The Yeast Phosphatidylinositol Kinase Homolog TOR2 Activates RHO1 and RHO2 via the Exchange Factor ROM2

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Summary

The Saccharomyces cerevisiae phosphatidylinositol kinase homolog TOR2 is required for organization of the actin cytoskeleton. Overexpression of *RHO1* or *RHO2*, encoding Rho-like GTPases, or *ROM2*, encoding a GDP/GTP exchange factor for RHO1 and RHO2, suppresses a *tor2* mutation. Deletion of *SAC7*, a gene originally identified as a suppressor of an actin mutation, also suppresses a *tor2* mutation. SAC7 is a novel GTPase-activating protein for RHO1. ROM2 exchange activity is reduced in a *tor2* mutant, and overexpression of ROM2 lacking its PH domain can no longer suppress a *tor2* mutation. Thus, TOR2 signals to the actin cytoskeleton through a GTPase switch composed of RHO1, RHO2, ROM2, and SAC7. TOR2 activates this switch via ROM2, possibly via the ROM2 PH domain.

Introduction

The Saccharomyces cerevisiae TOR1 and TOR2 proteins were originally identified as the targets of the immunophilin–immunosuppressant complex FKBP–rapamycin (Heitman et al., 1991; Cafferkey et al., 1993; Kunz et al., 1993; Helliwell et al., 1994). They are the founding members of a novel family of structurally homologous signaling proteins implicated in cell cycle control (Keith and Schreiber, 1995; Zakian, 1995). This family also includes the yeast MEC1, TEL1, and RAD3; the Drosophila MEI-41; and the mammalian FRAP/RAFT/mTOR, ATM, FRP1, and DNA-PK proteins. Each of these proteins contains a lipid kinase motif near its carboxyl terminus and is thus related to phosphatidylinositol (PI) 3- and PI 4-kinases. The demonstration that DNA-PK, the most distant member of the family, is an authentic protein kinase has led some to suggest that the TOR family members in general are protein kinases (Hartley et al., 1995; Hunter, 1995); however, it remains to be determined whether they are indeed lipid and/or protein kinases.

The putative PI kinase TOR2 has two essential functions (Kunz et al., 1993; Helliwell et al., 1994; Zheng et al., 1995; Hall, 1996). One function is redundant with TOR1 and is required for signaling activation of translation initiation and, thereby, early G1 progression in response to nutrient availability (Barbet et al., 1996; Di Como and Arndt, 1996). This signaling pathway, like TOR itself, appears to be conserved in mammalian cells (Beretta et al., 1996; von Manteuffel et al., 1996). The

second essential function of TOR2 is unique to TOR2. We have shown recently that loss of the TOR2-unique function disrupts the cell cycle–dependent polarized distribution of the actin cytoskeleton (Schmidt et al., 1996). Overexpression of *CCT6/TCP20*, encoding a subunit of the TCP-1 chaperonin that is involved in the folding and assembly of actin structures, suppresses the lethality of a *tor2* mutation and partly restores polarized distribution of the actin cytoskeleton. These results suggested that the unique function of TOR2 is required for organization of the actin cytoskeleton. However, it is not known how TOR2 might signal to the actin cytoskeleton.

In the budding yeast Saccharomyces cerevisiae, the cell cycle–dependent asymmetric distribution of the actin cytoskeleton is essential for establishing and maintaining cell polarity and for targeting secretion to the growing bud (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Drubin, 1991). Like in mammalian cells, the organization of the actin cytoskeleton in yeast cells is controlled by Rho-like GTPases (Hall, 1994; Machesky and Hall, 1996; Ridley, 1996). Several Rho-like GTPases, including CDC42, RHO1, RHO2, RHO3, and RHO4, have been identified in yeast (Madaule et al., 1987; Adams et al., 1990; Johnson and Pringle, 1990; Ziman et al., 1991; Matsui and Toh-e, 1992), and most, if not all, control organization of the actin cytoskeleton and polarized cell growth. Cells lacking CDC42 are defective in the polarization of the actin cytoskeleton and arrest as large unbudded cells because of a failure in bud emergence (Adams et al., 1990; Johnson and Pringle, 1990; Ziman et al.,1991). Cells lacking RHO3 and RHO4 die with small buds, lyse, and also have a random actin distribution (Matsui and Toh-e, 1992b; Imai et al., 1996). RHO1 and possibly its nonessential homolog RHO2 have been implicated in cell wall synthesis and organization of the actin cytoskeleton and may thus control both cell shape and polarity. Cells lacking RHO1 stop growing as small-budded cells and lyse, and RHO1 is located along with cortical actin patches at the presumptive budding site, the bud tip, and the site of cytokinesis (Yamochi et al., 1994). Cells lacking RHO2 have no evident phenotype, but overexpression of *RHO2* can suppress a dominant negative *RHO1* mutation (Ozaki et al., 1996). RHO1 controls cell wall synthesis, as suggested by the lysis phenotype of a *rho1* mutant, in two different ways. First, RHO1 is a regulatory subunit of $\beta(1\rightarrow3)$ glucan synthase (Drgonova et al., 1996; Qadota et al., 1996). Glucan polymers are the major structural component of the yeast cell wall. Second, GTP-RHO1 binds and activates PKC1, which in turn controls glucan synthases via a MAP kinase cascade (Levin and Bartlett-Heubusch, 1992; Errede and Levin, 1993; Roemer et al., 1994; Nonaka et al., 1995; Igual et al., 1996; Kamada et al., 1996). RHO1 might also control the actin cytoskeleton. It colocalizes with actin patches, and human RhoA, which is highly homologous to RHO1 and mediates actin reorganization in mammalian cells, can substitute for RHO1 in yeast (Qadota et al., 1994). Furthermore, mutants lacking the PKC1-controlled MAP kinase MPK1/SLT2 have a delocalized actin cytoskeleton (Mazzoni et al., 1993).

Several regulators of RHO1 and RHO2 have been identified. BEM2 is a GTPase-activating protein (GAP) for RHO1 (Peterson et al., 1994) and probably also for RHO2. RDI1 is a GDP dissociation inhibitor for RHO1 (Masuda et al., 1994). ROM1 and ROM2 contain a GDP/GTP exchange domain and a pleckstrin homology (PH) domain, and at least ROM2 is a GDP/GTP exchange factor (GEF) for RHO1. Both *ROM1* and *ROM2* are multicopy suppressors of a dominant negative *RHO1* allele (Ozaki et al., 1996). However, the mechanism by which RHO1, RHO2, and their regulators (and ultimately cell shape and polarity) are controlled is not known.

Here, we show that TOR2 activates RHO1 and RHO2 via their exchange factor ROM2. Overexpression of *RHO1*, *RHO2*, or *ROM2* suppresses the lethality of a *tor2* mutation, and ROM2 GEF activity is severely reduced in a *tor2* mutant. Overexpression of a mutant ROM2 protein lacking its PH domain cannot suppress a *tor2* mutation, raising the possibility that the PI kinase homolog TOR2 signals to ROM2 via the ROM2 PH domain. In addition, we show that *SAC7*, a gene originally identified as a genomic suppressor of actin mutations (Dunn and Shortle, 1990), encodes a novel GTPase-activating protein for RHO1. Loss of SAC7 restores growth in cells lacking TOR2, indicating that SAC7 antagonizes the activation of RHO1 by ROM2 and TOR2. Thus, TOR2 is an upstream component in a signaling pathway controlling polarized cell growth and possibly cell shape.

Results

Disruption of *SAC7* Restores Growth in a *tor2* Mutant

Previous results indicated that TOR2 is required for organization of the actin cytoskeleton (Schmidt et al., 1996). The first indication of the nature of this requirement came from our isolation of *SAC7*, as follows. In studies to be described elsewhere (M. B. and M. N. H, unpublished data), we have identified recessive mutations in either one of two genes, termed *ROT1* and *ROT2*, that suppress the growth defect of a *tor2* mutant. *SAC7* was isolated as a dosage suppressor of the synthetic lethality conferred by the combination of our suppressor mutations in *ROT1* and *ROT2*. *SAC7* (suppressor of actin) was originally identified as an extragenic suppressor of the temperature-sensitive growth defect conferred by the actin mutation *act1-4* (Dunn and Shortle, 1990). Null alleles of *SAC7* restore growth in *act1-1* and *act1-4* mutants that are defective in the polarized distribution of the actin cytoskeleton. At low temperatures, strains disrupted in *SAC7* are not viable and display aberrant actin assembly. These findings led Dunn and Shortle to suggest that SAC7 is involved in the assembly and function of actin and might have actin-severing activity.

We asked if a *SAC7* disruption, like *rot1* and *rot2* mutations, could also restore growth in a *tor2* mutant that is defective in the polarized distribution of the actin cytoskeleton. The *SAC7* gene was deleted in our wild-type strain JK9-3da (see Experimental Procedures). The obtained *sac7* strains grew normally at 30°C and 37°C and were very severely impaired for growth at 15°C,
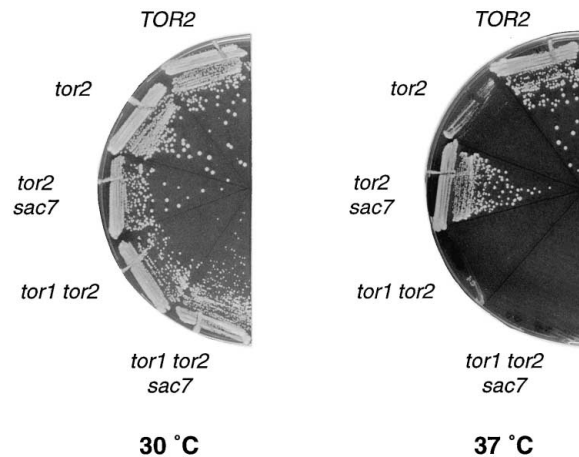

Figure 1. Deletion of *SAC7* Suppresses the Growth Defect of a *tor2*^ts Mutant but Not of a *tor1 tor2*^ts Double Mutant

Wild-type *TOR2* (JK9-3da), *tor2*^ts (SH121), *sac7 tor2*^ts (AS103-2a), *tor1 tor2*^ts (SH221), and *sac7 tor1 tor2*^ts (AS114-4c) cells were streaked out on YPD and incubated at 30°C or 37°C. *tor2*^ts and *tor1 tor2*^ts cells are not viable at 37°C.

similar to what has been described previously (Dunn and Shortle, 1990). The *sac7* strain AS100-6a was crossed to *tor2*^ts strain AS100-3c to generate diploid AS103, and the segregants from 18 tetrads derived from AS103 were examined for growth on rich medium at the nonpermissive temperature (37°C) for the *tor2*^ts mutation. While *tor2*^ts segregants were not viable at this temperature, *sac7 tor2*^ts double mutants grew almost like *TOR2* wild-type cells (Figure 1). The *SAC7* deletion also suppressed a *tor2* null allele (data not shown); *sac7 tor2*^ts segregants cleared of the plasmid-borne *tor2*^ts allele were still viable. We next tested whether loss of *SAC7* could also restore growth in a *tor1 tor2*^ts double mutant. The *sac7* strain AS109-19c was crossed to *tor1 tor2*^ts strain SH221. The resulting diploid strain (AS114) was sporulated and dissected at 30°C, and segregants from 30 tetrads were examined for growth at 37°C. As shown in Figure 1, *sac7 tor1 tor2*^ts cells were not viable at 37°C. Growth curves and FACS analysis showed that after shift to the nonpermissive temperature, *sac7 tor1 tor2*^ts cells died within one generation as large unbudded cells with a 1n DNA content, as do *tor1 tor2*^ts cells (data not shown). These results show that disruption of *SAC7* suppresses the loss of the TOR2-unique function but not the loss of the TOR1-shared function and suggest that SAC7 is involved in the TOR2-mediated control of the actin cytoskeleton. The finding that a *SAC7* deletion suppresses a *tor2* null allele suggests that SAC7 is downstream of TOR2.

SAC7 Encodes a Novel Rho-Type GTPase-Activating Protein

The resequencing of the *SAC7* gene by the yeast genome sequencing project revealed that *SAC7* is larger than previously published (Dunn and Shortle, 1990). The new *SAC7* open reading frame contains 1965 nucleotides, extended by 763 bp at the 5′ end and by 400 bp at the 3′ end, and predicts a protein of 654 amino acids

GAP subdomain

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       Sac7   151 PIVVAKCGAY L..KANGLET SGIFR.IAGN GKRVKALQYI FSSPPDYGTK FNDWET....  203
       Bag7    76 PVVIAKSGKY L..KENALDT TGIFR.IAGS NKRVRELQAV FSKPPDYGRK FEGWCD....  128
       Bem2  1981 PTIVVKLLEE IELRG..LDE VGLYR.IPGS IGSINALKNA FDEEGA.T.. .DNSFTLED 2032
       Bem3   927 PSVVYRCLEY L.YKNRGIQE EGIFR.LSGS STVIKTLQER FDKEYDVDLC RYNESIEAKD  984
       Rga1   805 PMILSVCIDF IESDEENMRS EGIYRKSGSQ .LVIEEIEKQ FSAWKVQQ.. .NTETPNIL   859
       Lrg1   755 PIIIDELISS L..SQMDMSV EGIFR.KNGN IRRLRELTAT IDSNPTEAP. .DFSK....   804
        Bcr  1068 PYIVRQCVEE IERRG..MEE VGIYRSVSGV ATDIQALKAA F..DVNNK.. .DVSVMMSE 1118
   Chimaerin   122 PMVVDMCIRE IESRG..LNS EGLYR.VSGF SDLIEDVKMA FDRDGEKA.. .DISVNMYE  174
    P50rhogap   260 PIVLRETVAY LQAHA..LTT EGIFRRSSAN TQVVREVQQK Y......... ..NMGLPVDF  305
   Consensus       P.øø......ø......l...GIfR...g. ......l.. f
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GAP subdomain

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       Sac7   204 .......YT VHDVASLLRR YLNNLAEPLI PLSLYEQFRN PLRSRPRILR HMLTHEVSHP  255
       Bag7   129 .......FN VHDIATLLKR YLNSLSEPLV PLALYDIFRN PILENPKINE H.........  171
       Bem2  2033 DR.....WFE VNAIAGCFKM YLRELPDSLF SHAMV..... .......... .NDFTDLAIK 2071
       Bem3   985 DEASPSLYIG VNTVSGLLKL YLRKLPHLLF GDE....... ..........  .QFLSFKRV 1025
       Rga1   860 TE.....QD LNVVTGVLKR YLRKLPNPIF TFQIYEPLMR LVKSKKMMEN LPFVGGKLSL  913
       Lrg1   805 .......EN AIQLSALLKK FIREQPQPIL STDLYELWIK A......... ..........  837
        Bcr  1119 .......MD VNAIAGTLKL YFRELPEPLF TDEFY..... ..........  .PNFAEGIAL 1154
   Chimaerin   175 .......D INIITGALKL YFRDLPIPLI TYDAY..... ..........  .PKFIESAKI  209
    P50rhogap   306 DQ.....YNE LHLPAVILKT FLRELPEPLL TFDLY..... ..........  .PHVVGFLNI  344
   Consensus       .. v..ø...lk. ylr.lpeplø ....y
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GAP subdomain

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       Sac7   316 EELFVILSND TKQLTIYLLD LLSL.....F ARQSQFNLMS GRNLAAIFQP SILSHPQHDM  370
       Bag7   180 EDIYMLLPQQ NRHLILYLAA LLNL.....F ARNEKKNLMS ASNLAAIVQP SLLSHPKDEM  234
       Bem2  2089 LLQK..LPTC YYQTLKRIVF HLN.....KV HQHVVNNKMD ASNLAIVFSM SFINQEDLAN 2141
       Bem3  1044 LIESGLVPHA NLSLMYALFE LLV.....RI NENSKFNKMN LRNLCIVFSP TLNIFISMLQ 1097
       Rga1   930 ILED..LPRE HYRVLRVLSE HIE.....KV TRYSHWNRMT LYNLALVPAP GLIRDFSGEK  983
       Lrg1   850 LLIYSLLPTY NRNLLEALLS FLHWTSSFSY IENEMGSKMD IHNLFTVITP NILYLRHKEI  910
        Bcr  1167 LLLS..LPEA NLLTFLFLLD H.K.....RV AEKEAVNKMS LHNLFTVFGP TLLRFSEKES 1219
   Chimaerin   222 ALKL..LPPA HCETLRYLMA HLK.....RV TLHEKENLMN AENLGIVFGP TLMRSPELDA  274
    P50rhogap   356 VLQT..LPEE NYQVLRFLTA FLV.....QI SAHSDQNKMT NTNLAVVFGP NLLWAKDAAI  408
   Consensus       Lp.. ....ø..l.. .l.......ø ......N.M....NL..vf.P .ll..
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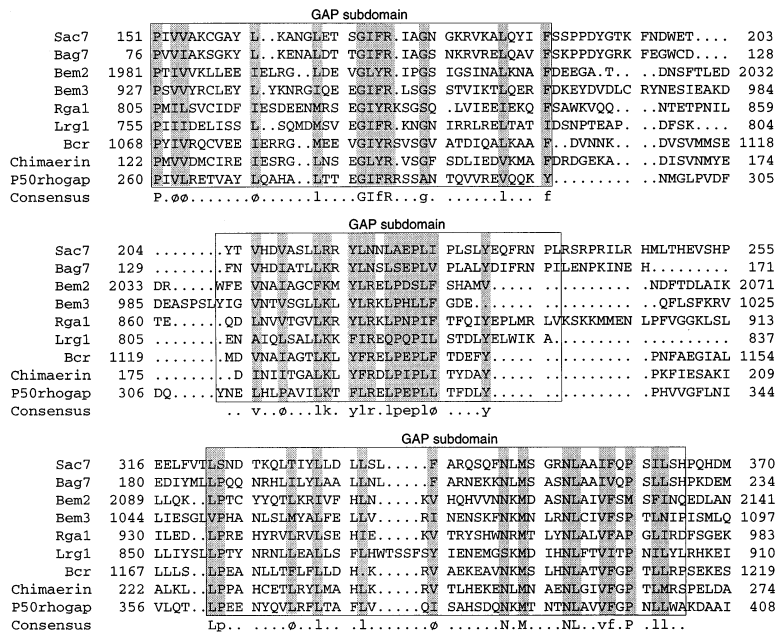
Figure 2. SAC7 Encodes a Novel Rho-Type GTPase-Activating Protein

An alignment of the Rho-GAP domains of the six known or predicted yeast Rho-type GAPs, SAC7, BAG7, BEM2, BEM3, RGA1, and LRG1 (Müller et al., 1994), human breakpoint cluster regions (Bcr) protein (Heisterkamp et al., 1985), human n-chimaerin (Hall et al., 1990), and p50rhoGAP (Barfod et al., 1993; Lancaster et al., 1994) is presented. Conserved residues are indicated by shading. The highly conserved subdomains are boxed.

with a molecular mass of 73.6 kDa. A homology search of the current EMBL and GenBank data bases with the extended *SAC7* open reading frame identified a domain in SAC7 (aa residues 151–370) that showed significant similarity to sequences common to Rho-type GTPase-activating proteins. An alignment of the GAP domain of SAC7 and that of other known Rho-GAPs from yeast and mammalian cells is shown in Figure 2. Evidence presented below indicates that SAC7 is indeed a Rho-type GAP. A search of the entire sequence of the yeast genome also identified a thus far uncharacterized yeast open reading frame (YOR134W) as encoding the closest SAC7 homolog. This structural homolog was termed BAG7. BAG7 is a protein of 409 aa with a predicted molecular mass of 46.2 kDa. BAG7 shows 42% overall identity to SAC7 and also contains a Rho-GAP domain that is 57% identical to the GAP domain of SAC7 (Figure 2).

The homology between SAC7 and Rho-GAPs led us to examine whether any of the other actin-related yeast Rho-GAPs (BEM2, BEM3, and RGA1) or BAG7 is also involved in TOR2 function (Zheng et al., 1993; Zheng et al., 1994; Stevenson et al., 1995; Chen et al., 1996). First, we determined whether a deletion of *BEM2*, *BEM3*, *RGA1*, or *BAG7* could suppress the lethality caused by a *tor2*[ts] mutation as does a *SAC7* deletion. Strains individually disrupted for *BEM2*, *BEM3*, *RGA1*, and *BAG7* were constructed (see Experimental Procedures) and crossed to a *tor2*[ts] mutant. Diploids (AS149, AS124, AS131, and AS130) were dissected and *tor2*[ts] segregants containing a GAP gene disruption were tested for growth at 37°C. Unlike disruption of *SAC7*, disruption of *BEM2*, *BEM3*, *RGA1*, or *BAG7* did not restore growth in a *tor2*[ts] mutant at the nonpermissive temperature (data not shown). Second, we determined whether overexpression of *BEM2*, *BEM3*, *RGA1*, or *BAG7* could suppress the growth of a *sac7 tor2*[ts] strain at 37°C. The *sac7 tor2*[ts] strains AS103-2a and AS147-1a were transformed with

the high copy number plasmids pBEM2, pBEM3, pRGA1, or pBAG7 (and pSAC7 and an empty vector as controls) and examined for growth at 37°C. While providing *SAC7* prevented growth in *sac7 tor2*[ts] cells at 37°C, *sac7 tor2*[ts] cells containing pBEM3, pRGA1, pBAG7, or the empty vector grew like the parental *sac7 tor2*[ts] strain (data not shown). The *sac7 tor2*[ts] cells containing pBEM2 gave an intermediate result of poor growth. Third, we determined whether overexpression of *BEM2*, *BEM3*, *RGA1*, or *BAG7* could suppress the growth defect of a *sac7* mutant at 15°C. The *sac7* strain MB75-1c was transformed with the high copy number plasmids pBEM2, pBEM3, pRGA1, or pBAG7 (and pSAC7 and an empty vector as controls) and examined for growth at 15°C. Overexpression of *BEM2*, *BEM3*, or *RGA1* did not suppress the growth defect of cells lacking *SAC7*. However, overexpression of *BAG7* completely suppressed the cold sensitivity of a *sac7* strain. Thus, BAG7 has at least partly overlapping function with SAC7 but neither BAG7, BEM3, nor RGA1 appeared to be involved in the function of TOR2. BEM2 may be involved in TOR2 function, as suggested by the poor growth of *sac7 tor2*[ts] cells containing pBEM2, although a *BEM2* disruption was not able to suppress a *tor2*[ts] mutation.

Overexpression of *RHO2* or *RHO1* Suppresses a *tor2* Mutation

Since *SAC7* has homology to Rho-type GTPase-activating proteins, we examined whether any of the known Rho-like GTPases in yeast are involved in TOR2 function. High copy number plasmids encoding the Rho-like GTPases (pCDC42, pRHO1, pRHO2, pRHO3, and pRHO4) or an empty vector were transformed into *tor2*[ts] strain SH121, and growth of transformants was monitored at 37°C. Whereas *tor2*[ts] cells transformed with pCDC42, pRHO1, pRHO3, pRHO4, or an empty vector were inviable at 37°C, *tor2*[ts] cells transformed with pRHO2 grew at 37°C, almost like wild-type cells (Figure
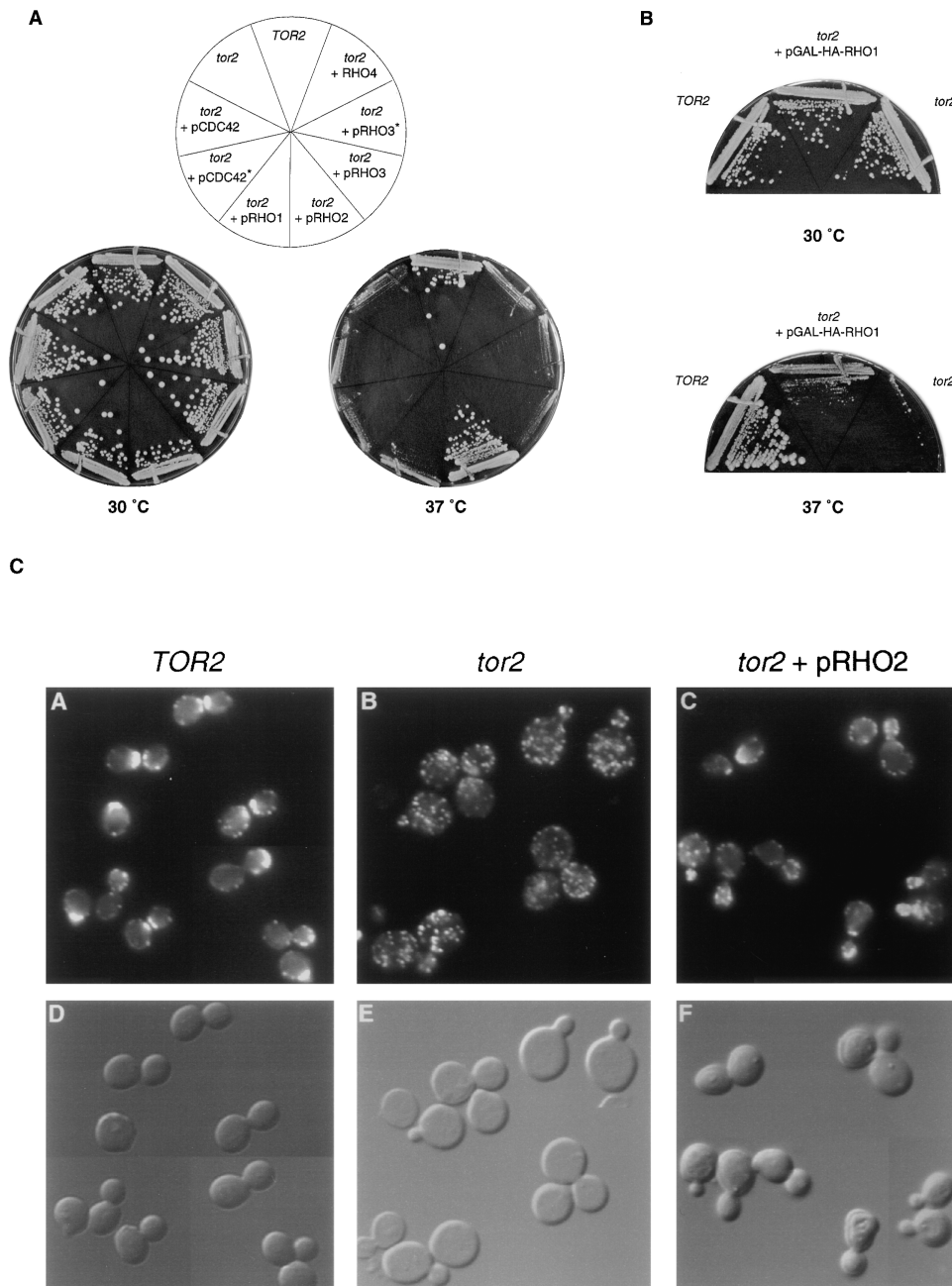
Figure 3. Overexpression of *RHO2* or *RHO1* Suppresses a *tor2* Mutation

(A) Wild-type *TOR2* (JK9-3da) cells; *tor2*[ts] (SH121) cells carrying an empty vector or pCDC42, pRHO1, pRHO2, pRHO3, pRHO3* (containing *RHO3*[val25]), or pRHO4; and *tor2*[ts] (AS129-1d) cells carrying pCDC42* (containing *CDC42*[val12]) were streaked out on YPD and incubated at 30°C or 37°C. *tor2*[ts] cells (SH121, AS129-1d) carrying an empty vector are not viable at 37°C.

(B) Wild-type *TOR2* (JK9-3da) and *tor2*[ts] (SH137) carrying an empty vector or pGAL-HA-RHO1 were streaked out on YPGal at 30°C or 37°C. *tor2*[ts] cells (SH137) carrying an empty vector are not viable at 37°C. The *tor2-37* allele was used in this experiment, because *tor2-21* is suppressed by galactose.

(C) Overexpression of *RHO2* suppresses the actin organization defect in *tor2*[ts] cells. Logarithmic cultures of wild-type *TOR2* (JK9-3da) (A and D) and *tor2*[ts] (SH121) cells carrying an empty vector (B and E) or pRHO2 (C and F) were grown at 30°C, shifted to 37°C for 8 hr, fixed, stained with TRITC-phalloidin, and observed by fluorescence (A–C) and Nomarski (D–F) microscopy. *tor2*[ts] cells carrying an empty vector have a random actin distribution.

3A). We also assessed if overexpression of constitutively activated alleles of the small GTPases or overexpression from a stronger promotor could restore growth in a *tor2*[ts] mutant. Overexpression of activated alleles of *CDC42* (pCDC42*) or *RHO3* (pRHO3*) did not suppress the lethality caused by loss of TOR2 (Figure 3A). We were unable to examine suppression by an activated *RHO1* allele because such an allele is extremely toxic. However,

galactose-induced overexpression of *RHO1* under control of the *GAL1* promotor (pGAL-HA-RHO1) weakly suppressed the lethality of a *tor2*[ts] mutation at 37°C (Figure 3B). Thus, specific overexpression of *RHO1* or *RHO2* suppressed the growth defect of a *tor2* mutant. This is in agreement with our finding that BEM2, a GAP for RHO1 and RHO2, may be involved in TOR2 function, whereas BEM3 and RGA1, GAPs for CDC42, are not involved in TOR2 function.

The high copy number plasmid pRHO2 was also transformed into *tor1 tor2*[ts] strain SH221. Overexpression of *RHO2* was not able to suppress the growth defect of a *tor1 tor2*[ts] mutant at nonpermissive temperature. Therefore, like disruption of SAC7, overexpression of *RHO2* suppressed loss of the TOR2-unique function but not loss of the TOR1-shared function.

To confirm that *RHO2* overexpression suppressed the loss of an actin-related TOR2-unique function, we examined the actin cytoskeleton in a *tor2* mutant overexpressing *RHO2*. Wild-type strain JK9-3da and *tor2*[ts] strain SH121 transformed with pRHO2 and an empty vector were grown at permissive temperature (30°C), shifted to nonpermissive temperature (37°C) for 8 hr, fixed, and stained with TRITC-phalloidin to visualize the actin cytoskeleton. In comparison to *tor2*[ts] cells carrying an empty vector, which exhibited a random distribution of actin in all phases of the cell cycle, *tor2*[ts] cells transformed with pRHO2 showed less severe defects in the organization of the actin cytoskeleton (Figure 3C). Taken together, the above findings suggest that RHO1 and RHO2, like SAC7, are involved in the actin-related TOR2 function and are downstream of TOR2 in a signaling pathway.

Overexpression of the GDP/GTP Exchange Factor ROM2 Suppresses the Growth Defect of a *tor2* Mutant

Since overexpression of *RHO1* or *RHO2* restored growth in a *tor2*[ts] mutant, we asked if overexpression of *ROM2*, encoding the GDP/GTP exchange factor (GEF) for RHO1 and RHO2, could also suppress a *tor2*[ts] mutation. The *tor2*[ts] strain SH121 was transformed with the high copy number plasmid pROM2, and transformants were monitored for growth at 37°C. As shown in Figure 6B, overexpression of *ROM2* restored growth of a *tor2*[ts] mutant at nonpermissive temperature. Overexpression of *ROM2* was not able to suppress the growth defect of a *tor1 tor2*[ts] mutant (data not shown).

We also examined if overexpression of *TOR2* could restore growth in a *rom2* mutant. *ROM2* was disrupted in our strain background (JK9-3da/α) to generate strain AS138-1c. The *rom2* strain AS138-1c had a growth defect at low temperatures (15°C, 24°C, and 30°C). However, in contrast to what has been reported previously for a *rom2* disruption in another strain background (Ozaki et al., 1996), our *rom2* strain grew on solid medium at 37°C almost like wild-type. AS138-1c cells (*rom2*) were transformed with the high copy number plasmid pTOR2, a plasmid bearing *ROM2*, or an empty vector, and streaked out on rich medium (YPD) at 30°C. The *ROM2* plasmid complemented the *rom2* mutation. However, neither the high copy number *TOR2* plasmid nor

the empty vector suppressed the growth defect of a *rom2* mutant (data not shown). This finding, combined with the above observations that high copy number *ROM2*, *RHO1*, or *RHO2* can suppress a *tor2* mutation, suggests that *ROM2* is downstream of *TOR2*.

SAC7 and *ROM2* Null Mutations Suppress Each Other

Our findings that deletion of *SAC7* and overexpression of *ROM2* suppress a *tor2* mutation suggest that the GAP homolog SAC7 and the GEF ROM2 have counteracting roles in mediating TOR2 function. To gain further evidence that SAC7 and ROM2 counteract each other, we examined whether *SAC7* and *ROM2* null mutations suppressed each other. First, the *rom2*, *sac7*, and *rom2 sac7* segregants from seven tetrads obtained by crossing *rom2* strain AS138-1c to *sac7* strain MB75-1c were streaked out on rich medium (YPD) at 30°C. Whereas *rom2* cells exhibited a severe growth defect at this temperature, *rom2 sac7* cells grew normally like *sac7* cells (data not shown). Second, *rom2*, *sac7*, and *rom2 sac7* strains (AS138-1c, MB75-1c, and AS139-3d, respectively) were streaked out on rich medium (YPD) at 15°C. Whereas *sac7* cells were severely impaired for growth at this temperature, *sac7 rom2* cells exhibited better growth and resembled *rom2* cells (data not shown). Thus, ROM2 and SAC7 indeed appear to antagonize each other, presumably by acting on the same Rho-like GTPase(s).

SAC7 Is a GAP for RHO1

The genetic and structural analyses of *SAC7* described above suggest that SAC7 is a GAP for RHO1 and/or RHO2. We investigated this possibility first by using the yeast two-hybrid system to detect an interaction between SAC7 and one of these RHOs. A DNA fragment encoding the full-length SAC7 protein was fused to *lexA*, and the resulting plasmid (pEG202::*SAC7*) was transformed into yeast strains that contained CDC42, RHO1, or RHO2 fused to the transcriptional activating domain of GAL4 (Stevenson et al., 1995). To facilitate the transport of these fusion proteins to the nucleus, all GTPases carried a C→S substitution at the C terminus, which removes the CAAX-box and prevents prenylation. It has been shown previously that RGA1 interacts with CDC42 in the two-hybrid system and that this interaction is specific for a CDC42 containing a G12V or a Q61L substitution, which traps the CDC42 protein in the GTP-bound form (Stevenson et al., 1995; Figure 4A). Thus, we also tested the interaction of SAC7 with CDC42[Q61L] and with the corresponding mutant RHO1 and RHO2 (RHO1[Q68H] and RHO2[Q65H]) proteins. Figure 4A shows that SAC7 strongly interacts with the GTP-bound activated RHO1[Q68H], but not with the unactivated form of RHO1. No interaction was detected between SAC7 and activated or unactivated CDC42 or RHO2. The finding that the putative GAP SAC7 interacts only with an activated form of RHO1 suggests that the interaction is physiologically relevant.

The above data suggest that SAC7 is a GAP for RHO1. To investigate this further, we performed GAP assays
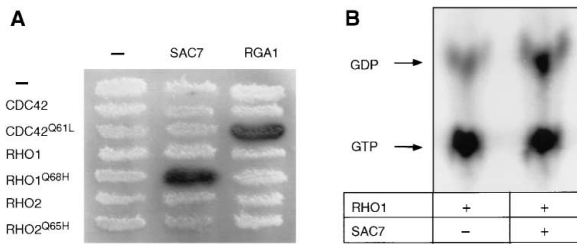
A

B

Figure 4. SAC7 Is a GAP for RHO1.

(A) SAC7 interacts with activated RHO1 in the two-hybrid system. Yeast strain EGY48 transformed with either pEG202 (−), pEG-202::*SAC7* (SAC7), or pEG202::*RGA1* (RGA1) and pJG4-5 (−), pJG4-5::*CDC42*^{C188S} (CDC42), pJG4-5::*CDC42*^{Q61L,C188S} (CDC42^{Q61L}), pJG4-5::*RHO1*^{C206S} (RHO1), pJG4-5::*RHO1*^{Q68H,C206S} (RHO1^{Q68H}), pJG4-5::*RHO2*^{C188S,C189S} (RHO2), or pJG4-5::*RHO2*^{Q65H,C188S,C189S} (RHO2^{Q65H}) and streaked out on SGal/Raffinose-URA-TRP-HIS/XGal plates at 30°C. Blue color (seen here as dark color) indicates a specific interaction in the two-hybrid system.

(B) SAC7 has GAP activity toward RHO1. Epitope-tagged RHO1 (+) was immunoprecipitated and allowed to bind to [α-^{32}P]GTP. Immunoprecipitated SAC7 (+) or a mock immunoprecipitate (−) was added, the mixture was briefly incubated, and the guanine nucleotides were extracted from RHO1 and separated chromatographically.

with SAC7 and RHO1 or RHO2 (see Experimental Procedures). Epitope-tagged RHO1 and RHO2 were immunoprecipitated and allowed to bind to [α-^{32}P]GTP. Immunoprecipitated epitope-tagged SAC7 or a mock immunoprecipitate were then added to the [α-^{32}P]GTP-bound RHO1 or RHO2. After a brief incubation, the guanine nucleotides were extracted and separated chromatographically. As shown in Figure 4B, addition of SAC7 enhanced the GTPase activity of RHO1 2.7-fold from a basal level already high in the absence of SAC7. No GAP activity of SAC7 toward RHO2 could be detected (data not shown). Thus, SAC7 is a GAP for RHO1.

TOR2 Is Required for Activation of ROM2

Our results so far have suggested that SAC7, RHO1, RHO2, and ROM2 are a GTPase switch activated by TOR2. One possibility for how TOR2 might activate this GTPase switch is that TOR2 activates ROM2. To test

this, we looked at whether the GDP/GTP exchange activity of ROM2 toward RHO1 is decreased in a *tor2* mutant strain compared to a *TOR2* wild-type strain. GDP/[^{35}S]GTPγS exchange activity toward RHO1 was measured in crude protein extracts prepared from *TOR2* wild-type and *tor2*, *rom2*, and *tor2 rom2* mutant strains (JK9-3da, SH121, AS138-1c, and AS146-7c, respectively) (see Experimental Procedures). As shown in Figure 5A, exchange activity on RHO1 was decreased to approximately 32% in extracts from a *tor2* mutant strain compared to extracts from a wild-type strain. With extracts from the *rom2* mutant, exchange activity was reduced to about 49%. In a *tor2 rom2* double mutant extract, exchange activity on RHO1 was reduced to approximately 21%, indicating that the decrease in GEF activity seen with the *tor2* strain was caused mainly by a loss of ROM2 GEF activity. The GEF activity remaining in the mutant extracts could be due, at least in part, to ROM1. Thus, TOR2 controls ROM2 activity, suggesting that TOR2 regulates RHO1 and RHO2 through ROM2.

To determine whether the decrease in ROM2 activity in a *tor2* mutant was caused by a decrease in the amount of ROM2 protein, a Western analysis was performed on protein extracts from wild-type and *tor2* mutant strains transformed with the pHA-ROM2 plasmid encoding a functional, epitope-tagged ROM2. As shown in Figure 5B, HA-ROM2 was equally expressed in wild-type and *tor2* mutant strains. Thus, the control of ROM2 by TOR2 was not at the level of ROM2 synthesis or stability.

The PH Domain of ROM2 Is Required for Suppression of a *tor2* Mutation

Like most of the GEFs for members of the Ras superfamily, ROM2 possesses a PH domain C-terminally adjacent to its GEF domain (Ozaki et al., 1996). PH domains have been shown to bind to phosphatidylinositol derivatives and have been implicated in the activation of signaling molecules (for reviews, see Lemmon et al., 1996; Shaw, 1996). We asked if the PH domain of ROM2 was necessary for suppression of a *tor2* mutation by high copy *ROM2*. A *ROM2* allele containing a PH domain deletion, *rom2*^{ΔPH}, was constructed (see Experimental Procedures) (Figure 6A). The deletion did not affect the ROM2
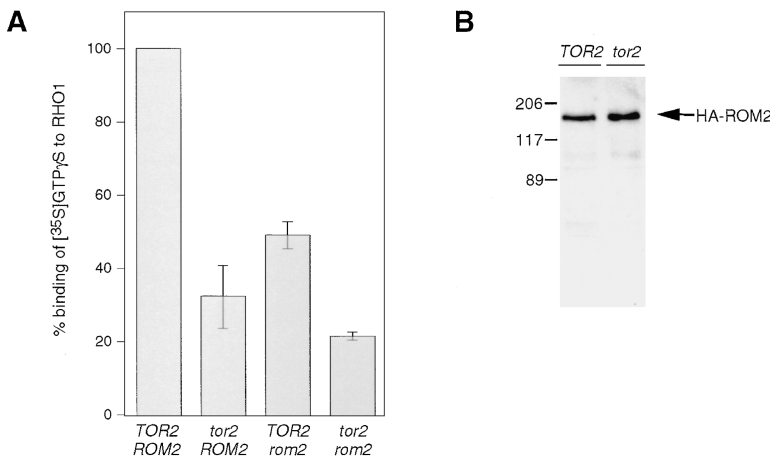
A

B

Figure 5. TOR2 Is Required for ROM2 GEF Activity

(A) GEF activity toward RHO1 is decreased in a *tor2* mutant strain. GDP/[^{35}S]GTPγS exchange activity toward immunoprecipitated RHO1 in crude protein extracts from *TOR2 ROM2* (JK9-3da), *tor2 ROM2* (SH121), *TOR2 rom2* (AS138-1c), and *tor2 rom2* (AS146-7c) was measured. All strains were grown at 30°C and shifted to 37°C for 8 hr before preparation of extracts.

(B) ROM2 is equally expressed in wild-type and *tor2* mutant strains at 37°C. Western analysis was performed on protein extracts from wild-type *TOR2* (JK9-3da) and *tor2* (SH121) mutant strains carrying a plasmid encoding HA-tagged ROM2. Equal amounts of total protein were loaded in each lane.
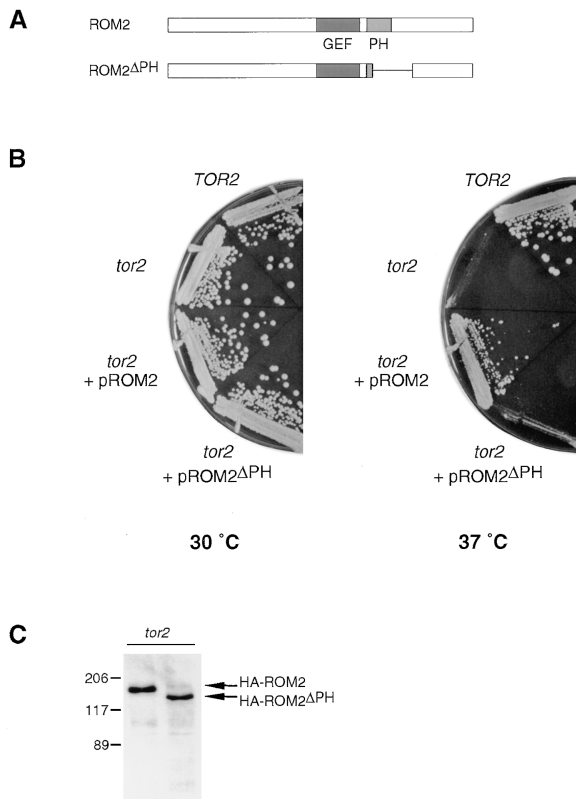
A

B

30 °C   37 °C

C

Figure 6. The PH Domain of ROM2 Is Required for Suppression of a *tor2* Mutation

(A) Schematic representation of wild-type ROM2 and mutant ROM2$^{\Delta PH}$ lacking amino acids 908–1083. ROM2 is 1356 amino acids; the GEF and PH domains are amino acids 660–856 and 875–1000, respectively.

(B) Wild-type *TOR2* (JK9-3da) cells, *tor2*^{ts} (SH121) cells carrying an empty vector or pROM2, or pROM2$^{\Delta PH}$ were streaked out on YPD and incubated at 30°C or 37°C. *tor2*^{ts} cells (SH121) carrying an empty vector are not viable at 37°C.

(C) ROM2$^{\Delta PH}$ is stably expressed in *tor2*^{ts} cells at 37°C. Western analysis was performed on protein extracts from *tor2*^{ts} strain SH121 transformed with either pHA-ROM2 or pHA-ROM2$^{\Delta PH}$, grown at 30°C and shifted to 37°C for 8 hr. HA-ROM2$^{\Delta PH}$ is approximately 20 kDa smaller than HA-ROM2. Equal amounts of total protein were loaded in each lane.

GEF domain, and the deleted residues were shown previously not to be required for at least basal level ROM2 GEF activity (Ozaki et al., 1996). The *tor2*^{ts} strain SH121 was transformed with pROM2 and pROM2$^{\Delta PH}$ (high copy number plasmids containing *ROM2* and *rom2*$^{\Delta PH}$, respectively) and monitored for growth on rich medium at 30°C and 37°C (Figure 6B). Whereas overexpression of wild-type *ROM2* restored growth at the nonpermissive temperature, overexpression of *rom2*$^{\Delta PH}$ did not suppress the growth defect of the *tor2*^{ts} mutant. Western blot analysis of whole cell extracts showed that the epitope-tagged ROM2$^{\Delta PH}$ protein (derived from functional, epitope-tagged ROM2) was stably expressed in the *tor2* mutant (Figure 6C). Thus, the PH domain of ROM2 was required to suppress the lethality of a *tor2*^{ts} mutation but was not required for the stability or synthe-
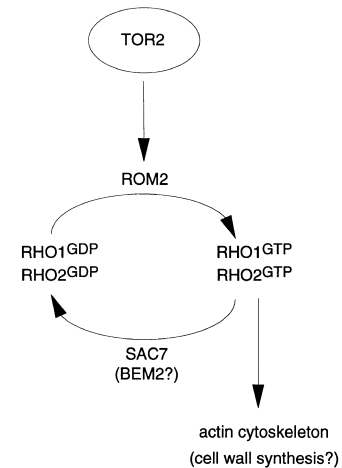

Figure 7. Model of TOR2 Signaling to the Actin Cytoskeleton through a Rho-Type GTPase Switch Composed of RHO1, RHO2, ROM2, and SAC7

See Discussion for further details.

sis of ROM2, suggesting that TOR2 might activate ROM2 via the ROM2 PH domain.

Discussion

We have shown previously that TOR2 is required for the cell cycle–dependent organization of the actin cytoskeleton (Schmidt et al., 1996). Here, we describe several findings concerning the mechanism by which TOR2 controls the actin cytoskeleton. First, overexpression of *RHO1* or *RHO2*, encoding two Rho-like GTPases, or *ROM2*, encoding the guanine nucleotide exchange factor for RHO1 and RHO2, suppresses the growth defect of a *tor2* mutant. *RHO2* overexpression also suppresses the actin organization defect of a *tor2* mutant. Second, deletion of *SAC7*, encoding a novel GTPase-activating protein for RHO1, suppresses *tor2* and *rom2* null mutations. Importantly, *SAC7* was originally identified as a second site suppressor of actin mutations that alter the polarized distribution of the actin cytoskeleton (Dunn and Shortle, 1990). Third, ROM2 GEF activity on RHO1 is reduced in a *tor2* mutant. Fourth, the PH domain of ROM2 is required for ROM2-mediated suppression of *tor2*. These findings suggest that TOR2 signals to the actin cytoskeleton by regulating a GTPase switch composed of RHO1, RHO2, ROM2, and SAC7 (Figure 7). Because any condition known or predicted to enhance the amount of activated GTPase (deletion of *SAC7* or overexpression of *RHO1*, *RHO2*, or *ROM2*) suppresses a *tor2* mutation, TOR2 presumably activates this GTPase switch. Furthermore, our finding that ROM2 GEF activity is reduced in a *tor2* mutant suggests that the PI kinase homolog TOR2 activates RHO1 and RHO2 by activating their exchange factor ROM2, possibly via the ROM2 PH domain. SAC7 and perhaps BEM2 down-regulate RHO1 and RHO2. It remains to be determined if TOR2 also controls cell wall synthesis via at least RHO1.

How does TOR2 activate ROM2? Our results suggest that this activation requires the TOR2 kinase domain

Table 1. Yeast Strains Used in This Study

Strain	Genotype
JK9-3da	*MAT**a** leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JK9-3dα	*MATα leu2-3,112 ura3-52 trp1 his4 rme1 HML**a***
JK9-3da/α	*MAT**a**/MATα leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1/trp1 his4/his4 rme1/rme1 HML**a**/HML**a***
SH121	JK9-3d**a** *ade2 tor2::ADE2-3/*YCplac111::*tor2-21*^{ts}
SH221	JK9-3d**a** *ade2 his3 HIS4 tor1::HIS3 tor2::ADE2-3/*YCplac111::*tor2-21*^{ts}
MB75-1c	JK9-3d**a** *sac7::kanMX*
AS100-6a	JK9-3d**a** *sac7::kanMX ade2*
AS100-3c	JK9-3dα *ade2 tor2::ADE2-3/*YCplac111::*tor2-21*^{ts}
AS103	JK9-3d**a**/α *ade2/ade2 sac7::kanMX/SAC7 tor2::ADE2-3/TOR2/*YCplac111::*tor2-21*^{ts}
AS103-2a	JK9-3d**a** *ade2 sac7::kanMX tor2::ADE2-3/*YCplac111::*tor2-21*^{ts}
AS109-19c	JK9-3dα *sac7::kanMX ade2 his3 TRP1 HIS4*
AS114	JK9-3d**a**/α *ade2/ade2 his3/his3 HIS4/HIS4 trp1/TRP1 sac7::kanMX/SAC7 tor1::HIS3/TOR1 tor2::ADE2-3/TOR2/*YCplac111:*tor2-21*^{ts}
AS114-4c	JK9-3d**a** *ade2 his3 HIS4 sac7::kanMX tor1::HIS3 tor2::ADE2-3/*YCplac111::*tor2-21*^{ts}
AS117-3c	JK9-3d**a** *bem2::URA3*
AS116-1d	JK9-3d**a** *bem3::LEU2*
AS149-1c	JK9-3d**a** *rga1::URA3 ade2*
AS115-3a	JK9-3d**a** *bag7::HIS3 his3 HIS4*
AS124	JK9-3d**a**/α *ade2/ade2 bem2::URA3/BEM2 tor2::ADE2-3/TOR2/*YCplac111::*tor2-21*^{ts}
AS131	JK9-3d**a**/α *ade2/ade2 bem3::LEU2/BEM3 tor2::ADE2-3/TOR2/*YCplac33::*tor2-21*^{ts}
AS130	JK9-3d**a**/α *ade2/ADE2 his3/his3 HIS4/HIS4 bag7::HIS3/BAG7tor2::ADE2-3/TOR2/*YCplac111::*tor2-21*^{ts}
AS149	JK9-3d**a**/α *ade2/ade2 rga1::URA3/RGA1 tor2::ADE2-3/TOR2/*YCplac111::*tor2-21*^{ts}
AS147-1a	JK9-3dα *ade2 sac7::kanMX tor2::ADE2-3/*YCplac33::*tor2-21*^{ts}
AS129-1d	JK9-3d**a** *ade2 tor2::ADE2-3/*YCplac33::*tor2-21*^{ts}
SH137	JK9-3d**a** *ade2 tor2::ADE2-3/*YCplac111::*tor2-37*^{ts}
AS138-1c	JK9-3dα *rom2::URA3*
AS139-3d	JK9-3dα *rom2::URA3 sac7::kanMX*
AS146-7c	JK9-3d**a** *ade2 rom2::URA3 tor2::ADE2-3/*YCplac111::*tor2-21*^{ts}

(Schmidt et al., 1996) and the ROM2 PH domain and is not at the level of ROM2 synthesis or stability. Furthermore, using the two-hybrid system, we did not detect a direct interaction between TOR2 and ROM2 (A. S., T. B., and M. N. H., unpublished data). Thus, an attractive and simple possibility is that TOR2, as a PI kinase, produces a phosphorylated phosphoinositide that binds the ROM2 PH domain and thereby activates ROM2. This signaling from TOR2 to ROM2 through a phosphorylated phosphoinositide intermediate could be direct, as TOR2,

Table 2. Plasmids Used in This Study

Plasmid	Characteristics and Source
pSAC7	= pMB7, *SAC7* in YEplac195 (2μ, *URA3*). Made by subcloning a 3.5 kb HindIII–EcoRI fragment from YEp13::*SAC7* into YEplac195.
pRGA1	= pRGA1.3, *RGA1* in YEp24 (2μ, *URA3*) (Stevenson et al., 1995)
pBEM2	= pPB415, *BEM2* in pTSV30A (2μ, *LEU2, ADE3*) (A. Bender, Indiana University, Bloomington)
pBEM3	= pPB547, *BEM3* in pSC113 (2μ, *LEU2*) (A. Bender, Indiana University, Bloomington)
pBAG7	= pAS33, *BAG7* in pSEY18 (2μ, *URA3*). Made by subcloning a 3.4 kb BglII fragment from cosmid pEOA986 (V. Benes, EMBL, Heidelberg, Germany) into the BamHI site of pSEY18.
pCDC42	= pPB102, *CDC42* in YEp24 (2μ, *URA3*) (Bender and Pringle, 1989)
pCDC42*	= YCp(CDC42SC^{val12}) (2μ, *LEU2*) (Ziman et al., 1991)
pRHO1	= *RHO1* in YEp24 (2μ, *URA3*) (Y. Takai, Osaka University Medical School, Japan)
pRHO2	= pC-186, *RHO2* (2μ, *URA3*) (Madaule et al., 1987)
pRHO3	= pYO324-RHO3 (2μ, *TRP1*) (Y. Matsui, University of Tokyo, Japan)
pRHO3*	= pRS314-RHO3^{val25} (*CEN, TRP1*) (Imai et al., 1996)
pRHO4	= pOPR4, *RHO4* (2μ, *TRP1*) (Matsui et al., 1992)
pGAL-HA-RHO1	= pTB230, expresses N-terminally HA-tagged RHO1 under the control of the *GAL1* promotor in pAS25 (pSEYC68galp, *CEN, URA3, GAL1* promotor, HA-tag)
pROM2	= pAS30, *ROM2* in YEplac195 (2μ, *URA3*). Made by subcloning a 6.3 kb BglII–HindIII fragment containing the entire *ROM2* gene from cosmid YSCL8039 (from ATCC, Rockville, Maryland)
pTOR2	= pJK6, *TOR2* in YEplac181 (2μ, *LEU2*)
pGAL-HA-SAC7	= pAS31, expresses N-terminally HA-tagged SAC7 under the control of the *GAL1* promotor in pAS24 (YCplac111galp, *CEN, LEU2, GAL1* promotor, HA-tag)
pGAL-HA-RHO2	= pAS35, expresses N-terminally HA-tagged RHO2 under the control of the *GAL1* promotor in pAS25 (*CEN, URA3, GAL1* promotor, HA-tag)
pHA-ROM2	= pAS32, encodes N-terminally HA-tagged ROM2 in YEplac195 (2μ, *URA3*)
pROM2^{ΔPH}	= pAS36, contains *rom2*^{ΔPH} in YEplac195 (2μ, *URA3*), made by an in-frame deletion of a 528 bp XbaI–SpeI fragment (amino acids 908–1083)
pHA-ROM2^{ΔPH}	= pAS37, same as pROM2^{ΔPH} but encodes N-terminally HA-tagged ROM2^{ΔPH}

RHO1, RHO2, and presumably active ROM2 are all localized at the plasma membrane (J. Kunz, U. Schneider, I. Stevenson, and M. N. H, unpublished data; A. S. and M. N. H., unpublished data; Yamochi et al., 1994). Another possibility is that the activation is by phosphorylation of ROM2, as has been suggested recently for the activation of the PH domain–containing protein kinase Akt/PKB (Kohn et al., 1996). TOR2 or another protein could be the activating protein kinase. TOR2 has not yet been demonstrated to be either a lipid or a protein kinase. Although we find the above models appealing, our data do not exclude other, more indirect mechanisms of ROM2 activation by TOR2.

What is upstream of TOR2 in controlling RHO1 and RHO2? TOR1 and TOR2 activate translation initiation in response to nutrients (Barbet et al., 1996; Di Como and Arndt, 1996). TOR2 might also control RHO1 and RHO2 and eventually cell polarity, in response to nutrients. Interestingly, under conditions of nitrogen starvation, S. cerevisiae undergoes filamentous growth as a foraging response (Kron and Gow, 1995). Filamentous growth is caused by a change in the normal pattern of polarized cell growth and is presumably elicited by an underlying change in the actin cytoskeleton (Mösch et al., 1996).

The pathway we propose above for yeast cells may be conserved in mammalian cells. Mammalian PI 3-kinase induces actin reorganization by Rac- and Rho-dependent pathways (Reif et al., 1996) and is also possibly upstream of mTOR (FRAP/RAFT) (Downward, 1995). Furthermore, mammalian RhoA can substitute for RHO1 in yeast cells (Qadota et al., 1994). Thus, TOR may be upstream of Rho-type GTPases in mediating actin changes in mammalian cells. However, it remains to be demonstrated that mTOR has a role in actin organization and that it is indeed upstream of a Rho-type GTPase.

Experimental Procedures

Strains, Plasmids, and Media
S. cerevisiae strains used in this work are listed in Table 1. All strains were isogenic JK9-3da derivates. Plasmids used in this study are listed in Table 2. The composition of rich medium (YPD, YPGal) and synthetic minimal medium (SD, SGal) complemented with the appropriate nutrients for plasmid maintenance was as described (Guthrie and Fink, 1991).

Fluorescence Microscopy
Cells were grown to early logarithmic phase, fixed in formaldehyde, and stained with TRITC-phalloidin (Sigma) to visualize actin as described (Benedetti et al., 1994).

Gene Disruptions
To disrupt SAC7, we replaced the entire open reading frame of SAC7 with a PCR-generated DNA fragment containing the kanMX2 module (Wach et al., 1994) flanked by 59 bp and 57 bp homologous to the region either directly upstream of the start codon or downstream of the stop codon of SAC7. BEM2 was disrupted by transformation of a 4 kb SspI–SalI fragment containing bem2::URA3 (Peterson et al., 1994). The bem2::URA3 disruption in our strain background (AS117-3c) conferred slow growth at all temperatures, but the growth defect was less severe at high temperature (37°C). BEM3 was disrupted by transformation of a 3 kb XhoI–BamHI fragment containing bem3::LEU2 (Zheng et al., 1994). RGA1 was disrupted by transformation of a 5.8 kb HindIII fragment containing rga1::URA3 (Stevenson et al., 1995). The bem3::LEU2 and rga1::URA3 disruptions did not confer any growth defect in our strain background (strains AS116-1d and AS149-1c, respectively),

as previously published for other strain backgrounds (Zheng et al., 1994; Stevenson et al., 1995). BAG7 was disrupted by replacing the entire BAG7 open reading frame with a PCR-generated DNA fragment containing the HIS3 module (A. Wach, and P. Philippsen, Biozentrum, Basel, Switzerland) flanked by 45 bp homologous to the region either upstream of the start codon or downstream of the stop codon of BAG7. A bag7::HIS3 strain (AS115-3a) grew normally at all temperatures. To disrupt ROM2, the internal 1.9 kb XbaI fragment of the ROM2 gene was replaced with a 1 kb XbaI fragment containing the URA3 gene. A 3.2 kb EcoRI fragment containing rom2::URA3 was transformed into yeast. All of the above disruptions were verified by Southern analysis.

Two-Hybrid Assays
The Interaction Trap two-hybrid system was used (Gyuris et al., 1993). Plasmids pEG202, pJG4-5, and yeast strain EGY48 were obtained from R. Brent (MGH, Boston). Plasmids pEG202::RGA1, pJG4-5::CDC42^{C188S}, pJG4-5::CDC42^{Q61L,C188S}, pEG202::RHO1^{C206S}, and pEG202::RHO2^{C188S,C189S} were obtained from C. De Virgilio (Botanisches Institut, University of Basel, Basel, Switzerland). pJG4-5::RHO2^{C188S,C189S} was constructed by subcloning an 0.8 kb EcoRI–XhoI fragment from pEG202::RHO2^{C188S,C189S} into pJG4-5. To construct pJG4-5::RHO2^{Q65H,C188S,C189S}, PCR-based mutagenesis was performed with the mutagenic primers 5′-GATACAGCGGGACATGAGGAATAT GAACG and 5′-CGTTCATATTCCTCATGTCCCGCTGTATC and the flanking primers 5′-AGCGACCTCATGCTATAC and 5′-CGTCAGCAG AGCTTCAC and pEG202::RHO2^{C188S,C189S} as template. A 0.8 kb EcoRI–XhoI fragment was excised and subcloned into pJG4-5. pJG4-5::RHO1^{C206S} was constructed by subcloning a 0.8 kb EcoRI– XhoI fragment from pEG202::RHO1^{C206S} into pJG4-5. To construct pJG4-5::RHO1^{Q68H,C206S}, the mutant RHO1^{Q68H} allele (plasmid p68-LEUe (Madaule et al., 1987)) was amplified by PCR with primers (5′-CGCTCGAGATGTCACAACAAGTTGGTA and 5′-GACACTCGAG CTATAACAAGACAGACTTCTTCTTC) that introduced XhoI cloning sites at the N terminus and C terminus of RHO1 as well as a single nucleotide change converting codon 206 from TGT (C) to TCT (S). The PCR product was digested with XhoI and cloned into pJG4-5. pEG202::SAC7 was constructed by subcloning a 2.5 kb SalI–XhoI fragment containing the entire SAC7 open reading frame from pAS31 into pEG202. To assess β-galactosidase activities, strain EGY48 containing the lexAop–lacZ reporter plasmid pSH18-34 (Gyuris et al., 1993) was cotransformed with either pEG202 or with a pEG202-derived plasmid expressing a LexA BD fusion protein and with pJG4-5 or a pJG4-5-derived plasmid expressing an AD fusion protein and four independent transformants of each combination were streaked out on SGal/Raffinose-URA-TRP-HIS/XGal plates.

SAC7 GAP Activity Assay
The GAP activity of HA-SAC7 was assayed by guanine nucleotide analysis by thin layer chromatography as described (Tanaka et al., 1991; Park et al., 1993), with slight modifications. All procedures were at 4°C unless stated otherwise. SAC7 was expressed in yeast from the GAL1 promotor as a fusion protein with a HA epitope at the N terminus (pAS31). The HA-SAC7 construct encoding epitope-tagged SAC7 complemented a sac7 deletion strain (A. S., unpublished data). RHO1 was expressed in yeast from the GAL1 promotor as a fusion protein with a HA epitope at the N terminus (pTB230). To prepare whole cell extracts, yeast cells were resuspended in extraction buffer I (50 mM Tris-HCl (pH 7.5) 50 mM NaCl, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol, and various protease inhibitors), lysed with glassbeads in a bead-beater, and centrifuged at 500 × g for 5 min to remove cell debris. HA-tagged SAC7 and HA-tagged RHO1 were immunoprecipitated from approximately 0.2 mg whole cell extract with anti-HA antibody (BabCO, Berkeley) and goat anti-mouse IgG-coupled protein A–Sepharose beads. As the control (− SAC7, Figure 4B), a mock immunoprecipitation was performed with the same anti-HA antibody but with an extract of cells lacking HA-tagged SAC7. After washing 5 times with extraction buffer I containing 500 mM NaCl and 0.5% Triton X-100 and once with extraction buffer I, the immune complex containing RHO1 was allowed to bind to 1 μCi [α-32P]GTP (Amersham) for 15 min at 25°C. The reaction was stopped on ice, mixed with the immune complex containing HA-tagged SAC7 (+ SAC7) or the control (− SAC7) in

extraction buffer I containing 2 mM DTT, 2 mM MgCl$_2$, and incubated for 5 min at 25°C. The beads were washed 3 times with extraction buffer I, and the nucleotides were eluted from RHO1 by incubating with 15 μl of nucleotide elution buffer (1% SDS, 20 mM EDTA) for 5 min at 65°C. The nucleotides were loaded onto a PEI cellulose plate (Merck) and chromatographed in 1 M LiCl. After autoradiography, the amount of each nucleotide was quantified using a Molecular Dynamics Phosphoimager. SAC7 GAP activity on functional, HA-tagged RHO2 was also assayed, as described above for RHO1. Identical results were obtained in two independent assays, for both RHO1 and RHO2.

GEF Activity Assay

The GEF activity of various strains toward RHO1 was assayed by measuring the binding of [^{35}S]GTPγS to RHO1 as described, with slight modifications (Yamamoto et al., 1990). All procedures were at 4°C, unless stated otherwise. Whole cell extracts from various yeast strains were prepared by resuspending cells in extraction buffer II (20 mM Tris-HCl (pH 7.5) 10 mM MgCl$_2$, 2.5 mM EDTA, 1 mM DTT, and various protease inhibitors), lysing with glass beads in a bead-beater, and removing cell debris by centrifugation at 500 × g for 10 min. HA-tagged RHO1 was immunoprecipitated from whole-cell extracts as described above. The immune complex containing RHO1 was resuspended in extraction buffer II and incubated with 1 μM [^{35}S]GTPγS (Amersham) in the presence of 100 μg of whole cell extract and 1 mM DTT, 0.75 mM L-α dimyristoylphosphatidylcholine for 5 min at 25°C. The reaction was stopped by adding 1 ml of ice-cold stop buffer (20 mM Tris-HCl, (pH 7.5), 25 mM MgCl$_2$, 100 mM NaCl). The diluted mixture was filtered through nitrocellulose filters presoaked in stop buffer, and the filters were washed twice with stop buffer. The radioactivity trapped on the filters was quantified by scintillation counting. The GEF activity on RHO1 was calculated by subtracting the background, i.e., the radioactivity trapped on filters in the same experiment except that the immunoprecipitation was done from extracts of cells lacking HA-tagged RHO1. The GEF activity on RHO1 in the mutant extract was expressed as percentage of the GEF activity on RHO1 in the wild-type extract.

Miscellaneous Methods

Whole cell extracts for SDS–PAGE and Western Analysis were prepared by resuspending cells in extraction buffer I, lysing them with glass beads in a bead-beater, and removing cell debris by centrifugation at 500 × g for 10 min. SDS–PAGE and Western analysis were done by standard methods. Yeast transformation was performed by the lithium acetate procedure (Ito et al., 1983). Escherichia coli strain DH5α was used for propagation and isolation of plasmids as described (Sambrook et al., 1989). Restriction enzyme digests and ligations were done by standard methods. All enzymes and buffers were obtained commercially (Boehringer Mannheim). The EMBL and GenBank data bases were searched for SAC7 homologs using the BLAST program (Devereux et al., 1984).

Acknowledgments

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