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A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*

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The *RHO1* gene in *Saccharomyces cerevisiae* encodes a homolog of the mammalian RhoA small GTP-binding protein, which is implicated in various actin cytoskeleton-dependent cell functions. In yeast, Rho1p is involved in bud formation. A yeast strain in which *RHO1* is replaced with *RhoA* shows a recessive temperature-sensitive growth phenotype. A dominant suppressor mutant was isolated from this strain. Molecular cloning of the suppressor gene revealed that the mutation occurred at the pseudosubstrate site of *PKC1*, a yeast homolog of mammalian protein kinase C. Two-hybrid analysis demonstrated that GTP-Rho1p, but not GDP-Rho1p, interacted with the region of Pkc1p containing the pseudosubstrate site and the C1 domain. *MKK1* and *MPK1* encode MAP kinase kinase and MAP kinase homologs, respectively, and function downstream of *PKC1*. A dominant active *MKK1-6* mutation or overexpression of *MPK1* suppressed the temperature sensitivity of the *RhoA* mutant. The dominant activating mutation of *PKC1* suppressed the temperature sensitivity of two effector mutants of *RHO1*, *rho1*(F44Y) and *rho1*(E45I), but not that of *rho1*(V43T). These results indicate that there are at least two signaling pathways regulated by Rho1p and that one of the downstream targets is Pkc1p, leading to the activation of the MAP kinase cascade.

Keywords: MAP kinase cascade/protein kinase C/Rho

Introduction

The Rho family belongs to the small G protein superfamily and consists of the Rho, Rac and Cdc42 subfamilies (Hall, 1994; Takai *et al.*, 1995). The Rho subfamily is composed of three highly homologous members: RhoA, RhoB and RhoC. Rho cycles between the GDP-bound inactive and GTP-bound active forms. Rho is activated by stimulatory GDP/GTP exchange proteins (GEPs), including Dbl, Smg GDS, Ost, Lbc and Tiam-1, whereas Rho is inactivated by an inhibitory GEP, Rho GDI, or by GTPase activating proteins (GAPs) including p190 and Rho GAP. Evidence is accumulating that Rho regulates through reorganization of actin cytoskeleton actin filament-dependent various cell functions, such as maintenance of cell morphology, formation of stress fibers and focal adhesions, cell motility, membrane ruffling, cytokinesis, cell aggregation and smooth muscle contraction. Although it remains to be clarified how Rho regulates these actin cytoskeleton-dependent cell functions, Rho has been shown to regulate various enzymatic activities, including phosphatidylinositol 3-kinase, phosphatidylinositol 4-phosphate 5-kinase and phospholipase D. On the other hand, it has also been shown that activated Rho is co-localized with the ERM family members (Ezrin, Radixin, Moesin) at the membrane ruffling area, cell–cell adhesion sites and cleavage furrows during cytokinesis (Takaishi *et al.*, 1995). The ERM family members have been proposed to play crucial roles in these cell functions through the regulation of actin filament–plasma membrane interactions (Tsukita *et al.*, 1994). Rho has also been shown to regulate the formation of focal adhesions where actin filaments are associated with the plasma membrane (Hall, 1994). However, a direct target molecule of Rho has not yet been definitely identified.

The yeast *Saccharomyces cerevisiae* grows by budding for cell division and the actin cytoskeleton plays a pivotal role in the budding process (Drubin, 1991). There is a strong correlation between the 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). A homolog of the mammalian *RhoA* gene, *RHO1*, has been identified to be an essential gene in *S.cerevisiae* (Madaule *et al.*, 1987). There is also a homolog of mammalian Rho GDI, *RDI1*, in this yeast (Masuda *et al.*, 1994). We have recently shown that cells of a temperature-sensitive mutant of *RHO1* or *RHO1*-depleted mutant stop growing with small-budded cells under the restrictive conditions (Yamochi *et al.*, 1994). Moreover, immunofluorescence microscope study indicates that Rho1p is localized at the cell periphery in the growth site where cortical actin patches are localized, including the presumptive budding site, the bud tip and the cytokinesis site (Yamochi *et al.*, 1994). These results suggest that *RHO1* is involved in the process of bud formation and that Rho1p in yeast also regulates reorganization of actin cytoskeleton, like in mammalian cells. However, a direct target molecule of *RHO1* has not yet been identified in yeast.

In the present work, we have isolated a dominant reversion mutant from a temperature-sensitive mutant strain in which the *RHO1* gene is replaced with mammalian *RhoA*. Molecular cloning of this gene has revealed that the mutation occurred in the *PKC1* gene, which is a yeast homolog of mammalian protein kinase C leading to the

activation of the MAP kinase cascade (Levin *et al.*, 1990; Herskowitz, 1995). Genetic studies indicate that Pkc1p is one of the downstream targets of Rho1p in *S.cerevisiae*.

Results

Isolation of a dominant suppressor mutation of the temperature-sensitive RhoA mutation

We have recently shown that the human *RhoA* gene suppresses the lethality of disruption mutation of the *RHO1* gene (Yamochi *et al.*, 1994). However, the suppression could not be seen at a higher temperature, such as 35°C. Therefore, a yeast strain which carries the *RhoA* gene in place of the *RHO1* gene shows a temperature-sensitive growth phenotype. Since this phenotype is recessive, this temperature sensitivity seems to be caused by a loss of function of *RhoA* at 35°C. It was presumed that a gain of function mutation of a downstream gene of *RHO1* suppressed the temperature sensitivity of the *RhoA* mutant. To isolate such a dominant mutation, a diploid strain, HNY85, which has homozygous *RhoA* mutations was constructed. In this strain, the human *RhoA* gene was expressed under the control of the promoter of the *RHO1* gene. To discriminate between an intragenic suppressor mutation and an extragenic suppressor mutation, one *RhoA* allele was linked with the *URA3* gene, whereas the other *RhoA* allele was linked with the *HIS3* gene in this diploid strain. About 5×10^8 cells were plated and UV irradiated under the conditions where the survival rate was ~10%. Three reversion mutants that grew at 35°C were isolated independently. Cells of one reversion mutant, HNY85-R, were sporulated and subjected to tetrad analysis. Seventeen complete tetrads were recovered and all of them showed 2 +:2 – segregation pattern in terms of growth phenotype at 35°C. Moreover, segregation of the temperature-resistant phenotype was not linked with the Ura^+ or His^+ phenotype. Therefore, it was concluded that a single extragenic dominant mutation suppressed the temperature-sensitive growth phenotype of the *RhoA* mutant. Genetic analysis of two other mutants indicated that these mutations occurred in the same gene.

To clone the dominant mutant gene, a genomic library was constructed from the genomic DNA of a haploid segregant of HNY-85-R, HNY100, in a centromeric vector YCp50 (Rose *et al.*, 1987). A *RhoA* mutant strain, HNY82, which did not carry the suppressor mutation was transformed with the genomic library and five independent transformants which grew at 35°C were isolated. Since all of these transformants showed the plasmid-dependent growth phenotype at 35°C, we concluded that the temperature sensitivity was suppressed by cloned yeast genomic DNAs. Moreover, plasmids recovered through *Escherichia coli* transformation conferred the ability to grow at 35°C on the *RhoA* mutant (Figure 1). Restriction mapping of the recovered plasmids showed that they could be grouped into three types, and that all of them had 0.6 and 2.2 kbp *Eco*RI restriction fragments in common. Therefore, one of the clones, YCp50-DS1, was selected and used for further analysis.

The suppressor gene is PKC1

The 2.2 kbp *Eco*RI restriction fragment of YCp50-DS1 was cloned and sequenced. A search with the obtained

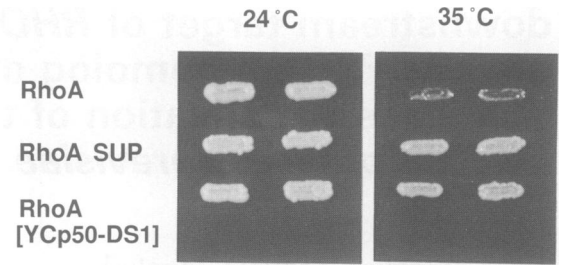

Fig. 1. The *RhoA* temperature-sensitive mutation is suppressed by the cloned dominant suppressor gene. Cells were streaked onto the YPDAU plate and incubated for 3 days at 24°C (left) or at 35°C (right). Strains are the parental, temperature-sensitive *RhoA* mutant, HNY81 (RhoA), the suppressor mutant, HNY100 (RhoA SUP) and HNY81 transformed with YCp50-DS1, which carried the dominant suppressor gene (RhoA [YCp50-DS1]).

sequence against the database revealed that the cloned fragment is a part of the *PKC1* gene which encodes a homolog of a conventional type of mammalian protein kinase C (Levin *et al.*, 1990). To examine whether the suppressor mutation occurred at the *PKC1* locus, the 5.2 kbp *Pst*I–*Xho*I DNA fragment from the wild-type *PKC1* gene was cloned into an integration vector pRS304 which carried the *TRP1* gene as a selection marker (Sikorski and Hieter, 1989). The constructed plasmid was cut with *Nhe*I located in the *PKC1* DNA and integrated into the *PKC1* locus of the *RhoA* mutant strain HNY78. The resultant transformant was crossed with the revertant strain, HNY102, and the diploid was sporulated and tetrad analyzed. All of the recovered complete tetrads (18 tetrads) showed 2 +:2 – segregation pattern for the temperature-sensitive growth phenotype and tryptophan auxotrophy. Moreover, the temperature-sensitive growth phenotype co-segregated with the TRP^+ phenotype in all of the tetrads. This result indicates that the *PKC1* locus is tightly linked with the suppressor mutation locus. To further verify that the *PKC1* gene was itself the suppressor gene, the 4.2 kbp *PKC1* DNA including the 562 bp upstream and 175 bp downstream non-coding regions was amplified by polymerase chain reaction (PCR) using the genomic DNA of the reversion mutant strain, HNY100, as a template. The amplified fragment was cloned into the centromeric vector pRS316. Since the resultant plasmid suppressed the temperature-sensitive growth phenotype of the *RhoA* mutant, and since the wild-type *PKC1* gene did not suppress the temperature-sensitive growth phenotype of the *RhoA* mutant even on a multicopy plasmid, we concluded that the *PKC1* gene was the suppressor gene itself.

The suppressor mutation occurred in the pseudosubstrate site of PKC1

Since the mutation was dominant, the mutation which occurred in *PKC1* seemed to be an activating mutation. To find out substitution of which residue was responsible for the dominant activating trait, the mutation site of *PKC1* was determined. The 260 bp *Nhe*I–*Msc*I fragment in the wild-type *PKC1* gene was replaced with the corresponding fragment of the mutant gene. A centromeric plasmid containing this chimeric *PKC1* gene suppressed the temperature-sensitive growth phenotype of the *RhoA* mutant. Therefore, the mutation site should be located in

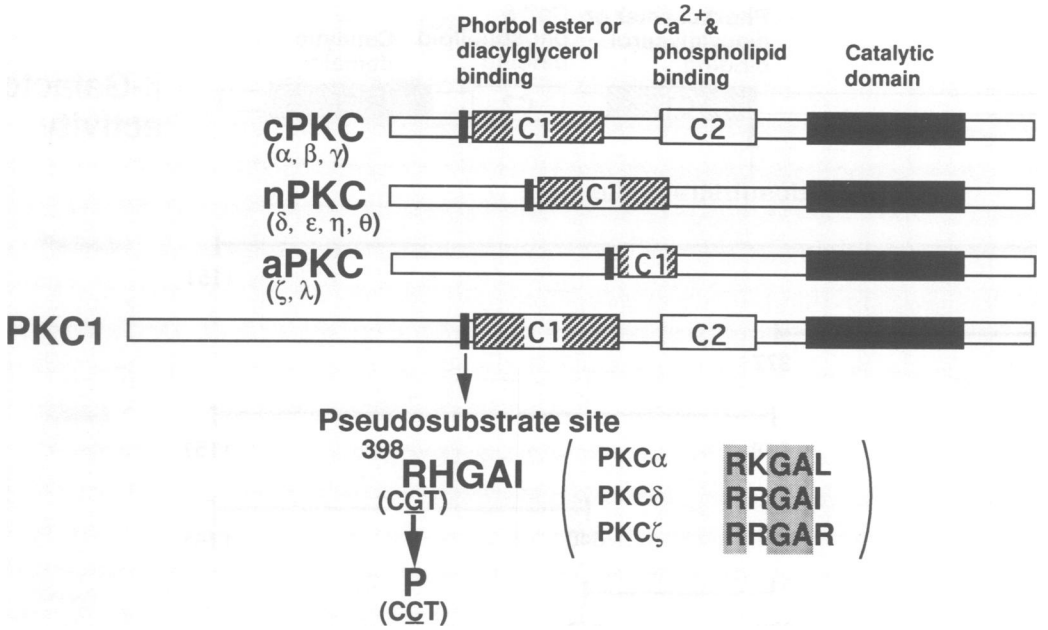

Fig. 2. Schematic representation of PKC1 and mammalian PKC isozymes and a substitution from arginine to proline at amino acid position 398 of Pkc1p. Identical residues between PKC1 and mammalian isozymes are shadowed.

this 260 bp fragment. This fragment encodes amino acid positions 328–414 of Pkc1p. Determination of the nucleotide sequence of this fragment revealed that CGT at nucleotide position 1193 (underlined) was mutated to CCT. This mutation caused an amino acid substitution (arginine to proline) at amino acid position 398 of Pkc1p. Interestingly, this amino acid is located in the pseudosubstrate site of Pkc1p that is RHGAI (Figure 2). This pseudosubstrate site is conserved in the PKC family members from yeast to mammals and it presumably keeps PKC family members in their inactive states when an activating signal is not present (Hug and Sarre, 1993). This result is consistent with the fact that the mutation was dominant activating.

Two-hybrid interactions between Rho1p and Pkc1p

Genetic results described above suggest that Pkc1p may be a downstream target of Rho1p. We tested whether Rho1p interacts with Pkc1p by the two-hybrid method (Fields and Song, 1989). If Pkc1p is actually a target of Rho1p, Pkc1p should interact specifically with GTP-Rho1p. Point mutations which keep Ras in the GTP-bound form (dominant active mutations) or the GDP-bound form (dominant negative mutations) have been reported (Bourne *et al.*, 1991; Farnsworth and Feig, 1991). One dominant activating mutation, Q68L, that corresponds to Q61L in Ras, and one dominant negative mutation, T24N, that corresponds to T17N in Ras, were made in the *RHO1* gene. Both genes inhibited cell growth when introduced into a wild-type strain on a single-copy plasmid, indicating that they actually behaved as dominant mutants *in vivo* (data not shown). Another type of mutation, the effector mutation, is known to inhibit the interaction of Ras with a target molecule (Farnsworth *et al.*, 1991). One such mutation, T42A, that corresponds to T35A in Ras, was made in the *RHO1*(Q68L) gene. The mutant *RHO1* genes and a wild-type *RHO1* gene were fused to the *LexA* DNA-binding domain of a two-hybrid vector pBTM116. To

DNA binding fusion	Transcriptional activation fusion	β-Galactosidase activity
RHO1 (Q68L)	PKC1	
RHO1 (Q68L, T42A)	PKC1	
RHO1 (T24N)	PKC1	
RHO1	PKC1	
RHO1 (Q68L)	Raf	
Ras (G12V)	PKC1	
Ras (G12V)	Raf	

Fig. 3. GTP-Rho1p interacts with Pkc1p(1–1151). Yeast strains expressing LexA-RHO1(Q68L), LexA-RHO1(Q68L, T42A), LexA-RHO1(T24N), LexA-RHO1 and LexA-Ras(G12V) were transformed with a plasmid encoding GAD-PKC1(1–1151). Yeast strains expressing LexA-RHO1(Q68L) and LexA-Ras(G12V) were also transformed with a plasmid encoding VP16-Raf. The resultant transformants were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity.

prevent association of these LexA–Rho1 fusion proteins with membranes, the carboxyl-terminal lipid modification site (CVLL) was deleted in these constructs. A DNA fragment of PKC1 which encodes full-length Pkc1p(1–1151) was fused with the transcriptional activating domain of *GAL4* (GAD) and this plasmid was transformed into yeast strains which carried LexA-RHO1(Q68L), LexA-RHO1(Q68L, T42A), LexA-RHO1(T24N) and LexA-RHO1. The resultant transformants were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for activity of β-galactosidase, which is a reporter enzyme of the two-hybrid method. As shown in Figure 3, GAD-PKC1(1–1151), but not VP16-Raf, interacted with LexA-RHO1(Q68L) to an extent similar to that of the interaction of LexA-Ras(G12V) with VP16-Raf, that is a target of Ras (Vojtek *et al.*, 1993). However, GAD-PKC1(1–1151) did not interact with LexA-RHO1(Q68L, T42A), LexA-RHO1(T24N), LexA-RHO1 or LexA-Ras(G12V). These

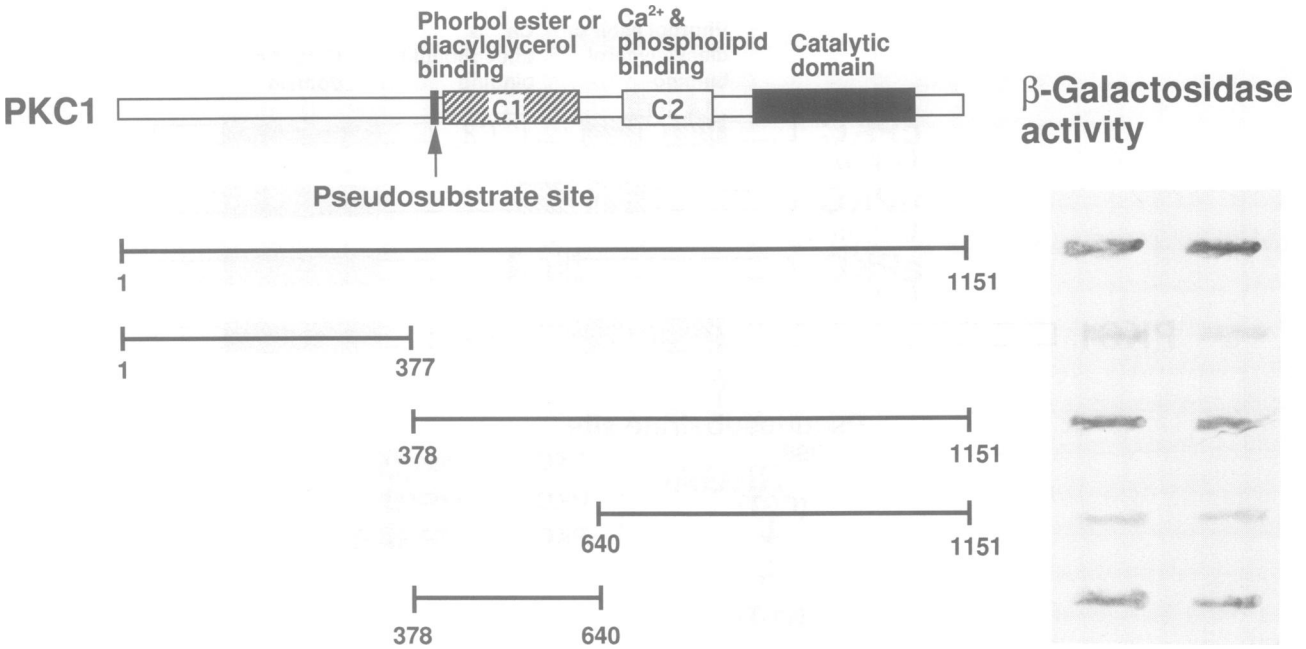

Fig. 4. GTP-Rho1p interacts with Pkc1p(377–640) containing the pseudosubstrate site and the C1 domain. DNA fragments of *PKC1* were fused with GAD and the resultant plasmids were transformed into the yeast strain expressing LexA-RHO1(Q68L). The resultant transformants were stained for β-galactosidase activity.

results indicate that Pkc1p specifically bound to GTP-Rho1p and that the T42A effector mutation inhibited this interaction.

The domain of Pkc1p that interacted with Rho1p was determined in the next experiment. DNA fragments of *PKC1* which encode various truncated Pkc1ps were fused with GAD and these GAD–PKC1 fusions were transformed into a yeast strain which carried LexA-RHO1(Q68L). The results of staining for β-galactosidase activity indicate that GTP-Rho1p interacted with the 264 amino acid region (377–640) of Pkc1p (Figure 4). Since this region of Pkc1p contains the pseudosubstrate site (amino acid positions 398–406) and the C1 regulatory domain (amino acid positions 415–532) (Levin *et al.*, 1990), the pseudosubstrate site may have some role in the Pkc1p–Rho1p interactions. Although we examined whether the R398P substitution affects binding of Pkc1p to Rho1p, GAD-PKC1(R398P)(1–1151) interacted with LexA-RHO1(Q68L) to an extent similar to that of the interaction of GAD-PKC1(1–1151) with LexA-RHO1(Q68L) (data not shown).

The RhoA mutant is impaired in the PKC1-mediated signaling pathway

The result that the *PKC1*(R398P) mutation suppressed the *RhoA* mutation suggests that the *RhoA* mutant is impaired in the *PKC1*-mediated signaling pathway. This point was examined genetically. *PKC1* is involved in the regulation of osmotic integrity: growth deficiency of the *pkc1* mutant can be suppressed by the presence of an osmotic stabilizer such as 1 M sorbitol in the medium (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). We therefore tested whether the *RhoA* mutant showed a similar phenotype. As shown in Figure 5A, the *RhoA* mutant grew at 35°C when 1 M sorbitol was present in the medium, suggesting that *pkc1* and *RhoA* are deficient in the common signaling pathway. Genetic studies have shown that one of the downstream signaling pathways of *PKC1* is the MAP kinase cascade which is composed of *BCK1*, *MKK1/MKK2* and *MPK1* in this order (Herskowitz, 1995). *BCK1*, *MKK1/MKK2* and *MPK1* are homologs of MAP kinase kinase kinase (MEKK), MAP kinase kinase (MEK) and MAP kinase (MAPK) in mammalian cells, respectively. To find out whether the *RhoA* mutant is deficient in the activation of the MAP kinase cascade, it was tested whether the activation or overexpression of a member of the MAP kinase cascade suppressed the temperature-sensitive growth phenotype of the *RhoA* mutant. As shown in Figure 5B, both dominant activating mutation of *MKK1* (*MKK1-6*) and overexpression of *MPK1* suppressed the temperature-sensitive phenotype of the *RhoA* mutant. These results indicate that the *RhoA* mutant is impaired in the activation of *PKC1* at 35°C that leads to the activation of the MAP kinase cascade.

RHO1 regulates another signaling pathway

It has been shown that the addition of 1 M sorbitol to the medium suppresses the lethality of the *pkc1* disruption mutant (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992), but not that of the *rho1* disruption mutant (Yamochi *et al.*, 1994). *PKC1*(R398P) or *MKK1-6* did not suppress the lethal phenotype of the disruption mutant of *RHO1* either (data not shown). These results suggest that *PKC1* is not a sole downstream target of *RHO1*. If there is another target of Rho1p in addition to Pkc1p in *S.cerevisiae*, there should be mutants of the *RHO1* gene specifically impaired in the interaction with either another target or Pkc1p. Based on this assumption, the *RhoA* mutant appears to be deficient only in the activation of the *PKC1*-mediated MAP kinase pathway. To address this question further, it was examined whether *PKC1*(R398P) suppressed the various temperature-sensitive mutants of

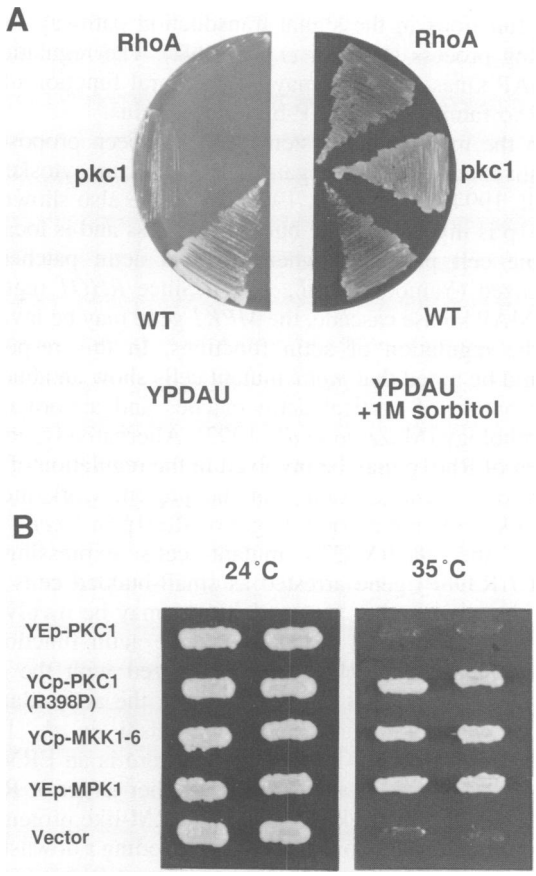

Fig. 5. The *RhoA* mutant is impaired in the *PKC1*-mediated signaling pathway. (**A**) The temperature-sensitive growth phenotype of the *RhoA* mutant is suppressed by 1 M sorbitol. The *RhoA* mutant, HNY78 (RhoA), the *pkc1* mutant, SYT11-12A (pkc1), and a wild-type strain, OHNY1 (WT), were streaked onto a YPDAU plate (left) and a YPDAU plate supplemented with 1 M sorbitol (right). The plates were then incubated for 2 days at 35°C. (**B**) Activation of the MAP kinase cascade suppresses the temperature sensitivity of the *RhoA* mutation. The temperature-sensitive *RhoA* mutant, HNY82, was transformed with various plasmids and the resultant transformants were streaked onto YPDAU plates. The plates were then incubated for 3 days at 24°C (left) or at 35°C (right). Plasmids are (from top) YEp352 carrying *PKC1* (YEp-PKC1), YCp50 carrying *PKC1*(R398P) [YCp-PKC1(R398P)], YCp50 carrying *MKK1-6* (YCp-MKK1-6), Yeplac195 carrying *MPK1* (YEp-MPK1) and YEp352 (Vector). YEp352 carrying *PKC1* (YEp352-PKC1), YCp50 carrying *MKK1-6* (YCp50-MKK1-6) and Yeplac195 carrying *MPK1* (p195-MPK1) are gifts from K.Irie and K.Matsumoto.

RHO1. We isolated three recessive temperature-sensitive mutations that occurred at the effector domain of *RHO1*. These are *rho1*(V43T), *rho1*(F44Y) and *rho1*(E45I). Strains which carried each of these mutations were transformed with YCp50-PKC1(R398P) and the resultant transformants were tested for growth at 35°C. As shown in Figure 6, YCp50-PKC1(R398P) suppressed the temperature sensitivity of the *rho1*(F44Y) or *rho1* (E45I) mutant, but not that of the *rho1*(V43T) mutant. This result indicates that the *rho1*(F44Y) and *rho1*(E45I) mutations, like the *RhoA* mutation, are specifically impaired in the activation of the *PKC1*-mediated MAP kinase pathway, whereas the *rho1*(V43T) mutation is impaired in the activation of at least another signaling pathway. Therefore, there should be another signaling pathway that is regulated by *RHO1*, in addition to the *PKC1*-mediated MAP kinase pathway.

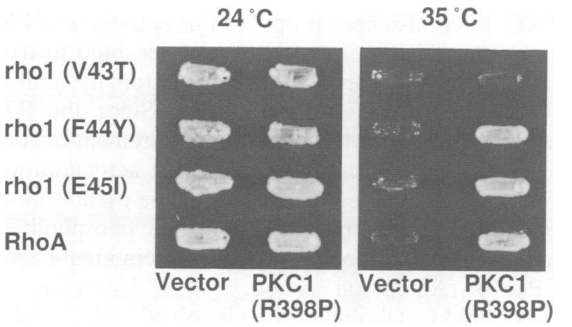

Fig. 6. The *PKC1*(R398P) mutation suppresses the temperature-sensitive growth phenotype of the *rho1* effector mutants. YCp50 (Vector) or YCp50-DS1 carrying *PKC1*(R398P) [PKC1(R398P)] was transformed into the *rho1* effector mutant strains, HNY93 [rho1(V43T)], HNY95 [rho1(F44Y)] and HNY97 [rho1(E45I)], and the *RhoA* mutant strain, HNY81 (RhoA). The resultant transformants were streaked onto YPDAU plate and the plates were then incubated for 2 days at 24°C (left) or at 35°C (right).

Discussion

Isolation of a dominant suppressor mutation from the *RhoA* mutant has revealed that a dominant active mutation of *PKC1*, *PKC1*(R398P), suppresses the *RhoA* mutation. This result indicates that Rho1p is an upstream regulator of Pkc1p and that RhoA is deficient in this function at 35°C. The results of genetic studies of *pkc1* and *rho1* mutants are very consistent with the conclusion that *PKC1* and *RHO1* function in the same signal transduction pathway. The *pkc1* mutants arrest as small-budded cells under the restrictive conditions and show a cell lysis phenotype that can be suppressed by an osmotic stabilizer such as 1 M sorbitol (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). We have seen the same phenotypes with *rho1* mutants (Yamochi *et al.*, 1994). Moreover, we have isolated the *PPZ2* gene encoding a type 1-related protein phosphatase as a multicopy suppressor of the *rho1–104* temperature-sensitive mutant (Musha *et al.*, unpublished results). The *PPZ2* gene has also been isolated as a multicopy suppressor of the *pkc1* mutant (Lee *et al.*, 1993a), indicating that *RHO1* is functionally relevant to *PKC1*.

The result that *PKC1*(R398P) suppressed the *RhoA*, *rho1*(F44Y) and *rho1*(E45I) mutations indicates that *PKC1* is a downstream component of *RHO1*. Moreover, the results of two-hybrid analysis strongly suggest that Pkc1p is a downstream target of Rho1p. The domain of Pkc1p that interacted with Rho1p was located to the 263 amino acid fragment from amino acid position 377 to 640 of Pkc1p. This domain contains the pseudosubstrate site and the C1 domain which has two zinc finger-like motifs (Levin *et al.*, 1990). It is noteworthy that the domain of Raf which interacts with Ras is also located at the amino-terminal region adjacent to the zinc finger-containing domain (Chuang *et al.*, 1994). The R398P substitution identified in this study occurred at the pseudosubstrate site of Pkc1p. It has been reported that the alteration of the pseudosubstrate site causes the constitutive activation of PKC both in yeast Pkc1p (Watanabe *et al.*, 1994) and in mammalian PKC (Pears *et al.*, 1990). Based on these results, the pseudosubstrate site is presumed to bind to the catalytic domain and inhibit the constitutive activation

of PKC. It has also been proposed that activators of PKCs, such as diacylglycerol and phorbol ester, bind to the C1 domain and release the catalytic domain from the inhibitory function of the pseudosubstrate site. Since the R398P substitution of Pkc1p bypassed the requirement of Rho1p, Rho1p may also function as one of the activators which inhibit the negative regulatory role of the pseudosubstrate site. It has recently been reported that phospholipid or diacylglycerol does not activate the protein kinase activity of Pkc1p (Antonsson *et al.*, 1994). However, some phospholipid metabolite may be involved in the regulation of Pkc1p, since the *STT4* gene, encoding phosphatidylinositol 4-kinase, has been shown to function upstream of *PKC1* (Yoshida *et al.*, 1994). It remains to be clarified how Rho1p and phospholipid coordinately regulate the function of Pkc1p.

The results showing that Rho1p binds to Pkc1p suggest that GTP-Rho1p may activate the protein kinase activity of Pkc1p. However, we have not yet succeeded in activating Pkc1p by GTP-Rho1p in a cell-free system using Pkc1p immunoprecipitated from yeast lysate or purified from yeast cells overexpressing *PKC1*. It is possible that these Pkc1 proteins have already been activated *in vivo* or during immunoprecipitation. Further studies should be undertaken to clarify this point. Although Rho1p may bind to Pkc1p to activate its protein kinase activity, there is another possibility for the function of Rho1p: Rho1p may bind to Pkc1p to localize it to the cellular site of action, presumably the actively growing site of the plasma membrane including the bud tip or the cytokinesis site where Rho1p is localized (Yamochi *et al.*, 1994). In the case of the Ras–Raf interactions, this point has been controversial. It has been shown that targeting of c-Raf-1 to the plasma membrane by adding the CAAX lipid modification site of Ras bypasses the requirement of Ras for the activation of c-Raf-1 (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). However, we have recently shown that GTP-Ras activates the protein kinase activity of B-Raf in a cell-free system where there are no membrane components (Yamamori *et al.*, 1995). We are currently investigating whether Rho1p functions to activate the protein kinase activity of Pkc1p or to localize Pkc1p to the site of action.

Genetic studies indicate that *PKC1* regulates the MAP kinase pathway (Lee and Levin, 1992; Irie *et al.*, 1993; Lee *et al.*, 1993b). The results that an activated mutant gene of *MKK1* (*MKK1-6*) or overexpression of *MPK1* suppressed the *RhoA* mutation suggest that the *RHO1* gene regulates the MAP kinase pathway through *PKC1*. It has been established that Raf protein kinase, a target of Ras in mammalian cells, is also an upstream regulator of a MAP kinase cascade (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992). In the yeast *Schizosaccharomyces pombe*, Ras1p also regulates the MAP kinase pathway through Byr2p which is a homolog of MEKK (Wang *et al.*, 1991). Recently, the target proteins of other Rho family members, including Cdc42 and Rac1, have been reported in mammalian systems (Manser *et al.*, 1994; Martin *et al.*, 1995). These target proteins are protein kinases that are homologous to yeast Ste20p protein kinase. Although it has not been shown whether the Ste20p-like protein kinases regulate a MAP kinase cascade in mammalian cells, it was established that Ste20p in yeast is an upstream regulator of the MAP kinase cascade that functions in the signal transduction pathway for the mating process (Leberer *et al.*, 1992). The regulation of a MAP kinase cascade may be a general function of Ras or Rho family small GTP-binding proteins.

In the mammalian system, Rho has been proposed to be involved in the reorganization of actin cytoskeleton (Hall, 1994; Takai *et al.*, 1995). We have also shown that Rho1p is involved in the budding process and is localized at the cell periphery where cortical actin patches are localized (Yamochi *et al.*, 1994). Since *RHO1* regulates the MAP kinase cascade, the *MPK1* gene may be involved in the regulation of actin functions. In this respect, it should be noted that *mpk1* mutant cells show an abnormal distribution of cortical actin patches and abnormal cell morphology (Mazzoni *et al.*, 1993). Alternatively, another target of Rho1p may be involved in the regulation of actin functions. Genetic studies in the present work indicate that Pkc1p is not an only target of Rho1p in *S.cerevisiae*. Since the *rho1*(V43T) mutant cells expressing the *PKC1*(R398P) gene arrested as small-budded cells (data not shown), another target of Rho1p may be involved in the budding process that requires the actin function. In mammalian cells, RhoA is co-localized with the ERM family members that are regulators of the actin filament/plasma membrane association (Takaishi *et al.*, 1995). Although we do not know whether there is an ERM-like protein in *S.cerevisiae*, Pkc1p or another target of Rho1p might function to link Rho1p to an ERM-like protein. We are currently trying to isolate genes encoding a downstream component of Mpk1p or another target of Rho1p.

Materials and methods

Strains and media

Yeast strains used in this study are listed in Table I. An *E.coli* strain, DH5α, was used for the construction and propagation of plasmids. Yeast strains were grown in YPDAU medium that contained 2% glucose, 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco), 0.04% adenine and 0.02% uracil. Standard yeast genetic techniques were used as described previously (Sherman *et al.*, 1986). Yeast transformations were performed by the lithium acetate method (Ito *et al.*, 1983; Gietz *et al.*, 1992). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco). Amino acids were used to supplement SD medium when required.

Molecular biological techniques

Standard molecular biological techniques were used for the construction of plasmids, PCR and DNA sequencing (Sambrook *et al.*, 1989). PCR was performed using GeneAmp PCR System 2400 (Perkin Elmer) and DNA sequences were determined using ALFred DNA sequencer (Pharmacia Biotech, Inc.).

Construction of the temperature-sensitive RhoA mutants

The 1.8 kbp *Kpn*I–*Hin*dIII fragment containing the 582 bp fragment of the *RhoA* coding region, the 140 bp fragment of the transcriptional terminator of the *TDH3* gene (McAlister and Holland, 1985) and the 1.1 kbp fragment of the *URA3* gene in this order were cut out from a plasmid pKT10-GAL-RhoA-URA3. This fragment was cloned into the *Kpn*I–*Hpa*I site of pRS316-RHO1(KpnI) to replace the *RHO1* coding region with the RhoA-URA3 fragment to construct a plasmid pRS316-RhoA-URA3. The 3.2 kbp *Sac*I–*Sma*I fragment containing the *RhoA-URA3* fragment, along with the 1.0 kbp upstream and 0.4 kbp downstream non-coding regions of *RHO1*, was isolated from pRS316-RhoA-URA3 and this fragment was used to transform a strain DHNY101 to replace the *rho1*::*HIS3* allele with the *RhoA-URA3* fragment. The resulting transformants showed the Ura$^+$, His$^-$ and temperature-sensitive growth phenotypes, and one of the transformants was selected and named HNY78. Tetrad analysis indicates that the temperature-sensitive *RhoA* mutation was linked with the *URA3* gene. To make a strain, HNY81, in

Table I. Yeast strains used in this study

OHNY1	MATa *ura3 leu2 trp1 his3 ade2*
OHNY2	MATα *ura3 leu2 trp1 his3 ade2*
DHNY101	MATa *ura3 leu2 trp1 his3 ade2 rho1::HIS3* (YCp-LEU2-GAL1-RHO1)
HNY78	MATa *ura3 leu2 trp1 his3 ade2 rho1::RhoA-URA3*
HNY81	MATa *ura3 leu2 trp1 his3 ade2 rho1::RhoA-HIS3*
HNY82	MATα *ura3 leu2 trp1 his3 ade2 rho1::RhoA-HIS3*
HNY85	MATa/MATα *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 rho1::RhoA-URA3/rho1::RhoA-HIS3*
HNY85-R	MATa/MATα *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 rho1::RhoA-URA3/rho1::RhoA-HIS3 PKC1*(R398P)/*PKC1*
HNY93	MATa *ura3 leu2 trp1 his3 ade2 rho1*(V43T)
HNY95	MATa *ura3 leu2 trp1 his3 ade2 rho1*(F44Y)
HNY97	MATa *ura3 leu2 trp1 his3 ade2 rho1*(E45I)
HNY100	MATa *ura3 leu2 trp1 his3 ade2 rho1::RhoA-HIS3 PKC1*(R398P)
HNY102	MATα *ura3 leu2 trp1 his3 ade2 rho1::RhoA-HIS3 PKC1*(R398P)
SYT11-12A	MATa *ura3 leu2 his3 ade8 met3 stt1-1*
L40	MATa *trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*

Strains used in this study are isogenic except SYT11-12A and L40.

which *RhoA* is linked with *HIS3*, the *URA3* gene of HNY78 was disrupted with the 1.8 kbp fragment of the *HIS3* gene. HNY81 was crossed with a wild-type strain, OHNY2, to obtain the *MATα RhoA-HIS3* strain, HNY82. A diploid strain, HNY85, was made by crossing HNY78 with HNY82. HNY85 was used to isolate a dominant reversion mutant of the *RhoA* mutation.

Molecular cloning of PKC1(R398P)

A revertant strain, HNY100, was grown in YPDAU medium and the total genomic DNA was isolated according to the method described by Guthrie and Fink (1991). The DNA was partially digested with *Sau*3AI and the digested DNA was gel purified using Agarase I (Takara Co., Ltd) according to the manual recommended by the manufacturer. The isolated DNA fragments were ligated with the *Bam*HI-digested centromeric vector YCp50 (Rose *et al.*, 1987) and the ligation mixture was transformed into an *E.coli* strain DH5α. Approximately 15 000 transformants were obtained and pooled. Plasmid DNA prepared from this pool was transformed into the *RhoA* mutant strain, HNY81, and five transformants that grew at 35°C were obtained. Plasmid DNAs were recovered from these transformants through *E.coli* transformation. Recovered plasmids had an overlapping genomic DNA and conferred the ability to grow at 35°C on HNY81.

Plasmid construction for the two-hybrid method

The *RHO1* coding region was amplified by PCR using sense-strand and antisense-strand primers, GTGTGAATTCATGTCACAACAAGTTGGTAAC and TTAAGTCGACTCACTTCTTCTTCTTTTCAGT, respectively. The carboxyl-terminal lipid modification site of Rho1p, CVLL, was deleted in this construction. The amplified fragment was cut with *Eco*RI and *Sal*I (underlined), and cloned into the *Eco*RI–*Sal*I site of pBTM116 to make a fusion with the LexA DNA binding protein. Point mutations in the *RHO1* coding region, CAA to TTA substitution at nucleotide positions 202–204, ACT to GCT and CAA to TTA substitutions at nucleotide positions 124–126 and 202–204, respectively, and ACA to AAC substitution at nucleotide positions 70–72, were introduced into this *RHO1* gene by the method described previously (Higuchi, 1989) to make LexA-RHO1(Q68L), LexA-RHO1(Q68L, T42A) and LexA-RHO1(T24N), respectively. The occurrence of each mutation was verified by DNA sequencing. DNA fragments of *PKC1* were cloned into pGAD424 (Clontech Laboratories, Inc.) to make fusions with *GAL4* transcriptional activation domain (GAD). The 3482 bp *Sal*I–*Pst*I PCR fragment of *PKC1* was cloned into the *Sal*I–*Pst*I site of pGAD424 to make GAD-PKC1(1–1151). The 2340 bp *Hpa*I–*Pst*I fragment was deleted from GAD-PKC1(1–1151) to make GAD-PKC1(1–377). The 792 bp *Hpa*I–*Stu*I fragment was cloned into the *Sal*I(filled in)–*Pst*I(filled in) site of pGAD424 to make GAD-PKC1(378–640). The 1142 bp *Sal*I–*Hpa*I fragment was deleted from GAD-PKC1(1–1152) to make GAD-PKC1(378–1151). LexA–RHO1 fusion plasmids were transformed into a yeast strain L40 and the resultant transformants were transformed by GAD–PKC1 fusion plasmids. Cells of each transformant were placed on the nitrocellulose filter and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for β-galactosidase activity as described previously (Vojtek *et al.*, 1993).

Isolation of temperature-sensitive Rho1 effector mutants

The plasmid pRS316-RHO1 carries the 3.2 kbp *Sma*I–*Bgl*II fragment of the *RHO1* gene containing the 2.1 kbp upstream non-coding region, the 627 bp coding region and the 487 bp downstream non-coding region. There are two *Sna*BI sites in this 3.2 kbp fragment. The former site is located 9 bp upstream of nucleotide position 124 in the *RHO1* coding region, which corresponds to amino acid position 42 of Rho1p. This residue is threonine in the putative effector domain of Rho1p, YVPTVFENY (amino acid positions 39–47). The latter site is located 79 bp downstream of the stop codon of the *RHO1* coding region. The latter *Sna*BI site (TACGTA) was mutated to TACGTG in the plasmid pRS316-RHO1-m1 by the method described previously (Higuchi, 1989). To introduce a mutation at the effector domain, five sense-strand primers, p1 (GAAGTCTACGTANNNACTGTCTTTGAAAACTATG), p2 (GAAGTCTACGTACCANNNGTCTTTGAAAACTATGTA), p3 (GAAGTCTACGTACCAACTNNNTTTGAAAACTATGTAGCAG), p4 (GAAGTCTACGTACCAACTGTCNNNGAAAACTATGTAGCAGATG) and p5 (GAAGTCTACGTACCAACTGTCTTTNNNAACTATGTAGCAGATGTTG), were synthesized. The underlined sequence in each primer corresponds to the former *Sna*BI site. An antisense-strand primer, (CCCTCGAGGTCGACGGTATCG), which corresponds to the multi-cloning site sequence of pRS316 downstream of the *RHO1* gene, was also synthesized. Each sense-strand primer and antisense-strand primer was used for PCR using pRS316-RHO1-m1 as a template, and the amplified fragments were cut with *Sna*BI–*Hin*dIII. The resultant fragments were cloned into the *Sna*BI–*Hin*dIII site of pRS316-RHO1-m1. The 3.2 kbp *Sac*I–*Hin*dIII DNA fragments containing the effector mutations of *RHO1* were isolated and transformed into a strain DHNY101 to replace the *rho1::HIS3* allele with the *rho1* effector mutant alleles. From the resultant transformants, His^- and temperature-sensitive mutant clones were searched for and three such mutants, HNY93, HNY95 and HNY97, were isolated. Determination of the nucleotide sequence identified GTC to ACA mutation at nucleotide positions 127–129 (V43T amino acid substitution), TTT to TAT mutation at nucleotide positions 130–132 (F44Y amino acid substitution) and GAA to ATA mutation at nucleotide positions 133–135 (E45I amino acid substitution) in the *rho1* genes of HNY93, HNY95 and HNY97, respectively.

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