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Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*

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The *RHO1* gene encodes a homolog of the mammalian RhoA small GTP binding protein in the yeast *Saccharomyces cerevisiae*. Rho1p is localized at the growth site and is required for bud formation. Multicopy suppressors of a temperature-sensitive, dominant negative mutant allele of *RHO1*, *RHO1*(G22S, D125N), were isolated and named *ROM* (*RHO1* multicopy suppressor). Rom1p and Rom2p were found to contain a DH (Dbl homologous) domain and a PH (pleckstrin homologous) domain, both of which are conserved among the GDP/GTP exchange proteins (GEPs) for the Rho family small GTP binding proteins. Disruption of *ROM2* resulted in a temperature-sensitive growth phenotype, whereas disruption of both *ROM1* and *ROM2* resulted in lethality. The phenotypes of Δ*rom1*Δ*rom2* cells were similar to those of Δ*rho1* cells, including growth arrest with a small bud and cell lysis. Moreover, the temperature-sensitive growth phenotype of Δ*rom2* was suppressed by overexpression of *RHO1* or *RHO2*, but not of *CDC42*. The glutathione-*S*-transferase (GST) fusion protein containing the DH domain of Rom2p showed the lipid-modified Rho1p-specific GDP/GTP exchange activity which was sensitive to Rho GDP dissociation inhibitor. These results indicate that Rom1p and Rom2p are GEPs that activate Rho1p in *S.cerevisiae*.

Keywords: GDP/GTP exchange protein/Rho/*S.cerevisiae*

Introduction

The Rho family belongs to the small GTP binding 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, -B and -C. Evidence is accumulating that, through reorganization of the actin cytoskeleton, Rho regulates 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. However, it remains to be clarified how Rho regulates reorganization of actin filaments.

Rho has two interconvertible forms: GDP-bound inactive and GTP-bound active forms (Hall, 1994; Takai *et al.*, 1995). The GDP-bound form is converted to the GTP-bound form by the GDP/GTP exchange reaction which is stimulated by GDP/GTP exchange proteins (GEPs) and is inhibited by GDP dissociation inhibitors (GDIs). The GTP-bound form is converted to the GDP-bound form by the GTPase reaction which is regulated by GTPase activating proteins (GAPs). Two GDIs, including Rho GDI (Fukumoto *et al.*, 1990; Ueda *et al.*, 1990) and Ly/D4 GDI (Lelias *et al.*, 1993; Scherle *et al.*, 1993), and four GEPs, including Smg GDS (Yamamoto *et al.*, 1990; Kaibuchi *et al.*, 1991), Dbl (Hart *et al.*, 1991; Yaku *et al.*, 1994), Ost (Horii *et al.*, 1994) and Lbc (Toksoz and Williams, 1994) have so far been reported to be active on Rho. These GEPs, except Smg GDS, share two conserved domains: one is the DH (Dbl homologous) domain, which is supposed to possess the GDP/GTP exchange activity for Rho (Hart *et al.*, 1994), and the other is the PH (pleckstrin homologous) domain, which is found in many signaling and cytoskeletal proteins and is suggested to be responsible for localization to the plasma or organellar membranes (Musacchio *et al.*, 1993; Gibson *et al.*, 1994). Rho is found in both cytosol and membrane fractions. Rho in the cytosol fraction was shown to be complexed with Rho GDI, indicating that Rho in this fraction is in the inactive state (Takai *et al.*, 1995). Therefore, it is likely that Rho complexed with Rho GDI in the cytosol is released from the inhibitory action of Rho GDI and is activated by its GEPs at the plasma membrane. However, the GEPs for Rho themselves have been shown not to be sufficient to activate Rho complexed with Rho GDI (Kikuchi *et al.*, 1992; Yaku *et al.*, 1994). It remains to be clarified how Rho is activated.

The budding yeast *Saccharomyces cerevisiae*, possessing the Rho family members, including *RHO1*, *RHO2* (Madaule *et al.*, 1987), *RHO3*, *RHO4* (Matsui and Toh-E, 1992) and *CDC42* (Adams *et al.*, 1990; Johnson and Pringle, 1990), offers an attractive system to solve the problems described above, since a powerful molecular genetical approach is applicable. Cells of this yeast grow by budding for cell division, and the actin cytoskeleton plays a pivotal role in the budding process (Drubin, 1991). *RHO1* is a homolog of the mammalian RhoA gene and has been shown to be an essential gene in *S.cerevisiae* (Madaule *et al.*, 1987). We have shown recently that cells of *rho1* mutants stop growing with small budded cells under restrictive conditions (Yamochi *et al.*, 1994). Moreover, immunofluorescence microscopic studies indicate that Rho1p is localized at 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 also regulates reorganization of the actin cytoskeleton as in mammalian cells. We have shown recently that Rho1p performs its functions through at least Pkc1p, which is a homolog of mammalian protein kinase C, as a downstream target of Rho1p (Nonaka *et al.*, 1995). Concerning the upstream regulators of *RHO1*, recently we have purified a homolog of Rho GDI in yeast, cloned its gene and named it *RDI1* (Masuda *et al.*, 1994). However, the gene for a GEP for *RHO1* has not yet been cloned in yeast.

Accumulating evidence indicates that there is a small GTP binding protein cascade operating in the budding process in *S.cerevisiae* (Chant and Stowers, 1995). In the budding process, Rsr1p/Bud1p, a homolog of mammalian Rap1, selects the site for budding, Cdc42p initiates the budding site assembly, and then Rho1p promotes the bud formation. Therefore, in this cascade, it is presumed that the GTP-bound form of Rsr1p/Bud1p activates Cdc42p and then the GTP-bound form of Cdc42p activates Rho1p. In accordance with this model, it has been shown recently that the GTP-bound form of Rsr1p/Bud1p binds directly to Cdc24p, which is a GEP for Cdc42p (Zheng *et al.*, 1995a). However, the linkage between Cdc42p and Rho1p has not yet been established at a molecular level. To this end, the gene for a GEP for Rho1p should be cloned.

In the present work, we have cloned two homologous genes, *ROM1* and *ROM2*, as multicopy suppressors of a temperature-sensitive dominant negative mutation of *RHO1*. Genetic and biochemical studies indicate that Rom1p and Rom2p are Rho1p-specific GEPs in *S.cerevisiae*.

Results

Isolation of a temperature-sensitive dominant negative mutation of RHO1, RHO1(G22S, D125N)

It has been reported that an amino acid substitution of glycine at position 22 of Ras2p results in a dominant negative phenotype (Powers *et al.*, 1989). This glycine is located in the amino acid sequence, GXXXXGK, which is highly conserved among small GTP binding proteins of the Ras superfamily, including Rho1p. By analogy, it was presumed that a corresponding mutation of *RHO1* would also result in a dominant negative phenotype. An analogous mutation was introduced into *RHO1* by PCR mutagenesis, and the resultant *RHO1*(G22S) gene was cloned into pRS316 (*URA3*, CEN6). Since transformation of a wild-type yeast strain OHNY1 with pRS316-RHO1(G22S) gave no transformants, *RHO1*(G22S) seemed to behave as a dominant lethal mutation (data not shown). To make this *RHO1*(G22S) mutant gene amenable to genetic analyses, a temperature-sensitive mutation of *RHO1*(G22S) was searched for. Since a temperature-sensitive *RHO1*(G22S) protein is active at a lower temperature, a wild-type strain carrying the temperature-sensitive *RHO1*(G22S) gene would show a cold-sensitive growth phenotype. Such a mutation, *RHO1*(G22S, D125N), was isolated by the hydroxylamine mutagenesis method as described in Materials and methods. It is noteworthy that the aspartic acid at amino acid position 125 in Rho1p corresponds to GNKXD, which is a guanine base binding site conserved among small GTP binding proteins of the Ras superfamily. Cells of OHNY1 carrying pRS316-RHO1(G22S, D125N) arrested with tiny or small budded cells (60–70% of ~300 cells counted) at 25°C. This phenotype is similar to that of *rho1* mutants, indicating that *RHO1*(G22S, D125N) behaved as a dominant negative mutation at 25°C.

Isolation of ROM genes, multicopy suppressors of RHO1(G22S, D125N)

To isolate genes functionally relevant to *RHO1*, we isolated multicopy suppressors of *RHO1*(G22S, D125N). OHNY1 carrying pRS316-RHO1(G22S, D125N) and pRS315-RHO1(G22S, D125N) were transformed with yeast genomic DNA libraries constructed in episomal vectors, YEp13 (*LEU2*) and YEp24 (*URA3*), respectively. Transformants were selected on SD –leu –ura medium at 25°C for 4–10 days. Of 180 000 transformants screened, ~2000 transformants formed colonies at 25°C with various growing rates. Since many transformants which grew as quickly as wild-type cells had been found to possess a rearrangement or a mutation in the *RHO1*(G22S, D125N) gene itself, we selected transformants with slower growing rates, and these transformants were analyzed further. Of ~200 transformants tested, 105 transformants showed the library plasmid-dependent growth phenotype at 25°C. Library plasmids were recovered from these transformants through *Escherichia coli* transformation, and 94 clones were found to confer the ability to grow at 25°C on OHNY1 carrying pRS316-RHO1(G22S, D125N) or pRS315-RHO1(G22S, D125N). Restriction enzyme mapping and Southern blot analyses revealed that 50 and five clones encoded *RHO1* and *RHO2*, respectively. One clone encoded *PKC1*, which is a yeast homolog of protein kinase C and is one of the downstream targets of Rho1p (Nonaka *et al.*, 1995). The other 38 clones comprised five genes that turned out to be new or poorly characterized and were designated *ROM1*, *ROM3*, *ROM4*, *ROM5* and *ROM6* (*RHO1* multicopy suppressor). *ROM1* consisted of one clone, YEp24-ROM1, and was characterized further in this study.

YSCL8039.3 is homologous to ROM1 and functions as a ROM gene

The deletion mapping indicated that *ROM1* was located in the 3.8 kb *Sal*I–*Cla*I fragment (Figure 1). DNA sequencing of the fragment revealed that the *ROM1* coding region was truncated at its N-terminal region. Thus, the insert DNA encoded the truncated Rom1p, ΔN-Rom1p, of 1013 amino acid residues. Searches for sequence homology to ΔN-Rom1p using BLAST and FASTA programs and the GenBank database revealed that a predicted protein, encoded by *YSCL8039.3* (GenBank accession number U19103) on chromosome XII of *S.cerevisiae*, was significantly homologous to ΔN-Rom1p. A 5.3 kb DNA fragment, containing the 660 bp upstream region, 4071 bp open reading frame (ORF) and 550 bp downstream region of *YSCL8039.3*, was amplified by PCR and was cloned into YEp352. The resulting plasmid suppressed the cold-sensitive growth phenotype of OHNY1 carrying pRS315-RHO1(G22S, D125N) (Figure 2). Thus, *YSCL8039.3* was named *ROM2* and was characterized further with *ROM1*.

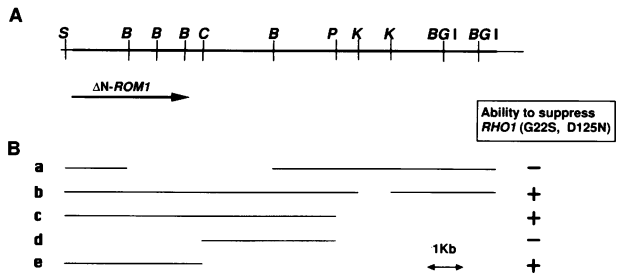

Fig. 1. Restriction enzyme map and deletion mapping of *ROM1*. (**A**) Restriction enzyme map of the insert DNA. The thick line represents the 12 kb original genomic DNA fragment cloned into YEP24-ROM1, whereas the thin lines represent portions of the vector YEp24. *S*, *Sal*I; *B*, *Bgl*II; *K*, *Kpn*I; *BG*I, *Bsr*GI; *P*, *Pvu*II; *C*, *Cla*I. The *Cla*I site is not unique in the insert DNA. The extent and direction of the *ROM1* ORF truncated at its N-terminal region (ΔN-*ROM1*) are shown by an arrow. (**B**) Deletion mapping of *ROM1*. Various deletion fragments of the insert DNA were cloned into YEp24 or YEp352. The constructed plasmids were transformed into OHNY1 carrying pRS315-RHO1(G22S, D125N) to examine their ability to suppress the cold-sensitive growth phenotype. a, YEp24-ROM1ΔBglII; b, YEp24-ROM1ΔKpnI; c, YEp352-ROM1[SalI–PvuII]; d, YEp352-ROM1[ClaI–PvuII]; e, YEp352-ROM1[SalI–ClaI].

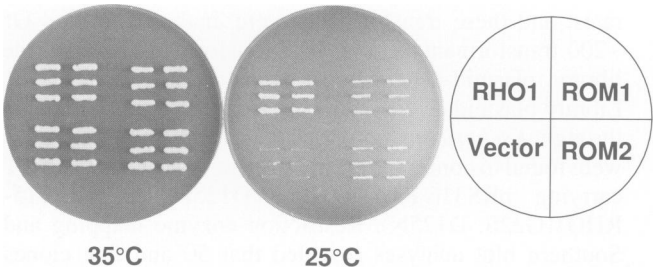

Fig. 2. Suppression of the cold-sensitive growth phenotype of the *RHO1*(G22S, D125N) mutant by overexpression of *ROM1* or *ROM2*. OHNY1 carrying pRS315-RHO1(G22S, D125N) was transformed with YEp24-RHO1 (RHO1), YEp24 (vector), YEp352-ROM1[ClaI–SalI] (ROM1) or YEp352-ROM2 (ROM2). The resultant transformants were streaked onto the SD-ura-leu plates and then incubated at 35°C (left) or at 25°C (right) for 2 days.

ΔN-Rom1p and Rom2p possess the DH and PH domains

Rom2p is a protein of 1356 amino acid residues with a predicted M_r of 153 kDa. The predicted amino acid sequences of ΔN-Rom1p and Rom2p were aligned optimally with 42% amino acid sequence identity (Figure 3). It should be noted that Rom2p is rich in serine (12.7%), resulting in many potential sites for phosphorylation by protein kinases. More importantly, ΔN-Rom1p and Rom2p possess a region of ~200 amino acid residues with significant sequence homology to the DH domain (Figures 3 and 4A). This DH domain is found to be present in many GEPs for the Ras superfamily members, including Cdc24p, Dbl, Ect2, Vav, mSos-1, Ras-GRF, Bcr (Boguski and McCormick, 1993), Abr (Heisterkamp *et al.*, 1993), Tiam1 (Habets *et al.*, 1994), Ost (Horii *et al.*, 1994), Lbc (Toksoz and Williams, 1994), Lfc (Whitehead *et al.*, 1995) and Tim (Chan *et al.*, 1994). Of these, Cdc24p (Zheng *et al.*, 1994), Dbl (Hart *et al.*, 1991, 1994; Yaku *et al.*, 1994), Tiam1 (Michiels *et al.*, 1995), Ost (Horii *et al.*, 1994) and Lbc (Zheng *et al.*, 1995b) have been shown to possess the GDP/GTP exchange activity for the Rho family members. The conservation of the DH domain varies from

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Rom2p     1                        MSETNVDSLG DRNDIYSQIF GVERRPDSFA TFDSDSHGDI
Rom2p    51 SSQLLPNRIE NIQNLNVLLS EDIANDIIIA KQRRRSGVEA AIDDSDIPNN EMKGKSSNYI
Rom2p   111 LSQQTNIKEV PDTQSLSSAD NTPVSSPKKA RDATSSHPIV HAKSMSHIYS TSNSASRQAK
Rom2p   171 HYNDHPLPPM SPRNEVYQKN KSTTAFVPKR KPSLPQLALA GLKKQSSFST GSASTTPTQA
Rom2p   231 RKSPLQGFGF FSRPSSKDLH EQHQHHQHIQ HNNINNHNNN NTNNNGAHYQ VGSSNSNYPQ

ΔN-Rom1p  1          SN VLLPHVRK.. .HSSPSLLSF S.KNSGSHMG DPNQLSTPPT PKSAGHTMEL
Rom2p   281 HSHSISSRSM SLNSSTLKNI ASSFQSKTSN SRKATQKYDI TSNPFSDPHH HHHHHSSNS

ΔN-Rom1p  49 HSSFNGKHSS SSTSSLFALE S...LKPQNR RSSNSSNHSS QYRRHTNQHQ RHHSRSKSSP
Rom2p   341 HSSLNNVHGS GNSSSVMGSS SNIGLGLKTR VSS-TSLALK RYTSVSGTSL SSPRRSSMTP

ΔN-Rom1p 106 ..VSVTEISM IKGTPLVYPA LLSLIAIKFK QTIQLGTHKK MGLLYRDSFT GKQAIDTLCL
Rom2p   400 LSASRPVMSA SSKKPQVYPA LLSRVATKFK SSIQLGEHKK DGLVYRDAFT GQQAVDVICA

ΔN-Rom1p 164 IIGSLDRNLG MLIGKSLEAQ KLFHDVLYDH GVRDSVLEIY ELSSESIF.. ....MAH...
Rom2p   460 IIRTSDRNLA LLFGRSLDAQ KLFHDVVYEH RLRDSPHEVY EFTDNSRFTG TGSTNAHDPL

ΔN-Rom1p 215 ....QSQSST S.......IA NTFSSSSSSV NS.LRTKT.. ..... ..... .....I
Rom2p   520 MLLPNSSSFN SGNHSYPNSG MVPSSSTSSL NSDQATLTGS RLHMSSSLSQ QKNPAAIHNV

ΔN-Rom1p 243 NGVFVPLTHC YSSTCSLEKL CYSISCPNRL QQQANLHLKL GGGLKRNISL ALDKEDDERI
Rom2p   580 NGVFTLLAEC YSPTCTRDAL CYSISCPRRL EQQARLNLKP NGGLKRNISM ALDDDDEEKP

ΔN-Rom1p 303 SWTNSVPKSV WESLSKQQIK RQEAIYELFT TEKKFVKSLE IIRDTFMKKL LETNIIPSDV
Rom2p   640 SWTSSVSKED WENLPKKEIK RQEAIYEVYI TEKNFVKSLE ITRDTFMKTL AETNIISADI

ΔN-Rom1p 363 RINFVKHVFA YINEIYSVNR EFLKALAQRQ SLSPICPGIA DIFLQYLPFF DPLLSYIASR
Rom2p   700 RKNFIKHVFA HINDIYSVNR RFLKALTDRQ RSSPVVRGIG DIVLRFIPFF EPFVSYVASR

ΔN-Rom1p 423 PYAKYLIETQ RSVNPNFARF DDEVSNSSLR HGIDSFLSQG VSRPGRYSLL VREIIHFSDP
Rom2p   760 PYAKYLIETQ RSVNPYFARF DDDMMSSSLR HGIDSFLSQG VSRPGRYMLL VKEIMKSTDP

ΔN-Rom1p 483 VTDKDDLQML MKVQDLLKDL MKRIDRASGA AQDRYDVKVL KQKILFKNEY VNLGLNNEKR
Rom2p   820 EKDKSDYEDL SKAMDALRDF MKRIDQASGA AQDRHDVKLL KQKILFKNEY VNLGLNDERR

ΔN-Rom1p 543 KIKHEGLLSR KDVNKTDASF SGDIQFYLLD NMLLFLKSKA VNKWHOHTVF QRPIPLPLLF
Rom2p   880 KIKHEGILSR KELSKSDGTV VGDIQFYLLD NMLLFLKAKA VNKWHOHKVF QRPIPLPLLF

ΔN-Rom1p 603 ICPAEDMPPI KRYVTENPNC SAGVLLPQYQ TSNPKNAIVF AYYGTKQQYQ VTLYAPQPAG
Rom2p   940 ACPGEDMPAL RKYIGDHPDC SGTVIQPEYN TSNPKNAITF LYYGAKQRYQ VTLYAAQYAG

ΔN-Rom1p 663 LQTLIEKVKQ EQKRLLDETK HITFKQMVGQ FFHSYINTNC VNDVLICSAG KILLVATNMG
Rom2p  1000 LQTLLEKIKQ GQAAIISKTE MFNVTKMSDR FF...DYTNK INSVTSCDGG RKLLIATNSG

ΔN-Rom1p 723 LFVLNYATSI N..... ..QKPVHLLH KISISQISVL EEYNVMILLI DKKLYGCPLD
Rom2p  1057 LYMSNIKRQQ NKDHRHKSSA FFSTPIQLVQ RNNITQIAVL EEFKSIILLI DKKLYSCPLS

ΔN-Rom1p 772 VIDDAENADF LFRKNSKVLF KYVAMFKDGF CNGKRIIMIA HHFLHTVK.L LIVNPLIFDF
Rom2p  1117 LIEAEGNGTS FFKKHHKELI NHVSFFAEGD CNGKRLIVTA HSSSHSIKYF EHEHPLLAEK

ΔN-Rom1p 831 N...SGNFKK NLKAGLVDFS VDSEPLSFSF LENKICIGCK KNIKILNVPE VCDKNGFKMR
Rom2p  1177 NGSGSGN.KK SLKKKITEVI FDSEPVSISF LKANLCIGCK KGFQI..... ..VSISQNAH

ΔN-Rom1p 888 ELLNLHDDKV LANMYKETFK VVSMFPIKNS TFACFPELCF FLNKQGKREE TKGCFHWEGE
Rom2p  1229 ESLLDPADTS LEFALRDTLK PMAIYRVGNM FLLCYTEFAF FVNNQGWRKK ESHIIHWEGE

ΔN-Rom1p 948 PEQFACSYPY IVAINSNFIE IRHIENGELV RCVLGNKIRM LKSYAKKILY CYEDPQGFEI
Rom2p  1289 PQKFAIWYPY ILAFDSNFIE IRKIETGELI RCVLADKIRL LQTSTQEILY CYEDYRGYDT

ΔN-Rom1p 1008 IGLLNF
Rom2p  1349 VASLDFWG
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Fig. 3. Amino acid sequences of the ΔN-Rom1p and Rom2p. The predicted amino acid sequences of ΔN-Rom1p and Rom2p are shown in single-letter code. Two sequences are aligned optimally on the basis of residue identity indicated with an asterisk (*). The DH domains are shown in bold letters and the PH domains are underlined.

20 to 30% identity (40–60% similarity) among these proteins and ΔN-Rom1p/Rom2p, and is 72% identity (86% similarity) between ΔN-Rom1p and Rom2p. Like most of these GEPs, ΔN-Rom1p and Rom2p possess a PH domain C-terminally adjacent to the DH domain (Figures 3 and 4B), which is found in many cytoskeletal and signaling proteins (Musacchio *et al.*, 1993; Gibson *et al.*, 1994).

Disruption of both ROM1 and ROM2 results in lethality

ROM1 and *ROM2* were disrupted with *TRP1* and *HIS3*, respectively, by one-step gene replacement. The *ROM1*/Δ*rom1*::*TRP1* diploid strain, DIOY10B, and the *ROM2*/Δ*rom2*::*HIS3* diploid strain, DIOY20B, were sporulated and subjected to tetrad analysis at 24°C. With regard to

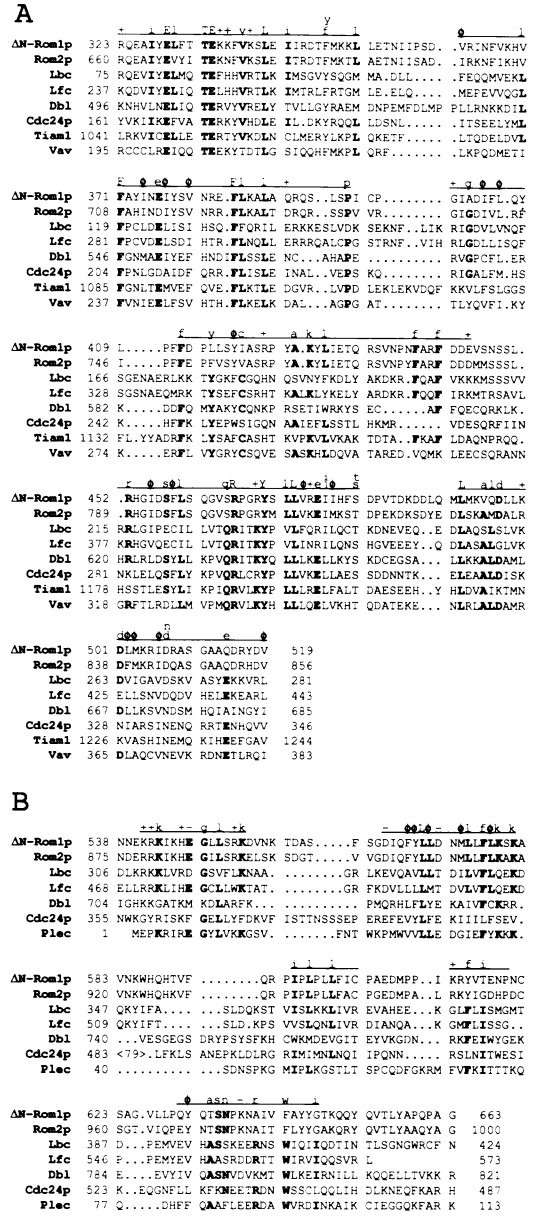

Fig. 4. Alignments of the DH and PH domains in ΔN-Rom1p, Rom2p and other proteins. Amino acid sequences were aligned by the Edit Align program (Hirosawa *et al.*, 1993) and further optimized by eye. Identical amino acids present in more than half of the proteins are shown in bold. Dots (.) indicate gaps introduced to maintain the alignment. A large insertion (79 amino acid residues) in the PH domain of Cdc24p has been removed and the missing number is given in blankets (< >). The amino acid residues and symbols in the consensus lines represent recurring features; upper case (identical in all proteins), lower case (identical in more than half of the proteins), +/– (cationic/anionic in more than half of the proteins) and ϕ (hydrophobic in all proteins). Lines under the consensus lines represent the conserved alignment blocks. (**A**) The DH domains of ΔN-Rom1p, Rom2p, Lbc, Lfc, Dbl, Cdc24p, Tiam1 and Vav are aligned. (**B**) The PH domains of ΔN-Rom1p, Rom2p, Lbc, Lfc, Dbl, Cdc24p and pleckstrin (Plec, the first PH domain of two PH domains) are aligned.

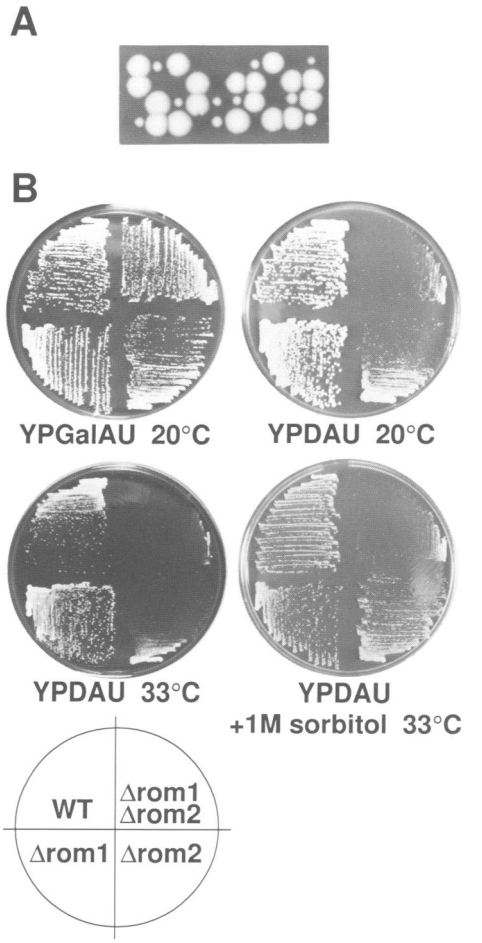

Fig. 5. Growth phenotypes of Δ*rom1*, Δ*rom2* and Δ*rom1*Δ*rom2* mutant cells. (**A**) Tetrad analysis of DIOY70B (Δ*rom1*::*TRP1*/*ROM1 ROM2*/Δ*rom2*::*HIS3*). Of 23 tetrads dissected, 10 tetrads are shown. Bigger segregants showed either Trp$^-$ His$^-$ (*ROM1 ROM2*) or Trp$^+$ His$^-$ (Δ*rom1*::*TRP1 ROM2*) phenotype, whereas smaller segregants showed a Trp$^-$ His$^+$ (*ROM1* Δ*rom2*::*HIS3*) phenotype. No Trp$^+$ His$^+$ (Δ*rom1*::*TRP1* Δ*rom2*::*HIS3*) segregant was recovered. (**B**) Growth phenotypes of Δ*rom1*, Δ*rom2* and Δ*rom1*Δ*rom2* mutants under various growth conditions. Strains, IOS00α (*ROM1 ROM2*, WT), IOS10α (Δ*rom1*::*TRP1 ROM2*, Δrom1), IOS20a (*ROM1* Δ*rom2*::*HIS3*, Δrom2) and IOY73a (Δ*rom1*::*TRP1* Δ*rom2*::*HIS3* pRS316-P_{GAL1}-ROM2, Δrom1Δrom2), were streaked onto various plate media and the plates were incubated at 33°C for 2 days or at 20°C for 4 days. IOY73a carried pRS316-P_{GAL1}-ROM2 which expressed *ROM2* in the presence of galactose in the medium. Residual growth of IOY73a seems to be due to a time lag for the repression of the *GAL1* promoter.

the disruption of *ROM1*, 11 complete tetrads were recovered, all of them showed a 2 Trp$^+$:2 Trp$^-$ segregation pattern, and all of the Trp$^+$ segregants grew at 14 or 35°C as wild-type cells did, indicating that *ROM1* is not an essential gene. In contrast, the disruption of *ROM2* resulted in a temperature-sensitive growth phenotype. Nine complete tetrads were recovered and all of them showed a 2 His$^+$:2 His$^-$ segregation pattern. All of the His$^+$ segregants grew more slowly than the His$^-$ segregants at 20°C and did not grow at 33°C as described below.

To characterize the phenotypes of the Δ*rom1*Δ*rom2* double disruption mutant, a haploid strain, IOY10a (Δ*rom1*::*TRP1*), was mated with a haploid strain, IOY20α (Δ*rom2*::*HIS3*), and the resultant diploid strain, DIOY70B, was sporulated and subjected to tetrad analysis at 24°C (Figure 5A). In 22 tetrads out of a total of 23 tetrads dissected, 17, one and four tetrads showed tetratype, parental ditype and non-parental ditype segregation patterns, respectively, for the His and Trp phenotypes. Genotypes of inviable spore clones could be inferred from

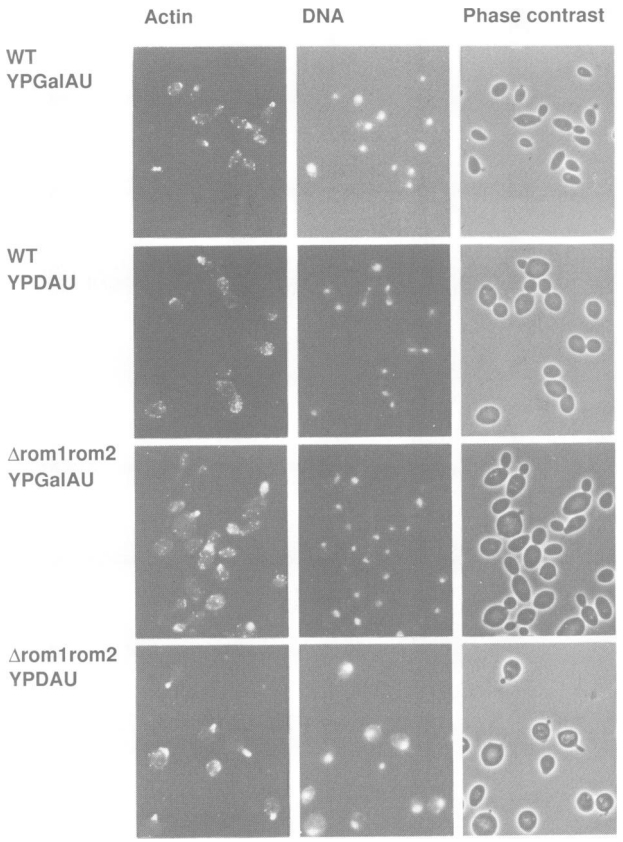

Fig. 6. Growth arrest of the Δ*rom1*Δ*rom2* mutant cells with a small bud. Cells of diploid strains, OHNY3 (WT) and DIOY73A (Δrom1Δrom2), were inoculated into YPGalAU or YPDAU medium and were cultured at 30°C for 12 h. Cells were fixed and double-stained with rhodamine–phalloidin for actin and with DAPI for DNA, then were subjected to microscopic observation. All fields were photographed at the same magnification.

those of viable spore clones. No His^+ Trp^+ segregant was recovered, suggesting that the Δ*rom1*Δ*rom2* double mutant is inviable. To examine whether *ROM1* and *ROM2* are required for vegetative cell growth, a conditional Δ*rom1*-Δ*rom2* mutant was constructed. The DIOY70B strain was transformed with pRS316-P_{GAL1}-ROM2 which expresses *ROM2* under the control of the *GAL1* promoter (P_{GAL1}). The resultant transformants were grown, sporulated and dissected on YPGalAU medium. From one ascus showing a tetratype segregation pattern for the His and Trp phenotypes, four strains, IOS00α (*ROM1 ROM2*), IOS10α (Δ*rom1*::*TRP1 ROM2*), IOS20a (*ROM1* Δ*rom2*::*HIS3*) and IOY73a (Δ*rom1*::*TRP1* Δ*rom2*::*HIS3* pRS316-P_{GAL1}-ROM2), were isolated. Growth phenotypes of these four strains were examined under various growth conditions. As shown in Figure 5B, the Δ*rom1* cells grew as wild-type cells did under all growth conditions examined, whereas the Δ*rom2* cells did not grow at 33°C. This temperature-sensitive growth phenotype of the Δ*rom2* mutant was partially suppressed in the presence of 1 M sorbitol in the medium in the same way as that of a *rho1* mutant was suppressed (Yamochi *et al.*, 1994). The Δ*rom1*Δ*rom2* cells did not grow at 20 or 33°C even in the presence of 1 M sorbitol in the medium, as was the case for the Δ*rho1* mutant, unless *ROM2* was expressed under the control of the *GAL1* promoter. Thus, we concluded that disruption of *ROM1* and *ROM2* in combination is lethal, and that *ROM1* and *ROM2* are functionally redundant.

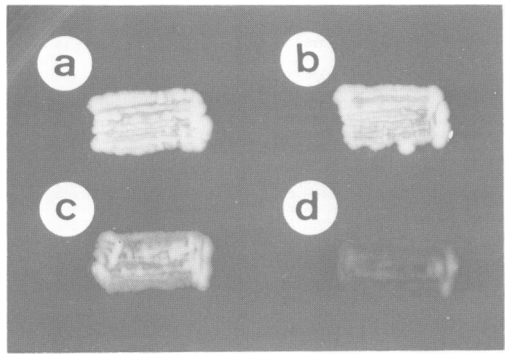

Fig. 7. Cell lysis phenotype of the Δ*rom1*Δ*rom2* mutant. Cells of strains IOS00α (*ROM1 ROM2*), IOS10α (Δ*rom1*::*TRP1 ROM2*), IOS20a (*ROM1* Δ*rom2*::*HIS3*) and IOY73a (Δ*rom1*::*TRP1* Δ*rom2*::*HIS3* pRS316-P_{GAL1}-ROM2) were streaked onto a YPDAU plate. This plate was incubated at 24°C for 15 h and then incubated further at 37°C for 3 h. The patches were stained with a chromogenic substrate, BCIP, of alkaline phosphatase at 37°C for 15 h. (**a**) IOS00α; (**b**) IOS10α; (**c**) IOS20a; (**d**) IOY73a.

The Δrom1Δrom2 mutant shows phenotypes similar to those of the Δrho1 mutant

Since *ROM1* and *ROM2* are multicopy suppressors of *RHO1*(G22S, D125N) and possess the DH domain, *ROM1* and *ROM2* may encode GEPs for Rho1p. If this is the case, the Δ*rom1*Δ*rom2* mutant would show phenotypes similar to those of the Δ*rho1* mutant. We have shown recently that Rho1p-depleted cells are arrested with a small bud (i.e. smaller than about one-third of its mother cell in diameter) (Yamochi *et al.*, 1994). To investigate this point, cells of a diploid strain, DIOY73A (Δ*rom1*::*TRP1*/Δ*rom1*::*TRP1* Δ*rom2*::*HIS3*/Δ*rom2*::*HIS3* pRS316-P_{GAL1}-ROM2), were grown under *ROM2*-expressing (YPGalAU) or non-expressing (YPDAU) conditions, along with cells of a wild-type strain OHNY3 as control. As shown in Figure 6, under the *ROM2*-non-expressing conditions, DIOY73A cells were arrested with a small bud or an abnormally elongated and abnormally shaped bud. Such cells accumulated from 50 to 80% in the DIOY73A culture in YPDAU medium, but not in that in YPGalAU medium or in the OHNY3 culture in either medium (>500 cells were counted for each culture). Arrested cells of DIOY73A possessed a single nucleus and, in spite of these morphological defects, showed almost normal localization of cortical actin patches and actin cables as did Δ*rho1* cells (Yamochi *et al.*, 1994). IOY20α (Δ*rom2*::*HIS3*) cells were also arrested with a small bud at 37°C (data not shown).

Another phenotype specific to the Δ*rho1* mutant is the cell lysis phenotype: the Δ*rho1* mutant cells release the intracellular materials into the medium without apparent cell lysis under the restrictive conditions (Yamochi *et al.*, 1994). This phenotype can be assayed by staining cells for alkaline phosphatase activity (which is normally localized at vacuoles in wild-type cells) released into the medium. As shown in Figure 7, cells of the Δ*rom1*Δ*rom2* mutant or the Δ*rom2* mutant released alkaline phosphatase as a result of cell lysis under the restrictive conditions. These results indicate that the Δ*rom1*Δ*rom2* mutant shows

phenotypes very similar to those of the Δ*rho1* mutant, suggesting that Rom1p/Rom2p and Rho1p function in the same signal transduction pathway.

The temperature sensitivity of the Δrom2 mutant is suppressed by overexpression of RHO1

If *ROM1* and *ROM2* function as upstream regulators of *RHO1*, it could be expected that overexpression of *RHO1* would suppress the temperature-sensitive growth phenotype of the Δ*rom2* mutant. A strain, IOY20α (Δ*rom2*::*HIS3*), was transformed with a multicopy plasmid bearing *ROM1, ROM2, CDC42, RHO1, RHO2, RHO3* or *RHO4* or with a vector as a control. As shown in Figure 8, the temperature sensitivity of the Δ*rom2* mutant was suppressed by either *RHO1* or *RHO2* and was weakly suppressed by either *RHO3* or *RHO4*, but not by *CDC42*. Since *RHO2* is a non-essential homolog of *RHO1*, these results suggest that a major function of Rom2p is to activate Rho1p. The temperature sensitivity of the Δ*rom2* mutant was also suppressed by *ROM1* (Figure 8), being consistent with the result that Δ*rom1* and Δ*rom2* were synthetically lethal.

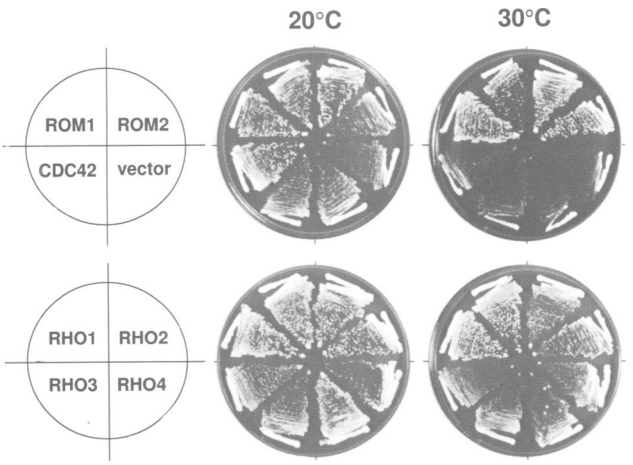

Fig. 8. Suppression of the temperature-sensitive growth phenotype of the Δ*rom2* mutant by overexpression of *RHO1*. IOY20α (*ROM1* Δ*rom2*::*HIS3*) was transformed with YEp352-ROM1[SalI–ClaI] (ROM1), YEp352-ROM2 (ROM2), YEp24 (vector), YEp13M4-CDC42 (CDC42), YEp24-RHO1 (RHO1), YEp24-RHO2 (RHO2), pYO324-RHO3 (RHO3) and pYO324-RHO4 (RHO4). Two independent transformants obtained from each transformation were streaked onto YPDAU plates which were incubated at 20°C for 4 days or at 30°C for 2 days.

Rom2p interacts with a dominant negative form of Rho1p in the two-hybrid system

The genetic and structural analyses of *ROM1* and *ROM2* described above suggested that Rom1p and Rom2p are GEPs for Rho1p. This possibility was examined by the two-hybrid method (Fields and Song, 1989). A dominant active mutation in Ras, Ras(Q61L), has been shown to keep Ras in the GTP-bound form (Bourne *et al.*, 1991), whereas two dominant negative mutations in Ras, Ras(T17N) and Ras(G15A), have been suggested to keep Ras in either the GDP-bound or nucleotide-free form (Chen *et al.*, 1994). Of these dominant negative mutants of Ras, only Ras(G15A) has been shown to form a stable complex with Cdc25 [a GEP for Ras (Martegani *et al.*, 1992)], even in the presence of GDP or GTP (Chen *et al.*, 1994). Since Cdc25 has been shown to form a stable complex only with the nucleotide-free form of wild-type Ras (Lai *et al.*, 1993), Ras(G15A) seems to be in the nucleotide-free form even in the presence of GDP or GTP. Recently, a nucleotide-free form of Cdc42 also has been shown to form a stable complex with Dbl, which is a GEP for Cdc42 (Hart *et al.*, 1994). A mutation corresponding to G15A, Q61L or T17N was introduced into *RHO1* or *CDC42*, and the resultant mutant alleles were fused to the LexA DNA binding domain (DBD_{LexA}) of a two-hybrid vector pBTM116. A DNA fragment encoding the full-length Rom2p was fused with the transcriptional activating domain of Gal4p (AD_{GAL}) and the resultant plasmid was transformed into yeast strains which carried the LexA fusion genes. The activity of β-galactosidase, which is a reporter enzyme of the two-hybrid system, was measured in each transformant. As described in Table I, AD_{GAL}-ROM2 interacted with DBD_{LexA}-RHO1(G22A) to an extent similar to that of the interaction of DBD_{LexA}-Ras(G12V) with VP16-Raf, which is a target of Ras (Vojtek *et al.*, 1993). AD_{GAL}-ROM2DH (Rom2p amino acid position from 623 to 917), which contains the Rom2p DH domain, also interacted with DBD_{LexA}-RHO1(G22A), indicating that Rom2p interacts with Rho1p through the DH domain (data not shown). However, AD_{GAL}-ROM2 did not interact with DBD_{LexA}-RHO1, DBD_{LexA}-RHO1(Q68L), DBD_{LexA}-RHO1(T24N), DBD_{LexA}-CDC42(G15A) or DBD_{LexA}-CDC42(T17N). These results suggest that Rom2p is a GEP for Rho1p, but not for Cdc42p.

The DH domain of Rom2p shows the Rho1p-specific GDP/GTP exchange activity

In the next experiment, we examined whether Rom2p actually shows the GDP/GTP exchange activity for Rho1p

Table I. Two-hybrid interactions between Rom2p and Rho1p

DNA binding domain fusions	Transcriptional activating domain fusions	Colony color	Units of β-galactosidase[a]
RHO1(G22A)	ROM2	blue	67 ± 9
RHO1(G22A)	vector	white	<1
RHO1	ROM2	white	<1
RHO1(Q68L)	ROM2	white	<1
RHO1(T24N)	ROM2	white	<1
CDC42(G15A)	ROM2	white	<1
CDC42(T17N)	ROM2	white	<1
Ras(G12V)	Raf	blue	55 ±6

[a]The values are average ± SE for three transformants.

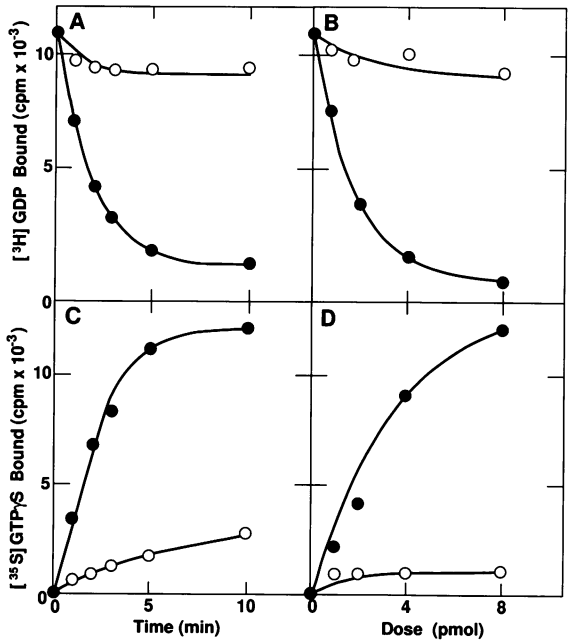

Fig. 9. GEP activity of GST–Rom2DH. The GEP activity to stimulate the dissociation of [^{3}H]GDP from or the binding of [^{35}S]-GTPγS to the lipid-modified form of Rho1p was assayed. (**A** and **B**) Dissociation of [^{3}H]GDP. (**C** and **D**) Binding of [^{35}S]GTPγS. (A and C) Time course (4 pmol of GST–Rom2DH or GST); (B and D) Dose–response (5 min). (●) GST–Rom2DH; (○) GST.

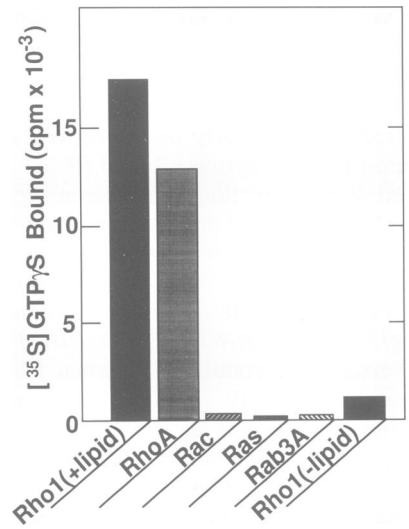

Fig. 10. Substrate specificity of GST–Rom2DH. The binding of [^{35}S]GTPγS to various small GTP binding proteins was assayed for 5 min in the presence of 4 pmol of GST–Rom2DH.

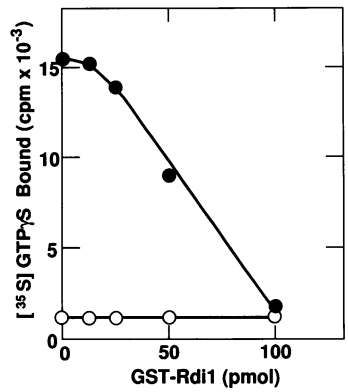

Fig. 11. Inhibition of the GEP activity of GST–Rom2DH by GST–Rdi1. The binding of [^{35}S]GTPγS to the lipid-modified form of Rho1p was assayed for 5 min in the presence of 4 pmol of GST–Rom2DH or GST and various amounts of GST–Rdi1. (●) GST–Rom2DH; (○) GST.

in vitro. A fusion protein, GST–Rom2DH, which is glutathione-*S*-transferase (GST) fused with amino acid positions 623–917 of Rom2p was expressed in *E.coli* and purified. This GST–Rom2DH possessed the DH domain and the first one-third of the PH domain of Rom2p. As shown in Figure 9A and B, this GST–Rom2DH stimulated the dissociation of GDP from the lipid-modified form of Rho1p in a dose- and time-dependent manner. Moreover, GST–Rom2DH stimulated the binding of [^{35}S]GTPγS to the lipid-modified form of Rho1p (Figure 9C and D). These results indicate that GST–Rom2DH is a GEP for Rho1p. The substrate specificity of GST–Rom2DH was examined further. As shown in Figure 10, GST–Rom2DH stimulated the binding of [^{35}S]GTPγS to the lipid-modified form of RhoA as well as to the lipid-modified form of Rho1p. This result is consistent with our finding that the expression of RhoA suppressed the *rho1* mutation (Yamochi *et al.*, 1994). GST–Rom2DH did not stimulate the binding of [^{35}S]GTPγS to the lipid-modified form of Rac1, Ki-Ras or Rab3A, indicating that Rom2p is a Rho1p- or RhoA-specific GEP.

We have shown previously that the post-translational lipid modifications of small GTP binding proteins are required for the GDP/GTP exchange activities of Smg GDS (Mizuno *et al.*, 1991) and Dbl (Yaku *et al.*, 1994). Consistent with these results, GST–Rom2DH did not stimulate the binding of [^{35}S]GTPγS to the non-lipid-modified form of Rho1p (Figure 10). GST–Rom2DH also did not stimulate the binding of [^{35}S]GTPγS to the non-lipid-modified form of Rho1p purified from *E.coli* (data not shown).

We have shown also that Rho GDI inhibits the GDP/GTP exchange reaction stimulated by Smg GDS (Kikuchi *et al.*, 1992) or Dbl (Yaku *et al.*, 1994). Recently, we have cloned a gene, *RDI1*, which is a yeast homolog of mammalian Rho GDI and have shown that the recombinant GST–Rdi1 is active on the lipid-modified form of Rho1p (Masuda *et al.*, 1994). As shown in Figure 11, GST–Rdi1 inhibited the GST–Rom2DH-stimulated binding of [^{35}S]GTPγS to the lipid-modified form of Rho1p. Therefore, the functional relationship between GEP and GDI seems to be conserved for Rho1p in *S.cerevisiae*.

Discussion

In this work we have isolated a temperature-sensitive dominant negative mutation of *RHO1*, *RHO1*(G22S, D125N), and have demonstrated that this type of mutation is valuable in the isolation of genes functionally relevant to *RHO1*. We have isolated six new genes (the *ROM* genes) as multicopy suppressors of *RHO1*(G22S, D125N) and have demonstrated that two of them, *ROM1* and *ROM2*, encode Rho1p-specific GEPs. These *ROM* genes could not be isolated as multicopy suppressors of a temperature-sensitive recessive mutation of *RHO1*

(H.Nonaka *et al.*, unpublished results). Therefore, this approach is an alternative method for the isolation of genes functionally relevant to *RHO1* and may be applicable to the study of other small GTP binding proteins.

The *Δrom1Δrom2* mutant was lethal and showed phenotypes very similar to those of the *Δrho1* mutant, including arrest with a small bud and cell lysis. The temperature sensitivity of the *Δrom2* mutant was suppressed by overexpression of *RHO1*, but not of *CDC42*. Moreover, the full-length Rom2p interacted with a dominant negative form of Rho1p, Rho1p(G22A), but not with the corresponding mutant of Cdc42p, Cdc42p(G15A), in the two-hybrid system. These results clearly indicate that *ROM1* and *ROM2* function as upstream regulators of *RHO1*. Consistent with the genetic results, Rom2p showed the Rho1p-specific GDP/GTP exchange activity *in vitro*. Although we have not yet tested whether Rom1p also shows the GDP/GTP exchange activity for Rho1p, the amino acid sequence similarity and functional redundancy of *ROM1* and *ROM2* suggest that Rom1p also possesses the Rho1p-specific GDP/GTP exchange activity.

The GDP/GTP exchange activity of Rom2p resides in the amino acid region 623–917 of Rom2p (Rom2DH), which contains the DH domain. This result is consistent with the finding that the GDP/GTP exchange activity of Dbl also resides in the DH domain-containing region (Hart *et al.*, 1994). The PH domain is located just downstream of the DH domain in Rom2p, and GST–Rom2DH lacks the C-terminal two-thirds of the PH domain. Therefore, the PH domain is not required for the GDP/GTP exchange activity of Rom2p. The PH domains have been shown to bind to phosphatidylinositol derivatives (Harlan *et al.*, 1994) or to the βγ subunits of a heterotrimeric G protein (Touhara *et al.*, 1994), and their functions are implicated in the translocation of the PH domain-containing proteins to the plasma or organellar membranes. Consistent with this model, it has been shown recently that the PH domain of Lfc is required for its activity in transforming NIH 3T3 cells and can be replaced functionally by an isoprenylation site which recruits this protein to the plasma membrane (Whitehead *et al.*, 1995). Since the GTP-bound form of Rho1p seems to be localized in the plasma membrane area of the growth site (Yamochi *et al.*, 1994), it is probable that Rom2p is also localized at the same site in order to activate Rho1p. The role of the PH domain in the intracellular localization of Rom2p currently is under investigation.

The functions of the regions of Rom1p or Rom2p other than the DH and PH domains are currently unknown. The amino acid sequences of these regions do not show significant homology to any known protein. Recently, Cdc24p has been shown to bind to Bem1p, which is also involved in the bud site assembly, through the C-terminal 75 amino acid region of Cdc24p (Peterson *et al.*, 1994). In mammalian systems, it has been reported that the N-terminal truncation is responsible for the transforming activity of the DH domain-containing proteins, including Dbl (Ron *et al.*, 1989), Vav (Katzav *et al.*, 1991) and Ost (Horii *et al.*, 1994), suggesting that the N-terminal regions of these proteins negatively regulate the transforming activity of these proteins. Thus, these regions of Rom1p and Rom2p may play some role in the regulation of activity of the DH domain.

We have demonstrated recently that there is a homolog of Rho GDI, *RDI1*, in *S.cerevisiae*, suggesting that there is a functional interaction between Rdi1p and Rom1p/Rom2p. It has been demonstrated in mammalian cells that Rho GDI is complexed with Rho in the cytosol (Kuroda *et al.*, 1992; Regazzi *et al.*, 1992). Moreover, Rdi1p has also been found to be complexed with Rho1p in the yeast cytosol (Koch *et al.*, in preparation). Based on these results, we have proposed that Rho complexed with Rho GDI is in the inactive state and that Rho should be released from Rho GDI for its activation (Takai *et al.*, 1995). It is most probable that the Dbl family members are involved in the activation process of Rho. However, no Dbl family members have circumvented the inhibitory activity of Rho GDI so far. In the present study, Rom2p also did not circumvent the inhibitory activity of Rdi1p. Therefore, there should be another factor which enables Rom1p/Rom2p to interact with and activate Rho1p complexed with Rdi1p. Recently, it has been reported that such a factor is present in the membrane fraction in neutrophils (Bokoch *et al.*, 1994). Isolation of this factor would be important in order to clarify the molecular mechanisms of activation of Rho1p and we are currently trying to isolate this factor genetically.

The GTP-bound form of Rsr1p/Bud1p has been shown to interact directly with Cdc24p (Zheng *et al.*, 1995a). This result is consistent with the physiological roles of these proteins: Rsr1p/Bud1p chooses the site for budding which is initiated by the activation of Cdc42p by Cdc24p. Since Rho1p seems to function after the establishment of bud site assembly that is regulated by Cdc42p, it is possible that Cdc42p might activate Rom1p/Rom2p directly or indirectly. Molecular cloning of *ROM1/ROM2* has enabled us to examine the regulatory mechanisms of Rom1p/Rom2p in the small GTP binding protein cascade regulating the budding process in *S.cerevisiae*.

Materials and methods

Strains, media and yeast transformations

Yeast strains used in this study are listed in Table II. An *E.coli* strain DH5α was used for construction and propagation of plasmids. Yeast strains were grown on YPDAU medium that contained 2% glucose, 2% Bacto-peptone (Difco Laboratories, Detroit, MI), 1% Bacto-yeast extract (Difco), 0.04% adenine sulfate and 0.02% uracil. YPGalAU medium contained 3% galactose, 0.2% sucrose, 2% Bacto-peptone, 1% Bacto-yeast extract, 0.04% adenine sulfate and 0.02% uracil. Yeast transformations were performed by the lithium acetate method (Gietz *et al.*, 1992). Transformants were selected on SD medium that contained 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco). The SD medium was supplemented with amino acids when required. Standard yeast genetic manipulations were performed as described (Sherman *et al.*, 1986).

Plasmid construction and other molecular biological techniques

Standard molecular biological techniques were used for the construction of plasmids, PCR and DNA sequencing (Sambrook *et al.*, 1989). Plasmids used in this study are listed in Table III. PCRs were performed using GeneAmp PCR System 2400 (Perkin Elmer) and DNA sequences were determined using the ALFred DNA sequencer (Pharmacia Biotech, Inc.). Point mutations were introduced by the PCR mutagenesis methods (Higuchi, 1989).

Isolation of a temperature-sensitive dominant negative mutation, RHO1(G22S, D125N)

pRS316-RHO1(G22S) was constructed by PCR mutagenesis and was found not to give any transformant when introduced into wild-type cells.

Table II. Yeast strains used in this study

Strains	Genotype
OHNY1	*MAT***a** *ura3 leu2 trp1 his3 ade2*
OHNY3	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2*
IOY10a	*MAT***a** *ura3 leu2 trp1 his3 ade2 Δrom1::TRP1*
IOY20α	*MAT*α *ura3 leu2 trp1 his3 ade2 Δrom2::HIS3*
IOS00α	*MAT*α *ura3 leu2 trp1 his3 ade2*
IOS10α	*MAT*α *ura3 leu2 trp1 his3 ade2 Δrom1::TRP1*
IOS20a	*MAT***a** *ura3 leu2 trp1 his3 ade2 Δrom2::HIS3*
IOY73a	*MAT***a** *ura3 leu2 trp1 his3 ade2 Δrom1::TRP1 Δrom2::HIS3 pRS316-*P_{GAL1}*-ROM2*
DIOY10B	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 ROM1/Δrom1::TRP1 ROM2/ROM2*
DIOY20B	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 ROM1/ROM1 ROM2/Δrom2::HIS3*
DIOY70B	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 Δrom1::TRP1/ROM1 ROM2/Δrom2::HIS3*
DIOY73A	*MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his3/his3 ade2/ade2 Δrom1::TRP1/Δrom1::TRP1 Δrom2::HIS3/Δrom2::HIS3 pRS316-*P_{GAL1}*-ROM2*
L40	*MAT***a** *trp1 leu2 his3 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ*

Strains used in this study are isogenic except L40.

This plasmid was mutagenized *in vitro* with hydroxylamine at 55°C for 24 h as described (Rose and Fink, 1987), and the resultant mutagenized DNA was used to transform a wild-type haploid strain, OHNY1. Transformants were grown on SD-ura medium at 35°C for 3 days and were replica-plated onto SD –ura medium that subsequently was incubated at 20°C for 5 days. Of 200 transformants screened, five transformants did not grow at 20°C. Plasmids were recovered from these cold-sensitive clones and were transformed back into OHNY1. All clones conferred the cold-sensitive growth phenotype on OHNY1. One of these clones was selected and the 2.2 kb *Sma*I–*Sac*I *RHO1*(G22S) DNA fragment was cleaved out and cloned into the *Sma*I–*Sac*I site of pRS315 (*LEU2*, CEN6). The resultant plasmid again conferred the cold-sensitive growth phenotype on OHNY1, indicating that the temperature-sensitive mutation occurred in the *RHO1*(G22S) gene, but not in the vector. The nucleotide sequence of the mutant *RHO1*(G22S) gene was determined, and one point mutation, G→A, was found at nucleotide position 373 of the *RHO1* ORF, consistent with the specificity of base changes by hydroxylamine mutagenesis. This mutation caused amino acid substitution from Asp to Asn at amino acid 125 of Rho1p. OHNY1 carrying the mutant plasmid, pRS316-RHO1(G22S, D125N) or pRS315-RHO1(G22S, D125N) was used for isolation of multicopy suppressors.

Determination of the nucleotide sequence of ROM1

The 3.8 kb *Sal*I–*Cla*I DNA fragment containing the *ROM1* gene was cloned into YEp352 to construct YEp352-ROM1[SalI–ClaI]. Nested deletions were made from both ends of the *ROM1* insert as described (Henikoff, 1984) and the DNA sequence of *ROM1* DNA portions of these deletion plasmids was determined. The *ROM1* DNA sequence reported in this work is based on the determination of DNA sequences of both strands of the *ROM1* DNA.

Disruption of ROM1 and ROM2

To disrupt *ROM1*, pUC19-Δrom1::TRP1 was cut with *Sal*I and *Eco*RI and the digested DNA was introduced into a diploid strain OHNY3. To disrupt *ROM2*, pUC19-Δrom2::HIS3 was cut with *Not*I and the digested DNA was introduced into a diploid strain OHNY3. The genomic DNA was isolated from each transformant and the proper disruption of *ROM1* or *ROM2* was verified by PCR (data not shown). A diploid strain in which one *ROM1* allele was disrupted was named DIOY10B, whereas a diploid strain in which one *ROM2* allele was disrupted was named DIOY20B. These strains were used for subsequent experiments.

Two-hybrid method

A strain L40 was transformed with derivatives of pBTM116 bearing wild-type or mutant forms of *RHO1* or *CDC42* fused to the LexA DNA binding domain (DBD_{LexA}). In these pBTM116 fusion plasmids, the carboxy-terminal lipid modification sites (CVLL in Rho1p and CTIL in Cdc42p) were deleted. A resultant transformant was transformed with a vector pACTII-HK or pACTII-HK-ROM2 in which *ROM2* was fused with the *GAL4* transcriptional activation domain (AD_{GAD}). Western blot analysis using a monoclonal antibody raised against the hemagglutinin epitope confirmed that cells carrying pACTII-HK-ROM2 expressed a protein of ~165 kDa, the expected size of the protein of Rom2p fused with AD_{GAD}. For quantitative assay, cells of each transformant were cultured in SD-trp-leu medium and the β-galactosidase activity was measured according to the ONPG assay method described (Guarente, 1983). For qualitative assay, 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 (Vojtek *et al.*, 1993).

Materials and chemicals for biochemical assays

The lipid-modified and non-lipid-modified forms of Rho1p were purified from the membrane and cytosol fractions of *Spodoptera frugiperda* cells, respectively, which were infected with the baculovirus carrying the *RHO1* gene as described (Matsuura *et al.*, 1987; Mizuno *et al.*, 1991). The lipid-modified forms of RhoA, Rac1 and Ki-Ras were purified similarly from the membrane fraction of the insect cells (Mizuno *et al.*, 1991; Ando *et al.*, 1992). The lipid-modified form of Rab3A was purified from bovine brain membranes as described (Kikuchi *et al.*, 1988). Recombinant yeast Rho GDI (Rdi1p) and Rom2DH were purified from overexpressing *E.coli* DH5α as GST fusion proteins using a glutathione–Sepharose 4B column (Pharmacia P-L Biochemicals Inc.) as described (Kikuchi *et al.*, 1992). The BA-85 (0.45 μm pore size) nitrocellulose filter was purchased from Schleicher & Schuell.

Assay for the GEP activity of Rom2p

The GEP activity of GST–Rom2DH was assayed by measuring the dissociation of [^{3}H]GDP from or the binding of [^{35}S]GTPγS to each small GTP binding protein (3 pmol). The dissociation of [^{3}H]GDP or the binding of [^{35}S]GTPγS was assayed at 25°C by the filtration method using nitrocellulose filters by measuring the radioactivity of [^{3}H]GDP or [^{35}S]GTPγS bound to each small GTP binding protein after incubation with GST–Rom2DH in a 100 μl reaction mixture as described (Yamamoto *et al.*, 1990).

Cytological techniques

Actin and DNA were stained with rhodamine–phalloidin (Molecular Probes, Inc., Eugene, OR) and 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co.), respectively, as described (Yamochi *et al.*, 1994). Stained cells were observed and photographed on Neopan Super Presto film (Fuji Photo film, Tokyo, Japan) using a Zeiss Axiophoto microscope (Carl Zeiss, Oberkochen, Germany). Cell lysis was examined by assaying the alkaline phosphatase activity using a chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma Chemical Co., St Louis, MO) as described (Paravicini *et al.*, 1992).

Accession numbers

The DDBJ/EMBL/GenBank accession number for the sequence reported in this paper is D83171.

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Table III. Plasmids used in this study

Plasmids	Characteristics
pRS315	*LEU2*, CEN6 (Sikorski and Hieter, 1989)
pRS316	*URA3*, CEN6 (Sikorski and Hieter, 1989)
pRS316-RHO1	*RHO1*, *URA3*, CEN6; made by inserting the 2.1 kb *Bsu*36I–*Bgl*II *RHO1* genomic DNA fragment containing the 1.1 kb upstream and 414 bp downstream regions into pRS316
pRS316-RHO1(G22S)	*RHO1*(G22S), *URA3*, CEN6; a derivative of pRS316-RHO1 in which the codon for amino acid position 22 of Rho1p, GGT (Gly), was mutated to TCA (Ser) by PCR mutagenesis
pRS316-RHO1(G22S, D125N)	*RHO1*(G22S, D125N), *URA3*, CEN6; a derivative of pRS316-RHO1(G22S) in which the codon for amino acid position 125 of Rho1p, GAT (Asp), was mutated to AAT (Asn) by hydroxylamine mutagenesis
pRS315-RHO1(G22S, D125N)	*RHO1*(G22S, D125N), *LEU2*, CEN6; made by inserting the 2.2 kb *Sma*I–*Sac*I *RHO1*(G22S, D125N) DNA fragment from pRS316-RHO1(G22S, D125N) into the *Sma*I–*Sac*I site of pRS315
YEp352	*URA3*, 2 μm (Hill *et al.*, 1986)
YEp24-RHO1	*RHO1*, *URA3*, 2 μm; isolated from a genomic library in YEp24 (Carlson and Botstein, 1982)
YEp24-RHO2	*RHO2*, *URA3*, 2 μm; isolated from a genomic library in YEp24 (Carlson and Botstein, 1982)
YEp24-ROM1	ΔN-*ROM1*, *URA3*, 2 μm; isolated from a genomic library in YEp24 (a gift of H.Ogawa) as a clone containing the 12 kb genomic DNA fragment of *ROM1*
YEp24-ROM1ΔKpnI	ΔN-*ROM1*, *URA3*, 2 μm; made by deleting the 0.9 kb *Kpn*I fragment from YEp24-ROM1
YEP24-ROM1ΔBglII	*URA3*, 2 μm; made by deleting the 4.0 kb *Bgl*II fragment from YEp24-ROM1
YEp352-ROM1[SalI–PvuII]	ΔN-*ROM1*, *URA3*, 2 μm; made by inserting the 7.5 kb *Sal*I–*Pvu*II fragment from YEp24-ROM1 into the *Sal*I–*Sma*I site of YEp352
YEp352-ROM1[ClaI–PvuII]	*URA3*, 2 μm; made by deleting the 3.8 kb *Cla*I–*Sal*I fragment from YEp352-ROM1[SalI–PvuII]
YEp352-ROM1[SalI–ClaI]	ΔN-*ROM1*, *URA3*, 2 μm; made by deleting the 3.6 kb *Cla*I–*Kpn*I (*Kpn*I site in the multicloning site of YEp352) fragment from Yep352-ROM1[SalI–PvuII]
YEp352-ROM2	*ROM2*, *URA3*, 2 μm; made by inserting the *Bam*HI–*Sal*I 5.2 kb *ROM2* PCR fragment containing 670 bp upstream and 450 bp downstream regions into the *Bam*HI–*Sal*I site of YEp352. The 5.2 kb *ROM2* DNA was amplified by PCR using an upstream primer 5′-GGCGGATCCGATGAGCACTCTGTCGGCGGG and a downstream primer 5′-GGCGTCGACATGCGGAAAATACACAGTACC. The underlined sequences are portions of the *ROM2* ORF
pRS316-P_{GAL1}-ROM2	P_{GAL1}-*ROM2*, *URA3*, CEN6; made by inserting the 4.1 kb *Bam*HI–*Sma*I DNA fragment containing the *ROM2* ORF from pACTII-HK-ROM2 into the *Bam*HI–*Sma*I site of pRS316-P_{GAL1} to place *ROM2* downstream of the promoter fragment of *GAL1*
YEp13M4-CDC42	*CDC42*, *LEU2*, 2 μm; isolated from a genomic library in YEp13M4 (Yamochi *et al.*, unpublished)
pYO324-RHO3	*RHO3*, *TRP1*, 2 μm; made by inserting the 1.8 kb *RHO3* fragment into the *Kpn*I–*Xho*I site of pYO324
pYO324-RHO4	*RHO4*, *TRP1*, 2 μm; made by inserting the 2.0 kb *RHO4* fragment into the *Kpn*I site of pYO324
pBTM116	DBD_{LexA}, *TRP1*, 2 μm; (Bartel *et al.*, 1993)
pBTM116-RHO1	DBD_{LexA}-RHO1, *TRP1*, 2 μm; (Nonaka *et al.*, 1995)
pBTM116-RHO1(Q68L)	DBD_{LexA}-RHO1(Q68L), *TRP1*, 2 μm; (Nonaka *et al.*, 1995)
pBTM116-RHO1(G22A)	DBD_{LexA}-RHO1(G22A), *TRP1*, 2 μm; made by inserting *RHO1*(G22A) into pBTM116 as described (Nonaka *et al.*, 1995)
pBTM116-RHO1(T24N)	DBD_{LexA}-RHO1(T24N), *TRP1*, 2 μm; (Nonaka *et al.*, 1995)
pBTM116-CDC42	DBD_{LexA}-CDC42, *TRP1*, 2 μm; made by inserting the 580 bp *Eco*RI–*Pst*I PCR fragment containing the *CDC42* ORF into the *Eco*RI–*Pst*I site of pBTM116
pBTM116-CDC42(G15A)	DBD_{LexA}-CDC42(G15A), *TRP1*, 2 μm; a derivative of pBTM116-CDC42. A codon for amino acid position 15 of Cdc42p, GGG (Gly), was mutated to GCG (Ala) by PCR mutagenesis
pBTM116-CDC42(T17N)	DBD_{LexA}-CDC42(T17N), *TRP1*, 2 μm; a derivative of pBTM116-CDC42. A codon for amino acid position 17 of Cdc42p, ACG (Thr), was mutated to AAC (Asn) by PCR mutagenesis
pACTII-HK	AD_{GAD}, *LEU2*, 2 μm; a derivative of pACTII. The sequence of the multicloning site of pACTII, 5′-CATATGGCCATGGAGGCCCCGGGGATCCGAATTCGAAGCTCGAGAGATCT-3′ was changed to 5′-CATATGGCCATGGGCGGATCCGGTACCCCCGGGGTCGAGAGATCT-3′ (Kohno *et al.*,unpublished results). The changed sequences are underlined
pACTII-HK-ROM2	AD_{GAD}-ROM2, *LEU2*, 2 μm; made by inserting the 4.1 kb *Bam*HI–*Sma*I PCR fragment containing the *ROM2* ORF into the *Bam*HI–*Sma*I site of pACTII-HK. The 4.1 kb *ROM2* DNA was amplified by PCR using an upstream primer 5′-GCGGGATCCATGAGCGAAACCAACGTTG-ACAGC and a downstream primer 5′-GCGCCCGGGTTAACCCCAGAAATCTAACGACGC. The underlined sequences are portions of the *ROM2* ORF
pGEX-ROM2DH	GST–ROM2DH; made by inserting the 884 bp *Hae*III–*Hae*III DNA fragment of *ROM2* containing the DH domain (amino acid positions 623–917 of Rom2p) into the *Sma*I site of pGEX2T
pUC19-ROM1	ΔN-ROM1; made by inserting the 3.8 kb *Sal*I–*Eco*RI DNA fragment from YEp352-ROM1[SalI–ClaI] encoding the ΔN-Rom1p into the *Sal*I–*Eco*RI site of pUC19
pUC19-Δrom1::TRP1	a derivative of pUC19-ROM1; made by replacing the 2.1 kb *Aff*II–*Bgl*II internal fragment of *ROM1* corresponding to amino acid positions 287–996 of ΔN-Rom1p with the 0.8 kb *TRP1* fragment
pUC19-(NotI-EcoRV-NotI)	made by inserting a (*Not*I–*Eco*RV–*Not*I) linker into the *Pvu*II–*Pvu*II site of pUC19
pUC19-ROM2	made by inserting the 2.4 kb *Xho*I(filled in)–*Bam*HI(filled in) PCR fragment corresponding to amino acid positions 358–1175 of Rom2p into the *Eco*RV site of pUC19-(NotI–EcoRV–NotI). The 2.4 kb *ROM2* DNA was amplified by PCR using an upstream primer 5′-GGCCTCGAGGGGAAGCTCGTCTAATATTGGGTTGG and a downstream primer 5′-GGCGGATCCCGGCCAATAATGGATGTTCATGCTCG. The underlined sequences are portions of the *ROM2* ORF
pUC19-Δrom2::HIS3	a derivative of pUC19-ROM2; made by replacing the 0.8 kb *Eco*RV–*Eco*RV internal fragment of *ROM2* corresponding to amino acid positions 713–988 of Rom2p with the 1.8 kb *HIS3* fragment

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