5D did not grow at 34°C whereas the rho3-1 strain YMR3732-2B grew poorly at 34°C (data not shown). The Ts+ growth phenotype of rho3-1 cells was recessive since introduction of RHO3 on a centromeric plasmid suppressed the Ts+ phenotype of the rho3-1 strain (data not shown).

Determination of the nucleotide sequence of the rho3-1 coding region revealed that nucleotide 593 on the coordinate of the RHO3 sequence (Matsui and Toh-e 1992a) was mutated from guanine to adenine. This mutation resulted in the substitution of glycine 198 for aspartate (Figure 1). The replacement of the sequence between the BstI and BamHI sites (Figure 1) in the wild-type RHO3 with the corresponding segment of the rho3-1 mutant gene resulted in a temperature sensitive allele of RHO3 (data not shown). This indicated that this mutation is responsible for the Ts+ phenotype. The substituted residue is not located in the conserved motifs for binding and hydrolysis of GTP, but it is highly conserved within the Ras superfamily. The corresponding residue in all members belonging to the Ras superfamily, with the exception of human K-ras2A, is glycine or asparagine. It is possible that this residue is important for the stability of small GTPases at elevated temperatures.

The cysteine residue in the CAAX motif at the C-terminus of the Ras-type and Rho-type GTPases is modified by prenylation and is important for the function of the GTPases (Barbacid 1987; Ziman et al. 1991). The C-terminal sequence of Rho3p, Cys-Thr-Ile-Met, corresponds to this motif (Figure 1). We constructed rho3<sup>30228</sup>, which encodes Rho3p terminating in Ser instead of Cys-Thr-Ile-Met (Figure 1). A rho3<sup>30228</sup> strain (YJR5-5A) grew normally at 25°C, but did not grow at 37°C (Figure 2A). Disruption of RHO4 enhanced the Ts+ phenotype of rho3<sup>30228</sup> cells; the rho3<sup>30228</sup> Δrho4 strain YJR5-5B did not grow at 34°C whereas the rho3<sup>30228</sup> strain YJR5-5A grew poorly at 34°C (data not shown). The Ts+ phenotype was recessive since YJR5-5A carrying RHO3 on a centromeric plasmid was Ts+ (data not shown).

As both rho3-1 and rho3<sup>30228</sup> were recessive alleles and overexpression of either alleles from the GAL1 promoter did not confer any dominant effect on cell growth (data not shown), we conclude that both rho3-1 and rho3<sup>30228</sup> mutations are loss-of-function mutations. These results indicate that the CAAX motif of Rho3p is required at elevated temperatures.
Western-blot analysis using anti-Rho3p antibodies revealed that the amounts of Rho3p in both the rho3-1 and rho3-22 strains were reduced at 37°C, suggesting that the reduced amounts of Rho3p might confer the temperature sensitivity of the rho3 strains. However, both the rho3-1 strain carrying rho3-1 on a multicopy plasmid and the rho3-22 strain carrying rho3-22 on a multicopy plasmid produced larger amounts of Rho3p than did the wild-type strain at 37°C. The temperature sensitivity of these strains were slightly weakened, comparing with the Ts- rho3 strains without the plasmids, but yet these rho3 strains with the plasmids still displayed the temperature-sensitive phenotype (data not shown). These results indicate that reduction of the Rho3p activity at nonpermissive temperature, rather than the reduced amount of Rho3p, is responsible for the temperature sensitivity of the rho3 strains.

The morphology of temperature-sensitive rho3 cells at nonpermissive temperatures: For elucidating the role of Rho3p, we observed the morphology of the Ts- rho3 mutant cells at nonpermissive temperatures. In this study, we examined the phenotypes of Ts- rho3 strains in the Δrho4 background, as well as in the RHO4 background, because it is possible that the Rho3p-related activity from RHO4 might affect the phenotypes of the Ts- rho3 strains.

Growth of both the rho3-1 Δrho4 strain YMR372-5D and the rho3-22 Δrho4 strain YJR3-5B arrested after about 6–8 hr from a shift to 37°C (Figure 2, B and C), and the viability of these cells began to be reduced at this time (data not shown). These Ts- rho3 Δrho4 cells became enlarged and rounded 6 hr after the shift to 37°C (Figure 3, B and E), and actin patches lied scattered throughout the cell surface and few actin cables were observed (Figure 3, D and G). In contrast, in the rho3 Δrho4 cells at 25°C, actin patches were concentrated in the bud and actin cables ran through the mother cell (data not shown) as observed in wild-type cells (Figure 3A; ADAMS and PRINGLE 1984; KILMARTIN and ADAMS 1994). Similar results were obtained with cells of the RHO4 background (i.e., using rho3-1 strain YMR372-2B and rho3-22 strain YJR3-5A), except that these rho3 strains required longer incubation (9–11 hr) at 37°C for cell growth arrest (data not shown). These observations indicate that the rho3 cells lost cell polarity at nonpermissive temperatures and grew more isotropically than wild-type cells. During incubation of the Ts- rho3 strains (strains YMR372-5D, YJR3-5B, YMR372-2B, and YJR3-5A) at 37°C, the fractions of unbudded cells, small-budded cells, and large-budded cells all were about 28–36% of total cells and the proportions did not vary noticeably. In this regard, the phenotype is different from that of mutants affected in bud emergence (e.g., the cdc42 mutant), which are arrested uniformly at telophase (SHIRAYAMA et al. 1994). The rho3-22 Δrho4 Δle1 cells (strain YJR13) that were arrested at telophase by the incubation at 11°C were shifted to 37°C. The fraction of the small-budded cells was <5% of total cells at the shift but became ~34% 8 hr after the shift to 37°C. These morphological observations strongly suggest that Rho3p is required to maintain cell polarity for bud growth but is not essential for the initiation of bud emergence.

Identification of a dose-dependent dominant rho3 mutation: For a complementary study, we introduced three mutations into RHO3 that might confer dominant effects. Glycine 25 was replaced by valine in rho3<sup>Val25</sup>, and aspartate 131 was replaced by alanine in rho3<sup>Ala131</sup>, producing mutant Rho3ps analogous to the constitutively activated products of the human H-ras<sup>Val12</sup> and H-ras<sup>Ala139</sup>, respectively (Figure 1). In rho3<sup>Asn89</sup>, threonine was replaced by asparagine, producing a mutant Rho3p analogous to the H-ras<sup>Asn17</sup> product that binds tightly to the GTP-GDP exchange factor for ras proteins (ras GEF), depletes ras GEF, and thus confers a dominant inhibitory phenotype (Figure 1; POWERS et al. 1989; BOURNE et al. 1991; BOGUSKI and MCCORMICK 1993).
For the overexpression of each of the mutated \textit{RHO3} genes in wild-type cells (strain YPH500), we introduced multicyclop plasmids carrying these mutant genes and plasmid carrying \textit{RH03}, and incubated at 30°C for 2 days or at 36°C for 5 days.

For the overexpression of each of the mutated \textit{RHO3} genes in wild-type cells (strain YPH500), we introduced multicyclop plasmids carrying these mutant genes and also constructed cells carrying the mutant genes expressed from the \textit{GAL7} promoter. Both methods for overexpression produced essentially the same results. Cells expressing either \textit{rho3}^{Val-25} or \textit{rho3}^{Aro-30} grew a little more slowly than cells expressing wild-type \textit{RHO3} (data not shown) and the morphology of the cells was normal.

In contrast, cells expressing \textit{rho3}^{Aro-131} displayed a Cs- phenotype (Figure 4A, sector f). Introduction of \textit{rho3}^{Aro-30} on a centromeric plasmid did not suppress the \textit{rho}3 defect, whereas both \textit{rho3}^{Val-25} and \textit{rho3}^{Aro-131} did, indicating that both \textit{rho3}^{Val-25} and \textit{rho3}^{Aro-131} retain \textit{RHO3} function but \textit{rho3}^{Aro-30} does not (data not shown). Western-blot analysis using anti-Rho3p antibodies revealed that almost the same amounts of Rho3p were produced from \textit{rho3}^{Val-25}, \textit{rho3}^{Aro-30}, \textit{rho3A}^{Aro-131}, and wild-type \textit{RHO3}, on a multicyclop plasmid (Figure 5), indicating that the \textit{rho3}^{Aro-131} mutation alters Rho3p qualitatively.

We replaced one of the \textit{RHO3} genes in the wild-type diploid strain YPH501 with \textit{rho3}^{Aro-131}, and cells were then sporulated and dissected. We obtained \textit{rho3}^{Aro-131} segregants only when spores were germinated at 34°C. The \textit{rho3}^{Aro-131} cells could not grow <30°C (Figure 4B, sectors c and d). The cold sensitivity of the \textit{rho3}^{Aro-131} cells was recessive because it was suppressed by one copy of \textit{RH03} (Figure 4B, sectors e and f), and \textit{rho3}^{Aro-131} diploid cells grew well at low temperatures (data not shown). These results indicate that \textit{rho3}^{Aro-131} is a dose-dependent dominant mutation.

Characterization of \textit{rho3}^{Aro-131}: In contrast to the dominant active mutation \textit{ras}^{Aro-119}, the corresponding \textit{CDC42}^{Aro-118} mutation is reported to act as a dominant inhibitory mutation like \textit{ras}^{Aro-17} (Ziman and Johnson 1994). Dominant inhibitory mutations confer almost the same phenotypes as those displayed by cells carrying loss-of-function mutation; e.g., \textit{CDC42}^{Aro-118} cells fail in cell polarization like \textit{cdc42-1} cells under restrictive conditions. In this context, \textit{rho3}^{Aro-131} is likely to be a dominant active mutation and not a dominant inhibitory one, because the phenotypes displayed by cells expressing \textit{rho3}^{Aro-131} were different from those displayed by \textit{rho3-1} cells and \textit{Delta}rho3 disruptants (see below and Matsui and Toh-e 1992b).

To test this hypothesis, we combined loss-of-function mutations with \textit{rho3}^{Aro-131}. Mutations in the effector domain of \textit{Ras} (e.g., \textit{H-ras}^{Ne-47}; Figure 1) suppress the effect of dominant active mutations (Barbacid 1987; Bourne et al. 1991; Boguski and McCormick 1993). \textit{rho3}^{Ne-47}, where proline 47 in the putative effector domain of Rho3p was replaced with serine, produced nonfunctional Rho3p; \textit{rho3}^{Ne-47} on a centromeric plasmid could not complement the \textit{Delta}rho3 deletion (data not shown). Both cells harboring \textit{rho3}^{Ne-47}, \textit{Aro-131} on a multicyclop plasmid and cells expressing \textit{rho3}^{Ne-47}, \textit{Aro-131} from the \textit{GAL7} promoter did not display cold sensitivity (Figure 4A, sector c). Western-blot analysis using anti-Rho3p antibodies revealed that almost the same amounts of Rho3p were produced from \textit{rho3}^{Ne-47}, \textit{rho3}^{Aro-131}, and \textit{rho3}^{Ne-47}, \textit{Aro-131} (data not shown). These results indicate that the putative effector domain is critical for \textit{RHO3} function and for the dominant effect of \textit{rho3}^{Aro-131}.

The loss-of-function mutation \textit{rho3}^{Aro-30} corresponding to \textit{ras}^{Aro-17}, also suppressed the Cs- phenotype; cells carrying \textit{rho3}^{Aro-30}, \textit{Aro-17} on a multicyclop plasmid grew normally at 15°C (Figure 4A, sector b). This result is analogous to that obtained with the dominant active mutation \textit{ras}^{Ne-47}, which is suppressed intramolecularly by the dominant inhibitory mutation \textit{ras}^{Aro-17} (Powers et al. 1989).

Moreover, \textit{rho3}^{Ne-228} and \textit{rho3-1} (i.e., \textit{rho3}^{Apo-108}), Ts- mutations producing loss-of-function, also suppressed the dominant effect of \textit{rho3}^{Aro-131}, cells overexpressing either \textit{rho3}^{Val-25}, \textit{rho3}^{Aro-30}, \textit{rho3A}^{Apo-131}, and wild-type \textit{RHO3}, on a multicyclop plasmid (Figure 5), indicating that the \textit{rho3}^{Apo-131} mutation alters Rho3p qualitatively.

We replaced one of the \textit{RHO3} genes in the wild-type diploid strain YPH501 with \textit{rho3}^{Aro-131}, and cells were then sporulated and dissected. We obtained \textit{rho3}^{Aro-131} segregants only when spores were germinated at 34°C. The \textit{rho3}^{Aro-131} cells could not grow <30°C (Figure 4B, sectors c and d). The cold sensitivity of the \textit{rho3}^{Aro-131} cells was recessive because it was suppressed by one copy of \textit{RH03} (Figure 4B, sectors e and f), and \textit{rho3}^{Aro-131} diploid cells grew well at low temperatures (data not shown). These results indicate that \textit{rho3}^{Aro-131} is a dose-dependent dominant mutation.

Characterization of \textit{rho3}^{Aro-131}: In contrast to the dominant active mutation \textit{ras}^{Aro-119}, the corresponding \textit{CDC42}^{Aro-118} mutation is reported to act as a dominant inhibitory mutation like \textit{ras}^{Aro-17} (Ziman and Johnson 1994). Dominant inhibitory mutations confer almost the same phenotypes as those displayed by cells carrying loss-of-function mutation; e.g., \textit{CDC42}^{Aro-118} cells fail in cell polarization like \textit{cdc42-1} cells under restrictive conditions. In this context, \textit{rho3}^{Aro-131} is likely to be a dominant active mutation and not a dominant inhibitory one, because the phenotypes displayed by cells expressing \textit{rho3}^{Aro-131} were different from those displayed by \textit{rho3-1} cells and \textit{Delta}rho3 disruptants (see below and Matsui and Toh-e 1992b).

To test this hypothesis, we combined loss-of-function mutations with \textit{rho3}^{Aro-131}. Mutations in the effector domain of \textit{Ras} (e.g., \textit{H-ras}^{Ne-47}; Figure 1) suppress the effect of dominant active mutations (Barbacid 1987; Bourne et al. 1991; Boguski and McCormick 1993). \textit{rho3}^{Ne-47}, where proline 47 in the putative effector domain of Rho3p was replaced with serine, produced nonfunctional Rho3p; \textit{rho3}^{Ne-47} on a centromeric plasmid could not complement the \textit{Delta}rho3 deletion (data not shown). Both cells harboring \textit{rho3}^{Ne-47}, \textit{Aro-131} on a multicyclop plasmid and cells expressing \textit{rho3}^{Ne-47}, \textit{Aro-131} from the \textit{GAL7} promoter did not display cold sensitivity (Figure 4A, sector c). Western-blot analysis using anti-Rho3p antibodies revealed that almost the same amounts of Rho3p were produced from \textit{rho3}^{Ne-47}, \textit{rho3}^{Aro-131}, and \textit{rho3}^{Ne-47}, \textit{Aro-131} (data not shown). These results indicate that the putative effector domain is critical for \textit{RHO3} function and for the dominant effect of \textit{rho3}^{Aro-131}.

The loss-of-function mutation \textit{rho3}^{Aro-30} corresponding to \textit{ras}^{Aro-17}, also suppressed the Cs- phenotype; cells carrying \textit{rho3}^{Aro-30}, \textit{Aro-17} on a multicyclop plasmid grew normally at 15°C (Figure 4A, sector b). This result is analogous to that obtained with the dominant active mutation \textit{ras}^{Ne-47}, which is suppressed intramolecularly by the dominant inhibitory mutation \textit{ras}^{Aro-17} (Powers et al. 1989).

Moreover, \textit{rho3}^{Ne-228} and \textit{rho3-1} (i.e., \textit{rho3}^{Apo-108}), Ts- mutations producing loss-of-function, also suppressed the dominant effect of \textit{rho3}^{Aro-131}, cells overexpressing either \textit{rho3}^{Val-25}, \textit{rho3}^{Aro-30}, \textit{rho3A}^{Apo-131}, and wild-type \textit{RHO3}, on a multicyclop plasmid (Figure 5), indicating that the \textit{rho3}^{Apo-131} mutation alters Rho3p qualitatively.
these results, we conclude that the \textit{rho3}^{Alo-131} mutation behaves as do dominant active mutations of the Ras superfamily.

**Morphology of \textit{rho3}^{Alo-131} cells:** Cells harboring \textit{rho3}^{Alo-131} expressed from the \textit{GAL7} promoter (p\textit{GAL7:rho3}^{Alo-131} strain \textit{YJR39A}), growing in galactose-containing medium at 30°, were shifted to 15°; 12 hr after the shift, the p\textit{GAL7:rho3}^{Alo-131} strain became elongated and bent (Figure 6D), whereas cells harboring wild-type \textit{RHO3} expressed from the \textit{GAL7} promoter (p\textit{GAL7:RHO3}) displayed almost normal morphology (Figure 6A). In the p\textit{GAL7:rho3}^{Alo-131} cells, actin patches were concentrated in the bud neck and/or in the mother cells, in addition to (or instead of) the bud, where wild-type cells preferentially concentrate actin patches. The positions of the actin patches often corresponded to the points of the cell surface elongated or bent in the p\textit{GAL7:rho3}^{Alo-131} cells (Figure 6E). A similar aberrant cell shape was observed in the \textit{rho3}^{Alo-131} strain. When the \textit{rho3}^{Alo-131} strain \textit{YJR38A} growing at 36° was shifted to 25°, the cells arrested growth 6 hr after the shift and became long, thin and bent (Figure 6B). The organization of actin filaments was aberrant as observed in the p\textit{GAL7:rho3}^{Alo-131} cells at nonpermissive temperatures (Figure 6C).

**Characterization of \textit{SRO6} and genetic interactions between \textit{SRO6} and \textit{RHO3}:** Previously, we identified \textit{SRO6} as a multicopy suppressor of \textit{rho3} (Matsu and Toh-e 1992b). The suppression activity was mapped to the region containing a \textit{PvuII} site (Figure 7). The nucleotide sequence of this region was identical to that of \textit{SEC4} (data not shown) and disruption of the \textit{SEC4} ORF by introducing a frameshift mutation at the internal HindIII site abolished the suppression activity (Figure 7, p\textit{SRO6ΔH}), indicating that \textit{SRO6} is \textit{SEC4}. In contrast, \textit{RHO3} did not serve as a multicopy suppressor of \textit{sec4} because neither a high dose of \textit{RHO3} nor expression of \textit{rho3}^{Alo-131} suppressed the Ts- phenotype of \textit{sec4} cells at 36° (data not shown). The possibility of a synthetic-
lethal interaction between RHO3 and SEC4 was tested as follows. The sec4-2 strain YJS4-2A was crossed with the rho3-1 Δ rho4 strain YMR3732-5D and the resulting diploid was analyzed by tetrad analysis. sec4-2, rho3-1, sec4-2 Δ rho4, and rho3-1 Δ rho4 segregants grew below 31°. However, sec4-2 rho3-1 and sec4-2 rho3-1 Δ rho4 segregants could not grow at 28° or above and also showed a Csc- phenotype; they grew very poorly below 20° (Figure 8A). These phenotypes indicate synthetic lethality between rho3-1 and sec4-2. The growth defect of sec4-2 rho3-1 segregants and sec4-2 rho3-1 Δ rho4 segregants at 28° and 20° was suppressed by either RHO3 or SEC4 on a centromeric plasmid (data not shown).

We tested whether the sec4-2 mutation suppressed the cold sensitive phenotype of pGAL7:rho3<sup>Δlo-131</sup> cells. pGAL7:rho3<sup>Δlo-131</sup> was introduced into sec4-2 cells and into isogenic wild-type cells (SEC4<sup>+</sup> cells). As observed in the experiments described above, SEC4<sup>+</sup> cells expressing rho3<sup>Δlo-131</sup> grew very poorly at 25° and were elongated. In contrast, sec4-2 cells expressing rho3<sup>Δlo-131</sup> grew well at 25° (Figure 8B) and were less elongated than the pGAL7:rho3<sup>Δlo-131</sup> SEC4<sup>+</sup> cells (data not shown). None of the sec1~sec23 mutations, except for sec4, suppressed the cold sensitivity of cells expressing rho3<sup>Δlo-131</sup> (data not shown).

The genetic interaction between RHO3 and SEC4 suggested the possibility that Rho3p might be involved in the secretory process. We tested whether the mutation in RHO3 conferred a secretion-defective phenotype by measuring secreted invertase activity of rho3 cells. No significant defect in invertase-secretion was detected in the Ts<sup><sub>rho3</sub></sup> cells (strains YMR3732-2B, YMR3732-5D, YJR3-5A, and YJR3-5B) incubated at 37° and in rho3<sup>Δlo-131</sup> cells (strain YJR3-8A) incubated at 25°, although the secretion of invertase was not detected in sec4-2 cells (strain YJS4-2A) 1 hr after the shift to 37° (data not shown). This result indicates that rho3 mutants do not show a Sec<sup>-</sup> phenotype.

**DISCUSSION**

**Mutations in RHO3 can cause temperature-sensitive growth:** The temperature-sensitive mutations rho3<sup>-1</sup> (rho3<sup>Δap-190</sup>) and rho3<sup>Δsc-228</sup> are loss-of-function mutations because they are recessive and have no dominant effects on cell growth, even when overexpressed. In contrast to the fact that both rho3<sup>-1</sup> cells and rho3<sup>Δsc-228</sup> cells could not grow at 37°, the Δ rho3 cells grow slowly at 37°. Thus, the presence of a nonfunctional Rho3p, rather than the lack of function per se, is inhibitory and the temperature-sensitive rho3 mutations differ from simple loss-of-function mutations. An analogous case has been reported in Dbf2p kinase. DBF2 and DBF20 encode a pair of functionally redundant kinases and deletion of DBF2 is lethal only when DBF20 is deleted (TAYLOR et al. 1991). However, dbf2 loss-of-function mutations are conditionally lethal even in the presence of the wild-type DBF20 gene. This may be because the nonfunctional Dbf2p sequesters Spo12p, a limiting factor for both the kinases, from Dbf20p (PARKES and JOHNSTON 1992; TAYLOR and JOHNSTON 1993). As a high dose of RH04 complements Δ rho3 (MATSUI and TOH-E 1992a), it is possible that Rho4p can substitute for Rho3p in the Rho3p pathway. The survival of Δ rho3 cells requires Rho4p since Δ rho3 Δ rho4 cells did not grow at 30° and above the temperatures (MATSUI and TOH-E 1992a). Therefore, it is highly conceivable that the nonfunctional Rho3p, which may still interact with the component(s) of the Rho3p pathway, prevents Rho4p from substituting for Rho3p in the pathway and Rho4p can replace Rho3p efficiently only in the absence of Rho3p.

**Characteristics of various mutations of RHO3:** Among various rho3 mutants (Figure 1), rho3<sup>Δlo-131</sup> is a dominant active mutation analogous to those reported for other members of the Ras superfamily, e.g., H-ras<sup>Δlo-115</sup> and CDC42<sup>Δa-12</sup> (BARRACID 1987; ZIMAN et al. 1991). However, the dose-dependence displayed by rho3<sup>Δlo-131</sup> is a novel characteristic, because the other dominant active mutations of the Ras superfamily are dominant, independent of the dose. The recessive phenotype of rho3<sup>Δlo-131</sup> (Figure 4B) suggests that Rho3p may require a factor to mediate coupling with a putative Rho3p effector to exert its function. Assuming that Rho3pAla-131 has less affinity for the unknown factor than
wild-type Rho3p, in the presence of wild-type Rho3p, an increased amount of Rho3pala-131 would be required for efficient coupling with the factor and thus with the effector, but in the absence of wild-type Rho3p, Rho3pala-131 can couple efficiently with these factors. It has been reported that rho GDI, which binds to Rho-type GTPases, has a potential activity of transferring the Rho-type GTPase in a membrane-attaching state to that in a cytosol-localizing state, and vice versa (Takai et al. 1992). A molecule required to transport Rho3p to the effector is a candidate for the unknown factor.

The RHO3 function is involved in cell polarity maintenance: Ts" rho3 cells lost cell polarity at nonpermissive temperatures and displayed defects in bud growth, indicating that Rho3p is required to maintain cell polarity for bud formation. By the following criteria, RHO3 mutants are different from those of CDC42, which is critical for the initiation of bud emergence. (1) Ts" cdc42 mutants are arrested uniformly as depolarized unbudded cells (Adams et al. 1990), whereas the fraction of unbudded cells in the Ts" rho3 cells at nonpermissive temperatures did not increase significantly. (2) The multibudded cells are accumulated in the culture of cells expressing dominant active mutant Cdc42 (Ziman et al. 1991), whereas cells expressing rho3ala-131 did not show the phenotype. These differences and the result that the synchronized Ts" rho3 cells could initiate bud emergence at 37° strongly suggest that Rho3p is not critical for the initiation of bud emergence.

In a previous study, we observed that Δrho3 Δrho4 cells died as lysed small-budded cells (Matsui and Toh-e 1992b). This terminal morphology is different from that of Ts" rho3 cells. However, when the osmolarity of the culture medium for the Δrho3 Δrho4 cells was adjusted to prevent the abortive cell lysis, the Δrho3 Δrho4 cells arrested as depolarized cells with a terminal morphology similar to that of the Ts" rho3 cells (Matsui and Toh-e 1992b). These results indicate that a primary defect caused by absence of RHO3 function is loss of cell polarity.

Rho3p is involved in determining the positions for surface growth: The locations at which actin patches are organized and exocytosis is executed are arranged dynamically under the control of cell polarity functions in order that the proper region of cell surface in the bud grows. The dose-dependent dominant active rho3 causes mislocalization of actin patches, and the cells become elongated and bent, often at the position where actin patches were concentrated (Figure 6, D and E). These phenotypes of cells expressing rho3ala-131 suggest that Rho3p plays an important role in directing the organization of the actin cytoskeleton and the localization of the machinery for exocytosis. In this context, it is noteworthy that RHO3 interacts genetically with SEC4. Sec4p is involved in exocytosis. The defect and acceleration of an upstream factor can be suppressed by an acceleration and a defect, respectively, of the downstream factor in the same pathway. In this study, we found that SEC4 can serve as a multicopy suppressor of rho3 and that the cold sensitivity of the rho3ala-131 mutant is suppressed by the sec4-2 mutation. These genetic interactions between SEC4 and RHO3 suggest that RHO3 functions upstream of SEC4. However, rho3 mutants did not display a Sec- phenotype, indicating that Rho3p does not play a direct role in secretion. The close relationship between the actin cytoskeleton and the vectorial transport of secretory vesicles has been observed in studies on yeast actin and actin-binding proteins Myo2p (myosin), Tpm1p (tropomyosin), and actin capping proteins (Novick and Botstein 1985; Johnston et al. 1991; Amatruda et al. 1992; Liu and Bretscher 1992). We interpret the genetic interaction between SEC4 and RHO3 to indicate that the RHO3 pathway functions upstream of the SEC4 pathway; that is, the RHO3 pathway is involved in the organization of the actin cytoskeleton that affects the SEC4 pathway for exocytosis.

During bud formation many factors are required, and protein-protein interactions among some of these factors have been reported. Cdc24p, the GEF for Cdc42p, which is required for cell polarity establishment, interacts not only with Cdc42p but also with Rsr1p/Bud1p, which is involved in the determination of the bud site (Sloat et al. 1981; Bender and Pringle 1989; Chant and Herskowitz 1991; Zheng et al. 1994, 1995). In addition, the C-terminal half of Bem1p interacts with Cdc24p (Peterson et al. 1994), and the SH3 domain of Bem1p binds the Boi proteins that are required for bud formation (Y. Matsui, R. Matsui, R. Akada, and A. Toh-e, unpublished results; L. Bender, H. Lo, H. Lee, J. Peterson, and A. Bender, Indiana University, personal communication). These protein-protein interactions strongly suggest that formation of a protein complex at the bud site (bud-site complex) is required for bud formation. The fact that RHO3 can serve as a multicopy suppressor of boi mutations (Y. Matsui, R. Matsui, R. Akada, and A. Toh-e, unpublished results; L. Bender, H. Lo, H. Lee, J. Peterson, and A. Bender, Indiana University, personal communication), along with the fact that BEM1 and CDC42 can serve as multicopy suppressors of rho3, suggests that Rho3p participates in the assembly of the complex. It has been reported in mammalian cells that Rho-type GTPases regulate the formation of the actin cytoskeleton during membrane ruffling and the assembly of focal adhesions, actin stress fibers, and microspikes (Ridley and Hall 1992; Ridley et al. 1992; Kozma et al. 1995). During the formation of focal adhesions, a protein complex including talin, vinculin, and integrin functions as an actin nucleation site (Burridge et al. 1988). It is likely that yeast Rho-type GTPases control the assembly of the protein complex that acts as an actin nucleation site to organize the actin cytoskeleton for bud growth. The bud-site complex is probably dynamic throughout the cell cycle to coordinate proper bud growth and must
be stabilized, dissociated, and reassembled according to the stages of bud growth (Lew and Reed 1995). We postulate a model in which RH03 function controls the stability and development of the bud site complex that finally tethers the cytoskeleton and secretory machinery for exocytosis.

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LITERATURE CITED


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