

Conditional lethality of a yeast strain expressing human *RHOA* in place of *RHO1*

(rho GTPase/functional conservation/specificity domain/osmotic integrity)

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ABSTRACT The yeast *RHO1* GTPase, which has 72% amino acid sequence identity with its human counterpart, *RHOA*, is essential for growth, although the reason has not been investigated. We report here that yeast strains that rely solely on expression of human *RHOA* in place of *RHO1* are able to grow at 23°C but grow neither at 37°C nor in the presence of 300 mM CaCl₂ even at 23°C. Measurements of steady-state protein levels indicate that inability to grow at the restrictive temperature is not due to instability of the protein. Homolog scanning with the two GTPases identified a small, 27-residue region of *RHO1* which, when substituted into *RHOA*, confers full function in yeast. This region corresponds to the α 3-helix loop 7 region of *RAS*; the same region was reported to determine specificity of function between GTPases of the RAB family, Sec4p and Ypt1p. By examining the phenotype of *RHOA* substitution strains at nonpermissive temperature, we found evidence suggesting that the normal function of *RHO1* is to maintain osmotic integrity.

Rho GTPases, which comprise one subfamily of ras-related GTP-binding proteins, are thought to act as molecular switches by cycling between two states (the active GTP-bound form and the inactive GDP-bound form) (1, 2). Transitions between these two states are controlled by regulator molecules, including GTPase-activating proteins, guanine nucleotide-exchange factors, and guanine nucleotide-dissociation inhibitors. Many such regulator molecules have been identified for mammalian rho GTPases (3). Evidence is accumulating that the rho family GTPases function in cell morphology (4), cell motility (5), cell adhesion (6, 7), cytokinesis (8), and smooth muscle contraction (9) through regulation of actin assembly (10–12).

In the yeast *Saccharomyces cerevisiae*, five members of the rho subfamily (*RHO1*, *RHO2*, *RHO3*, *RHO4*, and *CDC42*) have been identified (13–17). *RHO1* and *CDC42* are essential genes (13, 17). *RHO3* and *RHO4* are related functionally to each other and loss of both results in growth failure above 30°C (14, 15). *RHO2* is not essential for growth under any conditions tested (13). Of these rho-homologous genes, Cdc42p has been best studied to date both genetically and biochemically (16–20).

The functions of some of the rho subfamily proteins have been investigated by using conditional-lethal mutations. Failure of temperature-sensitive *cdc24* and *cdc42* mutations to form buds implicated Cdc24p and Cdc42p in bud organization (16), an idea supported in the case of Cdc42p by its localization in the growing tip of the bud (20). The amino acid sequences of Bem3p and Cdc24p showed them to be homologous to GTPase-activating proteins and guanine nucleotide-exchange factors, respectively, a result confirmed by direct biochemical analysis (21). In contrast, little is known about

the function of *RHO1* since no conditional-lethal mutants have yet been reported. A possible function of *RHO1* in the secretory process was suggested by the observation that Rho1p is found associated with the Golgi apparatus and post-Golgi vesicles (22).

Expression of a human homolog of *CDC42*, *CDC42Hs*, complements the temperature-sensitive growth of *cdc42-1* (23, 24). In contrast, although *RHO1* encodes a protein 72% identical with a human homolog (*RHOA*, the best-studied of three human RHO GTPases), functional conservation studies have not been reported. Fig. 1 shows the alignment of *RHOA* and Rho1p. They share common sequences in the GXXXXGK and DTAGQ GTPase consensus sequences and in the effector domains. Structural conservation is not so obvious in the C-terminal region; only 55% of the residues are identical in the sequences after the NKXDL consensus.

Since the human *RHOA* GTPase is required for cell proliferation (10), as is the yeast *RHO1* GTPase, we undertook the study of the functional similarities between yeast *RHO1* and human *RHOA*.

MATERIALS AND METHODS

Strains, Media, and Chemicals. The yeast strains used in this study are listed in Table 1. Yeast media and genetic analyses were described in ref. 27. To grow YOC706, both 2% glucose and 2% galactose were included as carbon sources. *Pfu* polymerase (Stratagene) was used in PCR.

Plasmids. pYO714 plasmid (Fig. 2) was constructed by insertion of a PCR amplified *RHOA* fragment into the *EcoRI*/*Bgl* II gap of pYO701, which consists of pRS314, the 0.2-kb *RHO1* promoter, and the 0.1-kb *RHO1* terminator. To amplify the *RHOA* fragment, the H14 plasmid (a gift from P. Madaule, Institut Pasteur, Paris) containing a 1.7-kb *RHOA* cDNA fragment was used as a template. pYO702 was constructed by insertion of a PCR-amplified *RHO1* gene into the same *EcoRI*/*Bgl* II gap of pYO701 and used as a positive control. Various chimeric genes of human *RHOA* and yeast *RHO1* were inserted into pYO701 after two-step PCR (28, 29). Primers used in the first PCR step were 36-mer oligonucleotides containing 18-mer oligonucleotides of each (*RHOA*/*RHO1*) nucleotide sequence. All of the chimeric genes were verified by DNA sequencing by the dideoxynucleotide chain-termination method (30) with a model 373A DNA sequencer (Applied Biosystems). pYO708 was constructed by insertion of the *RHO1*^{N46S} gene into pYO701 after site-directed mutagenesis with PCR. pYO774 was constructed by insertion of the PCR-amplified *RHO1* fragment between the *GAL1* promoter and the *CMK1* terminator of pYO773 (Y.O., unpublished data).

ADP Ribosylation Assay. Exponentially growing yeast cells were harvested and suspended in lysis buffer (100 mM sorbitol/20 mM Hepes, pH 7.2/50 mM potassium acetate/1 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol at a density of 1×10^9 cells per ml. The cells were broken by

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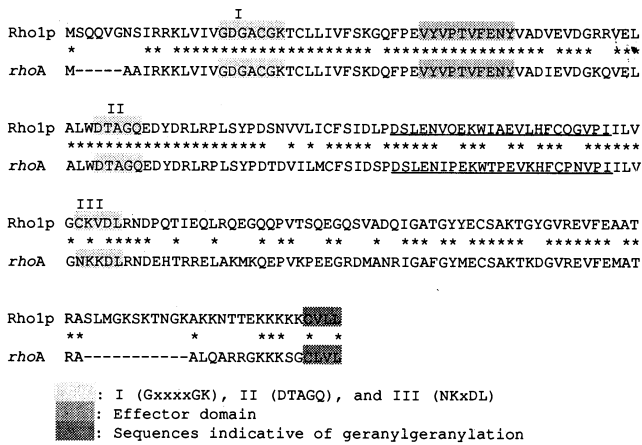


FIG. 1. Alignment of yeast Rho1p and human RHOA. Asterisks indicate identity between the two small GTPases. The α 3-helix and loop 7 region is underlined. Roman numerals I, II, and III indicate GTPase consensus sequences GXXXXGK, DTAGQ, and NKXDL, respectively.

agitation with glass beads (31). After centrifugation at low speed, the supernatant was used for the ADP ribosylation assay (32). NAD[adenylate-³²P] was purchased from ICN. After the ADP-ribosylation reaction, samples were analyzed by SDS/20% PAGE (33).

Cell Lysis Assay. Yeast strains were incubated on a yeast extract/peptone/dextrose (YPD) plate at 23°C for 3 days and shifted overnight to 37°C. The plate was then overlaid with an alkaline phosphatase assay solution as described (34). Colonies containing lysed cells turned blue within 1 h, while control colonies remained unstained even after 2 h.

RESULTS

Human RHOA Complements a Yeast rho1 Deletion Mutation at 23°C but Not at 37°C. Heterologous expression of RHOA GTPase in yeast was accomplished by constructing a single-copy plasmid harboring a human RHOA cDNA hooked up to the yeast RHO1 promoter. We introduced the RHOA-expressing plasmid (pYO714; Fig. 2) into a yeast strain (YOC706), which contained a rho1 deletion and a plasmid (pYO774) expressing the yeast RHO1 gene under control of the GAL1 promoter. The growth of the transformants was then tested on plates containing glucose but no galactose (YPD), where expression of yeast RHO1 was shut off. Fig. 3 shows that human RHOA complements the rho1 deletion mutation well at 23°C (Fig. 3A) but not at 37°C (Fig. 3B). Expression of RHOA in cells containing an intact RHO1 gene does not result in temperature-sensitive growth (see below); thus, the temperature sensitivity appears to be a failure of the heterologous gene to provide full function at 37°C. Overexpression of RHOA under the GAL1 promoter is toxic even to the wild-type cells (data not shown), so complementation under conditions of overexpression could not be tested.

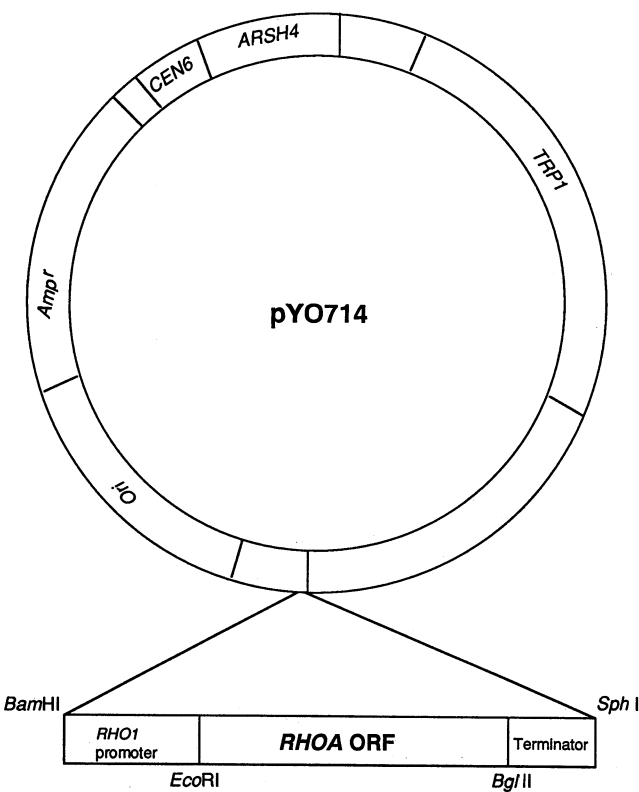


FIG. 2. pYO714 plasmid that expresses human RHOA in yeast. Start and stop codons of RHOA are located adjacent to the EcoRI site and the Bgl II site, respectively.

In addition to the temperature-sensitive phenotype, we found another recessive phenotype resulting from heterologous expression. The rho1 deletion strain harboring the RHOA expression plasmid (pYO714) cannot grow on a YPD plate containing 300 mM CaCl₂ (Fig. 3C). YOC725, which contains the rho1 deletion mutation and an integrated human RHOA gene on the chromosome, is also incapable of growing in the presence of high concentrations of CaCl₂. Wild-type yeast strains (whether or not they express RHOA) grow even on a YPD plate supplemented with 600 mM CaCl₂, while YOC725 fails to grow on a YPD plate supplemented with 300 mM CaCl₂ at 30°C. This effect is specific to Ca²⁺; YOC725 shows neither a Mg²⁺-, Mn²⁺-, Zn²⁺-, nor a Cu²⁺-sensitive phenotype (data not shown).

Expression of RHOA Protein in Yeast. The temperature sensitivity mentioned above could result from instability of the heterologously expressed protein at the restrictive temperature. To check this possibility, we directly examined levels of the RHOA protein using the RHO-specific ADP-ribosylation reaction catalyzed by botulinum C3 exoenzyme (22, 35). Yeast Rho1p could be labeled with NAD³²P and was shown to be a major ADP-ribosylated protein in yeast (22). Since Asn-46 in the effector domain of Rho1p is predicted to

Table 1. Yeast strains

Strain	Genotype	Source
YPH500	MAT α ade2 his3 leu2 lys2 trp1 ura3	Ref. 25
YOC706	MAT α ade2 his3 leu2 lys2 trp1 ura3 Δ rho1::HIS3 [pYO774]	This study
YOC724	MAT α ade2 his3 leu2 lys2 trp1 ura3 Δ rho1::HIS3 [pYO708]	This study
YOC725	MAT α ade2 his3 leu2 lys2 trp1 ura3 Δ rho1::LYS2 ade3::[pRHO1-rhoA:HIS3]	This study
YOC726	MAT α ade2 his3 leu2 lys2 trp1 ura3 Δ rho1::LYS2 ade3::[pRHO1-rhoA:HIS3] [pYO708]	This study
YS3-6D	MAT α ade8 his3 leu2 trp1 ura3	Ref. 26
SYT11-12A	MAT α ade8 his3 leu2 trp1 ura3 stt1-1	Ref. 26
DJTD2-16A	MAT α gal2 his4 leu2 trp1 ura3 cdc42-1	Ref. 16

All strains except those from other sources were constructed in this study from YPH500 by standard genetic methods.

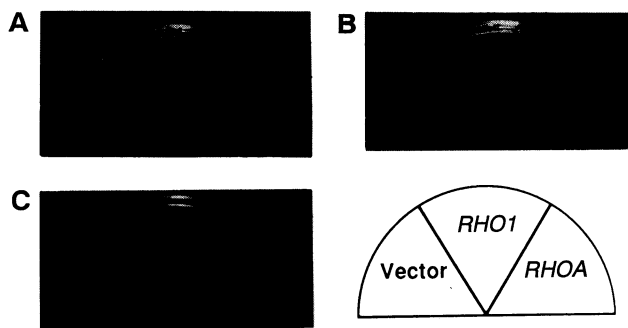


FIG. 3. Conditional complementation of $\Delta\rho 1$ by expression of human *RHOA*. Strain YOC706 was transformed with pYO701 (vector), pYO714 (*RHOA*), and pYO702 (*RHO1*). Growth of the transformants was tested after 3 days of incubation on a YPD plate at 23°C (A), on a YPD plate at 37°C (B), and on a YPD plate containing 300 mM CaCl_2 at 30°C (C).

be a site for ADP ribosylation (36), we constructed a mutation (*RHO1*^{N46S}) that encodes a functional protein that cannot be ADP ribosylated to serve as a negative control; indeed, in such a strain (YOC724), the Rho1p band was not observed (Fig. 4, lane 1). In cells expressing both the yeast *RHO1*^{N46S} and human *RHOA* (YOC726), we found that levels of the *RHOA* protein do not decrease noticeably after 6 h of incubation at 37°C (Fig. 4A). Since 53% of cells that rely solely on expression of *RHOA* already lost viability during the same period of incubation (Fig. 4B), we concluded that the temperature sensitivity of the cells expressing *RHOA* is not due to instability of the *RHOA* protein.

Chimeras of Human/Yeast RHO GTPases. To determine whether there are regions within the yeast *RHO1* protein that can provide to the human *RHOA* protein the ability to function in yeast at high temperature (or in the presence of 300 mM Ca^{2+}), we made precise reciprocal fusions between the coding sequences of *RHO1* and *RHOA* by using a PCR technique (28, 29). Using two conserved motifs, DTAGQ and NKXDL, as fusion points, we began by swapping each one-third of the *RHO1*/*RHOA* sequences. The ability of the fusion proteins to function was examined in two strains: one (YOC706) has a *RHO1* deletion as described above, and the

other (YOC725) contains in addition an integrated plasmid that expresses human *RHOA* (Fig. 5).

Chimeric genes in which two-thirds of *RHOA* sequence is replaced by yeast *RHO1* sequence all produce proteins that function well at 37°C (or with high Ca^{2+}) in YOC706. Surprisingly, one of them (1A1, in which only the central one-third is of human origin) fails to function well when coexpressed with intact human *RHOA* (i.e., in strain YOC725). One might restate this result as saying that the 1A1 chimera is dominant to the null but recessive to *RHOA*; clearly the 1A1 allele is less functional than *RHO1*⁺. This result is discussed further below.

Of three chimeric genes in which only one-third of *RHOA* sequence is replaced by *RHO1*, only one chimera (A1A) fully complements $\Delta\rho 1$ at 37°C (Fig. 5). Again, this chimeric allele does not function at 37°C in the strain that also expresses intact *RHOA* protein. Curiously, in this case the recessiveness to human *RHOA* is seen only at high temperature; the high Ca^{2+} -sensitive phenotype of the chimera is dominant.

To identify more precisely the region of *RHO1* that can confer full function to *RHOA*, we constructed more chimeras in which the fusion point was located between the DTAGQ and NKXDL motifs. This region contains the 27 residues of the $\alpha 3$ -helix and loop 7 region previously proposed to be a "specificity domain" of the small RAS-related GTPases. It should be noted that in the 27-residue region substituted, there are only 7 differences in the amino sequence encoded by yeast *RHO1* and human *RHOA*. This chimera [A1A($\alpha 3$ L7)], which encodes a protein with 27 residues of $\alpha 3$ helix and loop 7 derived from yeast and all other regions from the human *RHOA*, allows growth of both strains YOC706 and YOC725 at 37°C and in the presence of high Ca^{2+} at 30°C (Fig. 5). Thus the $\alpha 3$ -helix and loop 7 substitution results in full dominant function under all circumstances tested. The smaller substitutions [A1A($\alpha 3$) and A1A(L7)], have very little effect (Fig. 5).

Phenotype of Cells Relying Solely on *RHOA* Expression. We took advantage of the temperature sensitivity of human *RHOA* in yeast to study the rho GTPase function, since no conditional-lethal *rho* mutants had heretofore been available. Phenotypes of the YOC725 cells (which rely solely on

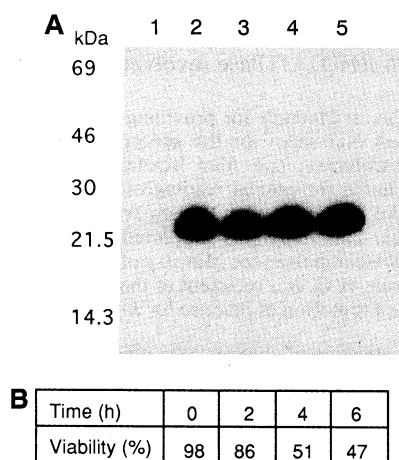


FIG. 4. Detection of the *RHOA* protein expressed in yeast. (A) Cells of YOC724 (lane 1) and YOC726 (lanes 2–5) were incubated at 37°C and harvested after 0 (lanes 1 and 2), 2 (lane 3), 4 (lane 4), and 6 (lane 5) h of incubation. A cell lysate equal to 5×10^7 cells was subjected to an ADP-ribosylation assay in a 100- μl reaction mixture. One-fifth of the reaction mixture was analyzed by SDS/20% PAGE and fluorography (2-h exposure). Positions of molecular size markers are shown on the left. (B) Percentages of viable cells in the culture were measured after incubation for the indicated times at 37°C.

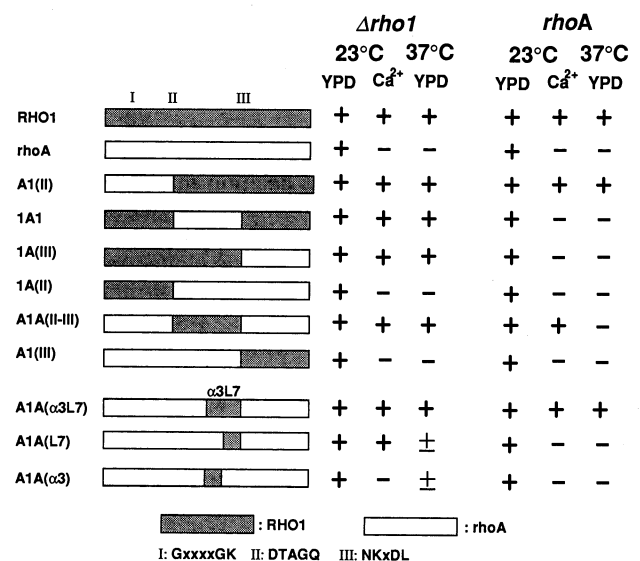


FIG. 5. Structure of chimeric proteins and their complementing ability in $\Delta\rho 1$ and *RHOA*-expressing cells. YOC706 ($\Delta\rho 1$) and YOC725 (*RHOA* $\Delta\rho 1$) were used in this experiment. Growth on YPD at 23°C, on YPD at 37°C, and on YPD containing 300 mM CaCl_2 at 30°C was examined after 3 days of incubation.

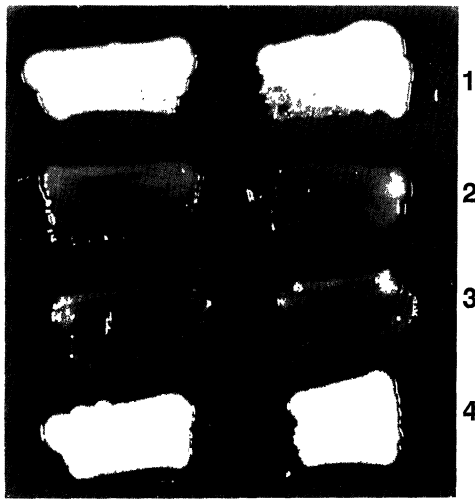


FIG. 6. Cells relying solely on *RHOA* cause cell lysis. After overnight incubation at 37°C, cells were stained with an alkaline phosphatase substrate: 1, wild-type (YS3-6D); 2, *RHOA* (YOC725); 3, *stt1/pkc1* (SYT11-12A); 4, *cdc42-1* (DJTD2-16A). 1 and 4 remain white, whereas 2 and 3 turn blue.

RHOA) were examined after incubation at the restrictive temperature. YOC725 cells stop growing after ≈ 6 h, but the bud sizes appear to be random. Flow cytometric analysis (data not shown) further confirms that the cells stop growing at all stages of the cell cycle. Since many cells looked fragile under the microscope, we tested an effect of osmotic stabilizer on their growth. We found that the temperature sensitivity of YOC725 is suppressed by the addition of 0.5 M sorbitol. The same condition does not suppress lethality of the *rho1* deletion mutation. To examine directly cell lysis, we used a simple plate overlay assay, which detected leakage of alkaline phosphatase from cells (34). Two temperature-sensitive strains were used as controls: *pkc1/stt1* causes cell lysis (26), and *cdc42-1* (16, 17) does not result in cell lysis. The wild-type control cells and the *cdc42-1* cells are unstained, whereas the YOC725 and *pkc1/stt1* cells turn blue (Fig. 6). These findings indicate that the function of rho GTPase is related to osmotic integrity in yeast.

DISCUSSION

We show above that human *RHOA* and yeast *RHO1* retain functional similarity in yeast, as might be expected from the 72% identity in amino acid sequence. Expression of human *RHOA* complemented the *rho1* deletion mutation at 23°C. Substitution of *RHO1* with *RHOA* was, however, insufficient either at high temperature (37°C) or in the presence of a high concentration (300 mM) of Ca^{2+} . We exploited this phenotype in two ways. First, we identified the region that specifies the functional difference between the two evolutionarily conserved GTPases. Second, we defined further the essential function of rho GTPase in yeast based on the observations that the cells lyse at high temperature and that the temperature-sensitive phenotype can be suppressed in the presence of 0.5 M sorbitol.

A Small Region Specifies Functional Differences Among the rho GTPases. Homolog scanning with the two GTPases identified a small, 27-residue region of *RHO1* that can confer full function to *RHOA*. Since substitution of the *RHO1* region corresponding to the $\alpha 3$ helix and loop 7 region into the *RHOA* sequence is sufficient to gain the *RHO1* functions, it is possible that the $\alpha 3$ helix and loop 7 region specifies the functional difference between *RHO1* and *RHOA* GTPases. This result can be put into the context of previous work,

which implicated this region in the functional difference between the *YPT1* and *SEC4* functions in yeast (37, 38). To this end, we suggest that the human *RHOA* cannot recognize an interacting molecule (e.g., guanine nucleotide-exchange factor or GTPase-activating protein or other target molecule) and that the corresponding molecules are associated with Ypt1p and Sec4p to specify their functional difference.

The chimeric genes were analyzed by using two yeast tester strains: a *rho1* deletion mutation and a strain that relies solely on the expression of human *RHOA*. The difference in complementation ability observed with these strains is an unexpected result, since expression of *RHOA* is recessive to *RHO1*. Our current supposition is that under some conditions the nonfunctional *RHOA* competes with the chimeric proteins by titrating out essential binding protein(s). Inability of *RHOA* to compete with Rho1p, as is deduced from recessiveness of *RHOA* to Rho1p, may be due to lower affinity of the heterologous protein. Alternatively, Rho1p may act as a multimer or in some complex in which more than one copy of Rho1p is included. In these circumstances, the wild-type Rho1p should form a homomultimer or be selectively incorporated into the complex.

Function of the Yeast rho GTPase. Cells relying solely on *RHOA* lyse in ordinary media at the restrictive temperature (Fig. 6). Furthermore, their temperature sensitivity is suppressed simply by the addition of 0.5 M sorbitol. This implies that substitution of *RHO1* with *RHOA* results in defects of osmotic integrity at the restrictive temperature. Since many mechanisms could account for defects of osmotic integrity, it is worthwhile to identify the pathway in which *RHO1* is involved. Our recent approach to isolate multicopy suppressors of the *rho1* deficiency has revealed that multiple copies of *PKC1*, a putative protein kinase C gene (39), suppressed the temperature sensitivity of the *RHOA* substitution strain (H.Q. and Y.O., unpublished result). *PKC1* itself functions in osmotic integrity, since a *PKC1* deletion mutation results in cell lysis (34, 40, 41) as is observed with the *RHOA* substitution strain. Genetic evidence is accumulating that a mitogen-activated protein kinase activation pathway is mediated by *PKC1* (42). Thus, it is likely that yeast *RHO1* functions somehow in the yeast protein kinase C pathway. A study of mammalian rho using C3 ADP ribosyltransferase also suggests that the *RHOA* protein plays a role downstream of protein kinase C (6). Further characterization of the other multicopy suppressors will be useful to reveal a genetic network with *RHO1* GTPase involved in osmotic integrity.

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