The EMBO Journal Vol.17 No.8 pp.2235–2245, 1998

Cell wall integrity modulates RHO1 activity via the exchange factor ROM2

Marc Bickle, Pierre-Alain Delley, Anja Schmidt and Michael N.Hall[1]

Department of Biochemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

[1]Corresponding author
e-mail: hall@ubaclu.unibas.ch

The essential phosphatidylinositol kinase homologue TOR2 of *Saccharomyces cerevisiae* controls the actin cytoskeleton by activating a GTPase switch consisting of RHO1 (GTPase), ROM2 (GEF) and SAC7 (GAP). We have identified two mutations, *rot1-1* and *rot2-1*, that suppress the loss of *TOR2* and are synthetic-lethal. The wild-type *ROT1* and *ROT2* genes and a multicopy suppressor, *BIG1*, were isolated by their ability to rescue the *rot1-1 rot2-1* double mutant. *ROT2* encodes glucosidase II, and *ROT1* and *BIG1* encode novel proteins. We present evidence that cell wall defects activate RHO1. First, *rot1*, *rot2*, *big1*, *cwh41*, *gas1* and *fks1* mutations all confer cell wall defects and suppress *tor2^{ts}*. Second, destabilizing the cell wall by supplementing the growth medium with 0.005% SDS also suppresses a *tor2^{ts}* mutation. Third, disturbing the cell wall with SDS or a *rot1*, *rot2*, *big1*, *cwh41*, *gas1* or *fks1* mutation increases GDP/GTP exchange activity toward RHO1. These results suggest that cell wall defects suppress a *tor2* mutation by activating RHO1 independently of TOR2, thereby inducing TOR2-independent polarization of the actin cytoskeleton and cell wall synthesis. Activation of RHO1, a subunit of the cell wall synthesis enzyme glucan synthase, by a cell wall alteration would ensure that cell wall synthesis occurs only when and where needed. The mechanism of RHO1 activation by a cell wall alteration is via the exchange factor ROM2 and could be analogous to signalling by integrin receptors in mammalian cells.

Keywords: actin cytoskeleton/glucosidase/GTPase/signal transduction/TOR2

Introduction

The *Saccharomyces cerevisiae* proteins TOR1 and TOR2 were originally identified genetically as the targets of the immunophilin–immunosuppressive complex FKBP–rapamycin (Heitman *et al.*, 1991; Kunz *et al.*, 1993; Cafferkey *et al.*, 1994; Helliwell *et al.*, 1994). They contain a lipid kinase domain near their C-terminus and are thus structurally related to PI 3- and PI 4-kinases. The kinase domain is essential for TOR function, although no kinase activity has been demonstrated for either TOR1 or TOR2 (Zheng *et al.*, 1995; Schmidt *et al.*, 1996). The mammalian counterpart of TOR1 and TOR2, which is also the target of FKBP-rapamycin, has protein kinase activity (Brown *et al.*, 1995; Brunn *et al.*, 1997).

TOR2 has two essential signalling functions (Kunz *et al.*, 1993; Helliwell *et al.*, 1994; Zheng *et al.*, 1995; Hall, 1996). One function is shared with TOR1 and is required for signalling activation of translation initiation and early G_1 progression in response to nutrients (Barbet *et al.*, 1996; Di Como and Arndt, 1996). Translation initiation and G_1 progression in mammalian cells are also controlled by TOR (mTOR/FRAP/RAFT), suggesting that this TOR signalling pathway is highly conserved (Beretta *et al.*, 1996; von Manteuffel *et al.*, 1996; Thomas and Hall, 1997). The second TOR2 function, which TOR1 is unable to perform, mediates the cell cycle-dependent organization of the actin cytoskeleton (Schmidt *et al.*, 1996, 1997). TOR2 signals to the actin cytoskeleton by activating a Rho-type GTPase switch.

In yeast, the organization of the actin cytoskeleton is controlled so as to optimize secretion toward regions of polarized growth (Adams and Pringle, 1984; Kilmartin and Adams, 1984). As in mammalian cells, the yeast cytoskeleton is regulated by a family of small GTPases comprising CDC42 (Johnson and Pringle, 1990), RHO1, RHO2 (Madaule *et al.*, 1987), RHO3 and RHO4 (Matsui and Toh-e, 1992b). During the G_1 phase of the cell cycle, growth occurs uniformly over the entire cell surface and cortical actin patches are distributed randomly. As cells progress into the cell cycle, CDC42 mediates bud emergence by controlling the concentration of actin cortical patches at the chosen bud site (Bender and Pringle, 1989; Adams *et al.*, 1990). RHO1, and possibly its non-essential homologue RHO2, are required for further bud growth (Yamochi *et al.*, 1994). RHO1 controls three different proteins which are important for cell wall synthesis and actin organization. It binds and activates β-1,3 glucan synthase which produces a major structural component of the cell wall (Drgonova *et al.*, 1996; Qadota *et al.*, 1996). It also binds and activates PKC1 (Nonaka *et al.*, 1995; Kamada *et al.*, 1996) which transcriptionally activates many cell wall biosynthetic enzymes, including β-1,3 glucan synthase (Shimizu *et al.*, 1994; Jiang *et al.*, 1995; Igual *et al.*, 1996; Madden *et al.*, 1997). PKC1 may also play a role in organizing the actin cytoskeleton (Mazzoni *et al.*, 1993). Lastly, RHO1 may control the actin cytoskeleton by binding BNI1 which binds profilin (Kohno *et al.*, 1996; Evangelista *et al.*, 1997). RHO3 and RHO4 are also important for bud growth; depleting them causes cells to lyse or arrest growth with small buds and delocalized actin (Matsui and Toh-e, 1992a).

Small GTPases exist in two states, a GTP-bound active state and a GDP-bound inactive state (Hall, 1990; Bourne *et al.*, 1991). GDP/GTP exchange factors (GEFs) catalyse the exchange of GDP for GTP and are thus G-protein activators. GTPase-activating proteins (GAPs) accelerate

the rate of GTP hydrolysis and are thus negative regulators. The mechanisms by which GEFs and GAPs are controlled are poorly understood.

The yeast cell wall is a dynamic structure that makes up 15–30% of the dry weight of the cell (Klis, 1994; Orlean, 1997). It is composed of three major components, mannoproteins, β-1,6-glucan and β-1,3-glucan, all cross-linked to each other and to chitin, an *N*-acetylglucosamine polymer (Kapteyn *et al.*, 1997; Kollar *et al.*, 1997). The cell wall determines cell shape, provides rigidity that counteracts the outward turgor pressure on the plasma membrane, and acts as a diffusion barrier delimiting the periplasmic space. Cell wall synthesis is a poorly understood, highly regulated process that is subject to cell growth and environmental cues.

We have shown recently that TOR2 activates RHO1 and RHO2 via their GEF ROM2 (Schmidt *et al.*, 1997). Here we show that cell wall damage also activates RHO1 via ROM2, independently of TOR2. Our results suggest that yeast cells have a cell wall monitoring system that activates cell wall synthesis. Such a monitoring system signals in response to cell wall alterations caused by duress and possibly also by normal cell wall remodelling.

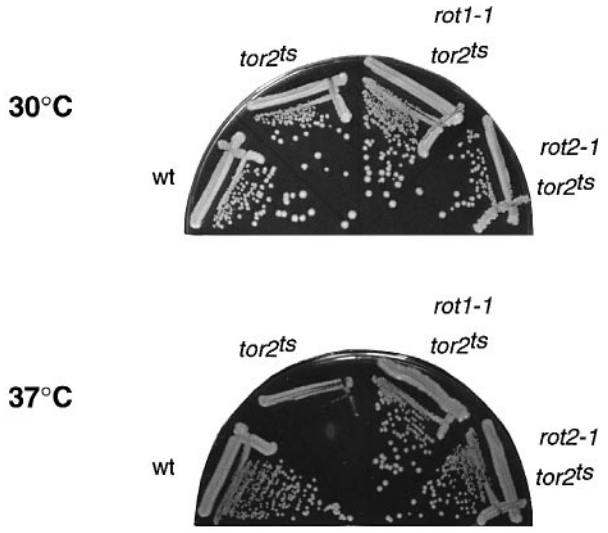

Fig. 1. Mutations in *ROT1* or *ROT2* suppress *tor2*^{ts}. Wild-type (wt) (MH272-1d**a**), *tor2*^{ts} (MB133-2b), *rot1-1 tor2*^{ts} (MB136-2d) and *rot2-1 tor2*^{ts} (MB137-1d) cells were streaked on YPD and incubated at 30 and 37°C. *rot1-1 tor2*^{ts} and *rot2-1 tor2*^{ts} cells, but not *tor2*^{ts} cells, are viable at 37°C.

Results

Mutations in ROT1 and ROT2 suppress the loss of TOR2

TOR2 has at least two essential functions. One is redundant with TOR1 and controls translation initiation; the other is unique to TOR2 and controls the actin cytoskeleton (Kunz *et al.*, 1993; Helliwell *et al.*, 1994; Barbet *et al.*, 1996; Schmidt *et al.*, 1996). To gain insight into the TOR2-unique function, we selected second-site suppressors of *tor2*^{ts} mutations in a *TOR1* background. The *TOR1 tor2*^{ts} mutants were defective only in the TOR2-unique function, and temperature sensitive for growth. Approximately 300 spontaneous mutants able to grow at 37°C on rich medium were isolated (see Materials and methods). Eight mutants containing suppressors that were able to suppress a *tor2*-null allele were chosen for further study (see Materials and methods). The eight mutants were back-crossed to the parental *tor2*^{ts} strain and diploids were sporulated and dissected. The suppressor mutations were all recessive and segregated 2:2, indicating that a single nuclear locus was affected in each case. The eight mutants were then crossed to each other and diploids were sporulated and dissected. The suppressor mutations were linked to one of two loci which were named *ROT1* and *ROT2* for reversal of TOR2 (*rot1* was isolated six times and *rot2* twice). We chose one mutant from each segregation group (*rot1-1*, MB136-2d; *rot2-1*, MB137-1d) for further study. The *rot1-1* and *rot2-1* mutations suppressed both the growth defect and the actin organization defect of the *tor2*^{ts} mutant (Figure 1 and data not shown). Interestingly, a *tor2*^{ts} diploid heterozygous for *rot1-1* and *rot2-1* (MB66) was viable at 37°C, indicating non-allelic non-complementation (data not shown).

To determine whether the rot mutations could suppress a loss of the TOR-common function, *rot1-1 tor2*^{ts} and *rot2-1 tor2*^{ts} strains were crossed to a *tor1 tor2*^{ts} mutant (strain SH221). None of the *tor1 tor2*^{ts} segregants was viable at 37°C, indicating that the *rot* mutations could suppress the loss of only the TOR2-unique function (data not shown).

Isolation of ROT1, ROT2, BIG1 and SAC7 by suppression of rot1-1 rot2-1 synthetic lethality

The *rot1-1* and the *rot2-1* mutations were introduced into our wild-type strain background to generate strains MB134-1a and MB135-4b, respectively. As expected from the wild-type-like growth of the *rot1-1 tor2*^{ts} and the *rot2-1 tor2*^{ts} mutants at all temperatures, the *rot1-1* and *rot2-1* mutations conferred no growth phenotype in a wild-type background at 24, 30 or 37°C (see Materials and methods for scoring of the *rot* mutations). However, a combination of *rot1-1* and *rot2-1* in an otherwise wild-type background (MB145-3b) conferred a recessive, temperature-sensitive growth defect (see Materials and methods). We took advantage of this recessive synthetic lethal phenotype to clone *ROT1* and *ROT2*. Both a high-copy-number (2μ) and a single-copy-number (CEN) plasmid library were screened for rescue of the temperature-sensitive growth phenotype of the *rot1-1 rot2-1* mutant. Four open reading frames and a tRNA gene which conferred growth at 37°C were identified (see Materials and methods): *YMR200w*; *YBR229c*; *YHR101c*; *SAC7*; and a Gln tRNA [tQ(CUG)M]. When present in a single copy, only *YMR200w* and *YBR229c* were able to suppress completely the synthetic lethality (Figure 2). *YMR200w* and *YBR229c* were subsequently shown, by genetic linkage, to encode *ROT1* and *ROT2*, respectively (see Materials and methods). This assignment was confirmed by transforming the single copy *YMR200w* and *YBR229c* plasmids into the *rot1-1 tor2*^{ts} and *rot2-1 tor2*^{ts} mutants. *YMR200w* was able to complement only *rot1-1*, and *YBR229c* was able to complement only *rot2-1* (data not shown).

The high-copy-number suppressor of the *rot1-1 rot2-1* synthetic lethality *YHR101c*, a weak suppressor, was named *BIG1* (bad in glucose, see below). The high-copy-number suppressor *SAC7* showed strong homology to Rho-type GTPase-activating proteins (GAPs). *SAC7* was

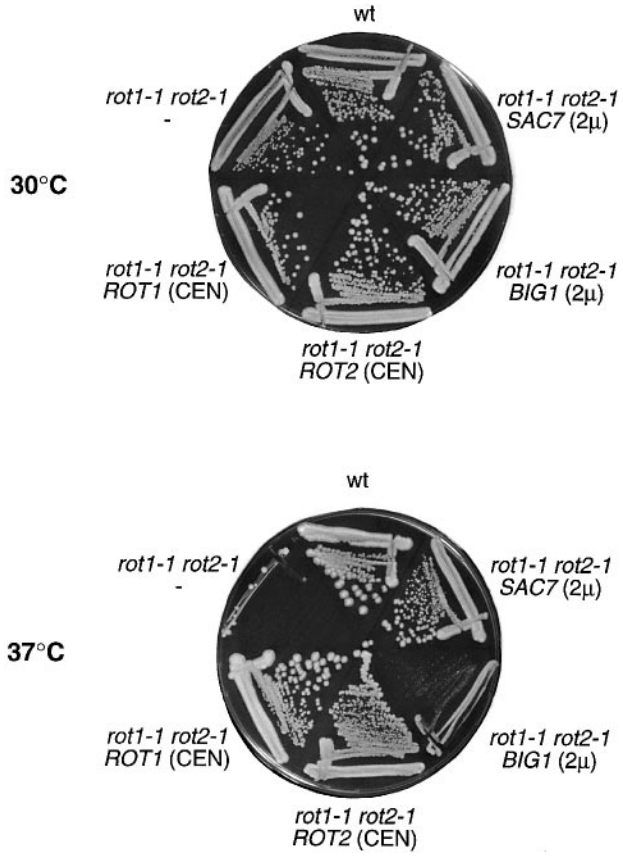

Fig. 2. *ROT1, ROT2, BIG1* and *SAC7* suppress the temperature-sensitive growth defect of a *rot1-1 rot2-1* double mutant. *rot1-1 rot2-1* (MB145-3b) cells, transformed with either empty plasmid (–; YEplac195), pMB2 (*ROT1 CEN*), pMB6 (*ROT2 CEN*), pPAD24 (*BIG1* 2μ), or pMB7 (*SAC7* 2μ), and wild-type (wt) (MH272-1d**a**) cells were streaked on YPD and incubated at 30 and 37°C. *rot1-1 rot2-1* mutants are viable at 37°C if they express *ROT1*, *ROT2*, *BIG1* or *SAC7*.

shown indeed to encode a GAP, for RHO1, as described elsewhere (Schmidt *et al.*, 1997). The remaining high-copy-number suppressor encoded a Gln tRNA and was not pursued further.

ROT1 and BIG1 were novel proteins with one putative transmembrane domain each and no homologue in the DDBJ/EMBL/GenBank database. Upon resequencing the 3′-end of *ROT1*, we observed an additional thymidine at position 758 compared with the sequence in the database. Our sequence changed the last five predicted amino acids and extended the predicted protein by 58 amino acids. The extended ROT1 protein consisted of 316 amino acids, with a calculated molecular weight of 34 kDa. The predicted BIG1 protein, taking into account an 87-nucleotide intron in *BIG1*, was 335 amino acids with a molecular weight of 37 kDa. ROT2 (predicted 954 amino acids, 110 kDa) showed strong homology to glucosidases, and Trombetta *et al.* (1996) have reported recently that *YBR229w* (*GLS2/ROT2*) encodes glucosidase II. Glucosidase II is an ER protein that removes the two α-1,3 linked glucose residues of N-linked oligosaccharides after glucosidase I has removed the terminal α-1,2 linked glucose residue (Kilker *et al.*, 1981; Saunier *et al.*, 1982; Herscovics and Orlean, 1993). Mammalian glucosidase II has been shown to be important, along with calnexin, calreticulin and the UDP:Glc glycoprotein glucosyltransferase, for monitoring the maturation of glycoproteins in the ER (quality control) (Bergeron *et al.*, 1994). A similar quality control mechanism may also exist in yeast (Parlati *et al.*, 1995).

The *ROT1*, *ROT2* and *BIG1* open reading frames were disrupted with PCR-generated cassettes (see Materials and methods). *ROT1* was essential for growth; *rot1*-null (*rot1::kanMX2*) spores germinated and arrested growth within one cell cycle as medium- or large-budded cells. A strain containing *ROT1* under control of the *GAL* promoter (MB121-1b) (see Materials and methods) also arrested growth, when shifted from galactose to glucose medium (Figure 7A). Thus, the original recessive *rot1-1* allele, which did not confer a growth defect, was not a null allele. *ROT2* was not essential, as reported previously (Trombetta *et al.*, 1996). A *ROT2* deletion (*rot2::HIS3MX6;* strain MB86-1b) conferred no obvious growth defect and suppressed a $tor2^{ts}$ mutation (MB104-2a), as observed for the original *rot2-1* mutation (Figure 3A). *BIG1* was important but not essential for growth. Disruption of *BIG1* (*big1::HIS3MX6*) caused a severe growth defect; *big1* cells (PA5-7b) formed microcolonies after 8 days on YPD medium (Figure 7A). The *big1* cells were very large and often unbudded and multinucleate. The poor growth of *big1* cells was partly suppressed on media containing galactose as a carbon source or 1 M sorbitol (data not shown). The *BIG1* disruption did not suppress a $tor2^{ts}$ mutation (data not shown). However, a $tor2^{ts}$ mutant containing *BIG1* under control of the *GAL* promoter (PA31-7b) was viable at 37°C on glucose (data not shown). Thus, although not originally identified as a suppressor of $tor2^{ts}$, loss of function of *BIG1* could also suppress $tor2^{ts}$. The difference in suppression by the two *big1* alleles is presumably due to the fact that the *BIG1* disruption caused a severe growth defect on its own, whereas the *GAL-BIG1* allele did not (see Materials and methods).

Loss of ER glucosidase activity suppresses $tor2^{ts}$

ROT2 was identified by Trombetta *et al.* (1996) as glucosidase II. To examine the link between suppression of $tor2^{ts}$ and glucosidase activity in the ER, we searched for a yeast homologue of mammalian glucosidase I. Lack of glucosidase I activity should also abolish trimming by glucosidase II as the enzymes act sequentially. The nearest homologue to mammalian glucosidase I in yeast is *CWH41*, which has been reported to be an ER protein involved in β-1,6 glucan synthesis (Jiang *et al.*, 1996). We constructed a *cwh41* $tor2^{ts}$ strain (MB119) and tested for growth at 37°C. We found that $tor2^{ts}$ was suppressed by the loss of *CWH41* (Figure 3A). To determine whether CWH41 is indeed glucosidase I, we constructed *cwh41* $sec18^{ts}$ (PA37-5c) and *rot2* $sec18^{ts}$ (PA36-2d) strains; the $sec18^{ts}$ mutation was necessary to block glycoproteins in the ER and thereby prevent heterogeneous outer chain carbohydrate addition that obscures small changes in electrophoretic mobility (Esmon *et al.*, 1984). These strains were shifted to 37°C to impose the ER block, labelled with [^{35}S]Met and lysed, and the glycoprotein carboxypeptidase Y (CPY) was immunoprecipitated. CPY from the *cwh41* $sec18^{ts}$ and *rot2* $sec18^{ts}$ mutants showed reduced motility compared with CPY from the $sec18^{ts}$ strain (PA33-

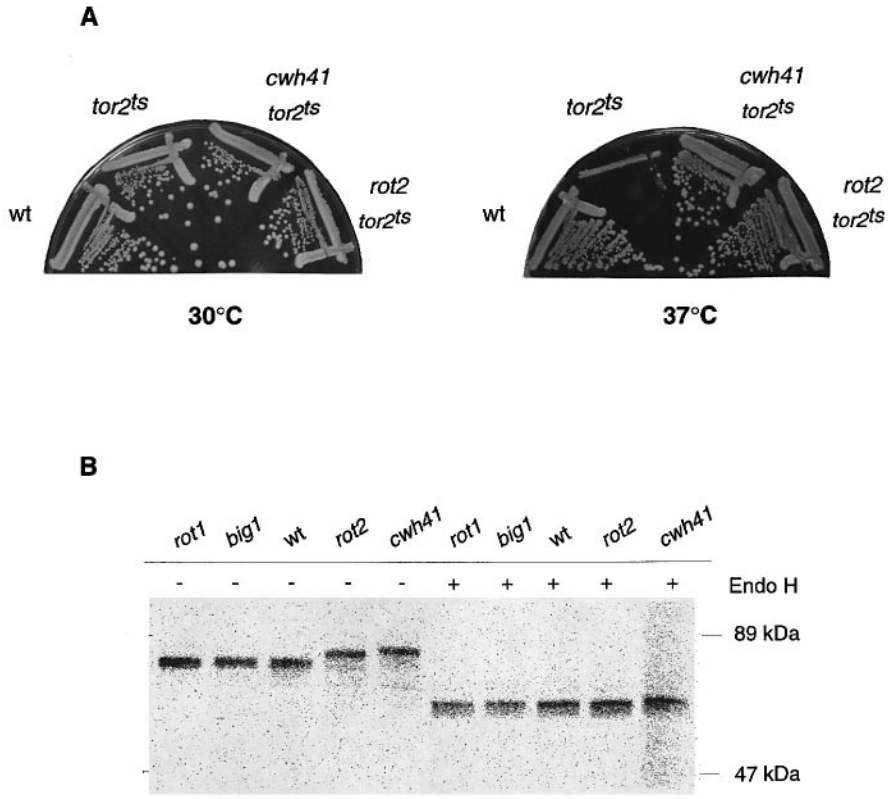

Fig. 3. Loss of ER glucosidase I (CWH41) or II (ROT2) suppresses *tor2*^{ts}. (**A**) Wild-type (wt) (MH272-1d**a**), *tor2*^{ts} (MB133-2b), *cwh41 tor2*^{ts} (MB119) and *rot2 tor2*^{ts} (MB104-2a) cells were streaked on YPD and incubated at 30 and 37°C. *cwh41tor2*^{ts} and *rot2 tor2*^{ts} mutants are viable at 37°C, *tor2*^{ts} mutants are not viable at 37°C. (**B**) Cultures of wild-type (*sec18-1*; PA33-6a), *rot1* (*rot1 sec18-1*; PA38-2a), *rot2* (*rot2 sec18-1*; PA36-2d), *big1* (*big1 sec18-1*; PA35-19d) and *cwh41* (*cwh41 sec18-1*; PA37-5c) cells were shifted from 24 to 37°C for 5 min, labelled for 1 h at 37°C and lysed, and carboxypeptidase Y (CPY) was immunoprecipitated. CPY from *cwh41* and *rot2* mutants migrates slower than CPY from wild-type cells or *rot1* and *big1* mutants. CPY from all strains migrates equally fast when treated with Endo H.

6a). The differences in mobility were due to differences in N-linked glycosylation as endo H-treated CPY from all three strains showed the same mobility (Figure 3B). These results suggest that CWH41 is a glucosidase, and confirm the glucosidase activity of ROT2. In agreement with this, Romero *et al.* (1997) and Simons *et al.* (1998) have found, while this study was in progress, that CWH41 is glucosidase I. Thus, loss of glucosidase activity in the ER correlates with suppression of *tor2*^{ts}.

We also examined whether ER glucosidase activity was affected upon depletion of ROT1 or BIG1. *GAL-ROT1 sec18*^{ts} (PA38-2a) and *GAL-BIG1 sec18*^{ts} (PA35-19d) strains were passaged in glucose medium at non-permissive temperature (see Materials and methods), and assayed for ER glucosidase activity as described above. In neither case was an effect on glucosidase activity observed (Figure 3B). Thus, because depletion of BIG1 suppresses *tor2*^{ts} but does not affect glucosidase activity, defects in ER glucosidases appear to be sufficient but not necessary for suppression of *tor2*^{ts}.

ROT1, ROT2 and BIG1 mutants have cell wall defects

Mutants defective in CWH41, like many other mutants altered in N-linked glycosylation, also have a cell wall defect (Klis, 1994; Jiang *et al.*, 1996). The cell wall is composed of three major components, mannoproteins, β-1,6-glucan and β-1,3-glucan, all cross-linked to each other and to chitin (Kapteyn *et al.*, 1997; Kollar *et al.*, 1997). *CWH41* mutants have been reported to have low levels of β-1,6-glucan (Jiang *et al.*, 1996). As a measure of cell wall composition, we examined the distribution and levels of chitin in *rot1-1*, *rot2-1* and *big1* (*GAL-BIG1*) mutants (strains MB134-1a, MB135-4b, and PA11-6a). First, *rot1-1*, *rot2-1* and *big1* cells were grown at 37°C in YPD (conditions under which *tor2*^{ts} is suppressed) and stained with calcofluor white to visualize chitin. The *rot1-1*, *rot2-1* and *big1* strains exhibited an altered distribution of chitin; unlike in wild-type cells, chitin staining was observed occasionally in bud tips and at elevated levels in the lateral wall of mother cells. Furthermore, the *rot1-1*, *rot2-1* and *big1* strains showed a random distribution of bud scars reflecting a random budding pattern. The bud scars of wild-type cells were aligned in rows reflecting a normal axial budding pattern (Figure 4A). Second, the chitin content in the different strains was measured by a colorimetric assay (Bulawa *et al.*, 1986). The *rot1-1*, *rot2-1* and *big1* cells had increased chitin levels (188, 176 and 173% of that of the wild type, respectively) in YPD at 37°C (Figure 4B). Thus, we conclude that the cell walls of *ROT1*, *ROT2* and *BIG1* mutants are abnormal. While this study was in progress, Simons *et al.* (1998) found that a *ROT2* (*GLS2*) mutant, like a *CWH41* mutant, has low levels of β-1,6-glucan, providing further evidence that *ROT2* mutants have a cell wall defect.

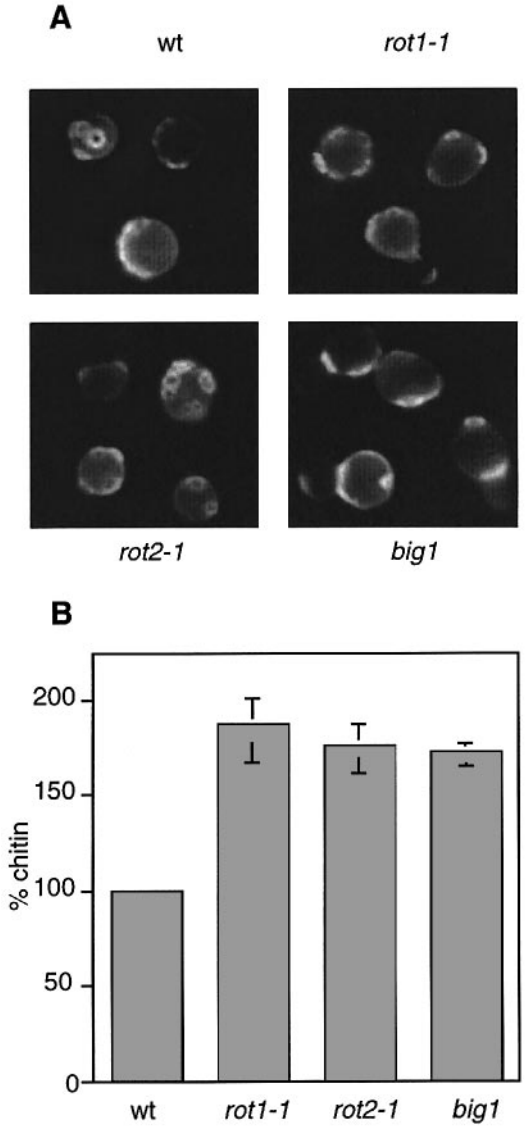

Fig. 4. *rot1-1*, *rot2-1* and *big1* mutants have altered cell walls. (**A**) Wild-type (wt) (MH272-1d**a**), *rot1-1* (MB134-1a), *rot2-1* (MB135-4b) and *big1* (PA11-6a) cells were grown in YPD at 37°C, stained with calcofluor white, and chitin was visualized by fluorescence microscopy. *rot1-1*, *rot2-1* and *big1* mutants have a random budding pattern and deposit chitin in the bud. (**B**) Wild-type (wt) (MH272-1d**a**), *rot1-1* (MB134-1a), *rot2-1* (MB135-4b) and *big1* (PA11-6a) cells were grown in YPD at 37°C and chitin levels were measured. *rot1-1*, *rot2-1* and *big1* mutants have more chitin than the wild type (188, 176 and 173% of wild type, respectively).

Cell wall defects suppress tor2^{ts}

Because *rot1*, *rot2*, *big1* and *cwh41* mutations all confer cell wall defects and suppress the loss of TOR2, we examined whether other cell wall defects could also suppress *tor2^{ts}*. First, we examined if destabilizing the cell wall by supplementing the growth medium with the detergent SDS (Igual *et al.*, 1996) suppressed the growth defect of a *tor2^{ts}* mutant. A *tor2^{ts}* mutant (MB133-2b) incubated at non-permissive temperature on rich medium supplemented with 0.005% SDS grew almost like wild type (Figure 5A). Second, we disrupted individually, in a *tor2^{ts}* mutant, three genes directly involved in specific aspects of cell wall assembly or synthesis: *GAS1*, *FKS1* and *CHS3* (see Materials and methods). *GAS1* encodes a GPI-anchored plasma membrane protein required for proper cell wall assembly (Popolo *et al.*, 1993, 1997; Ram *et al.*, 1995). *FKS1* encodes a component of the plasma membrane β-1,3 glucan synthase complex (Douglas *et al.*, 1994; Eng *et al.*, 1994; Castro *et al.*, 1995; Mazur *et al.*, 1995). *CHS3* encodes the putative catalytic subunit of the chitin synthase that produces 90% of cellular chitin (Shaw *et al.*, 1991; Cabib *et al.*, 1993). The *gas1 tor2^{ts}*, *fks1 tor2^{ts}* and *chs3 tor2^{ts}* double mutants (strains MB120, MB140 and MB147) were incubated at 37°C on rich medium. Deletions of *GAS1* and *FKS1* suppressed the lethality of *tor2^{ts}*. However, the suppression by *gas1* was weak, possibly because *gas1* itself caused a severe growth defect. Deletion of *CHS3* had no effect (Figure 5B). Thus, an SDS-induced defect or a specific defect in any one of the three main components of the cell wall, mannoproteins (*gas1*), β-1,3 glucan (*fks1*) and β-1,6 glucan (*cwh41*), suppressed the growth defect of a *tor2^{ts}* mutant. Taken together, these results suggest that disturbing the cell wall is sufficient to compensate for the loss of TOR2. A mutation in *CHS3* does not suppress *tor2^{ts}*, possibly because an increase in chitin levels, as observed in *rot*, *big1*, *gas1* and *fks1* mutants, may be necessary for suppression. Cell wall mutants in general appear to have an increase in chitin content that is essential to compensate for the primary cell wall defect (Kapteyn *et al.*, 1997; Popolo *et al.*, 1997).

Cell wall defects activate RHO1 via ROM2

Several lines of evidence suggested that mutations in *ROT1*, *ROT2* and *BIG1* activate RHO1. First, mutations in all three genes suppress *tor2^{ts}*, which is also suppressed by activation of RHO1 (Schmidt *et al.*, 1997). Second, the synthetic lethality of a *rot1-1 rot2-1* double mutation and the toxicity of a *big1*-null mutation are suppressed by overexpression of *SAC7*, which encodes a RHO1 GAP (Figure 2 and data not shown). Third, the growth defect conferred by disruption of *ROT1* or *BIG1* is partly suppressed by deletion of *ROM2*, which encodes one of two RHO1 GEFs (Figure 7A). The latter two findings suggest also that the growth defect of *rot1-1 rot2-1*, *rot1*-null, or *big1*-null strains is due to hyperactivation of RHO1. Hyperactivation of RHO1 is toxic (Madaule *et al.*, 1987). To determine if mutations in *ROT1*, *ROT2* and *BIG1* indeed activate RHO1, we assayed extracts from *rot1-1*, *rot2-1* and *big1* (*GAL-BIG1*) mutants (strains MB134-1a, MB135-4b and PA11-6a) for GEF activity toward RHO1 (see Materials and methods). GEF activity on RHO1 from *rot1-1*, *rot2-1* and *big1* strains was increased to 185, 183 and 135% of that of the wild type, respectively (Figure 6). Because mutations in *ROT1*, *ROT2* and *BIG1* all cause cell wall defects, we examined if other conditions that cause cell wall defects also activate RHO1. We assayed GEF activity in extracts from *cwh41*, *gas1* and *fks1* mutants and in an extract from an SDS-treated wild-type strain (strains MB132-2c, MB146-3d, MB144-2b and JK9-3d**a**) (see Materials and methods). GEF activity toward RHO1 in extracts from *cwh41*, *gas1*, *fks1* mutants and SDS-treated wild-type cells was 200, 178, 143 and 185% of the activity from SDS-untreated wild-type cells, respectively (Figure 6). Thus, cell wall defects activate RHO1 via a GEF activity.

We have shown previously that TOR2 activates RHO1

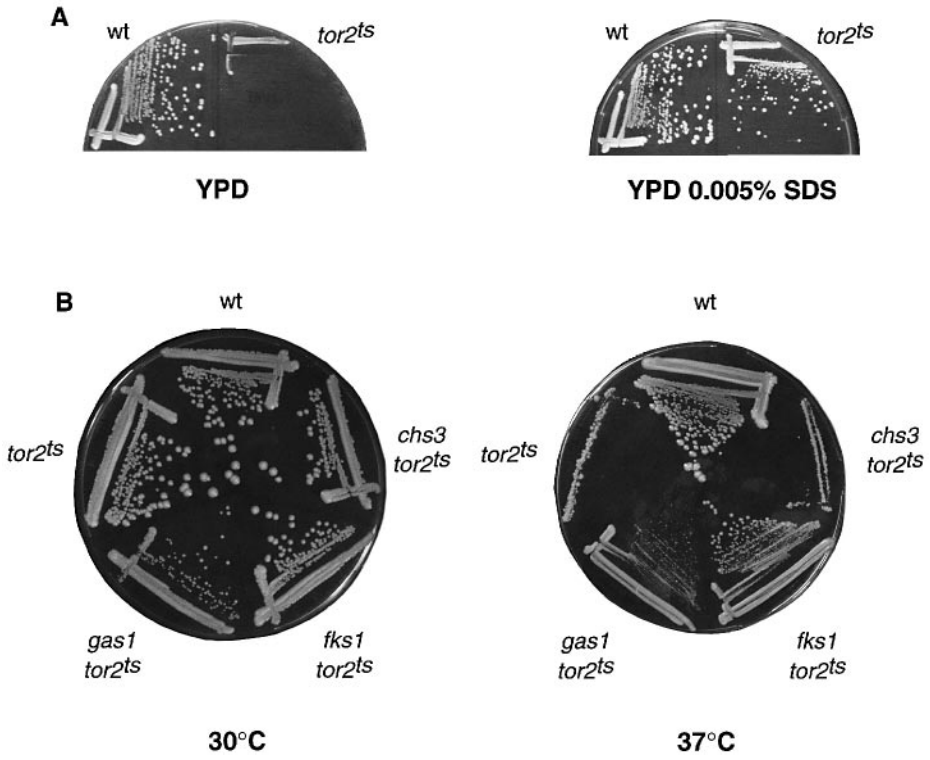

Fig. 5. Cell wall defects suppress *tor2*^{ts}. (**A**) Wild-type (wt) (MH272-1d**a**), *tor2*^{ts} (MB133-2b) cells were streaked on YPD and on YPD containing 0.005% SDS, and incubated at 37°C. *tor2*^{ts} mutants are viable on SDS-containing medium. (**B**) Wild-type (wt) (MH272-1d**a**), *tor2*^{ts} (MB133-2b), *gas1 tor2*^{ts} (MB120), *fks1 tor2*^{ts} (MB140) and *chs3 tor2*^{ts} (MB147) cells were streaked on YPD and incubated at 30 and 37°C. Deletion of *GAS1* or *FKS1*, but not *CHS3*, suppresses *tor2*^{ts}.

via the GEF ROM2 (Schmidt *et al.*, 1997). The findings that cell wall defects suppress a *tor2*^{ts} mutation and stimulate GEF activity toward RHO1, and that the growth defect conferred by disruption of *ROT1* or *BIG1* is suppressed, albeit weakly, by deletion of *ROM2* (Figure 7A) suggested that cell wall defects also activate RHO1 via ROM2. To investigate this further, we examined whether a *rom2* mutation prevents the suppression of *tor2*^{ts} by SDS. A *rom2 tor2*^{ts} double mutant (AS153-1a) grew very poorly on SDS-containing medium compared with *rom2* (AS152-1c) and *tor2*^{ts} (MB133-2b) single mutants (Figure 7B), indicating that ROM2 is required for the suppression by SDS. The residual growth of the *rom2 tor2*^{ts} double mutant on SDS could be due to stimulation of the other RHO1 GEF ROM1 (Ozaki *et al.*, 1996). Furthermore, we found that *rom2* also prevented the suppression of a *tor2* mutation by *rot2* (data not shown). Taken together, these results suggest that cell wall defects, like TOR2, also activate RHO1 via ROM2.

Our experiments do not directly address the issue of whether cell wall defects activate RHO1 via the GEF ROM1, or whether cell wall defects activate GTPases other than RHO1. However, cell wall defects may also activate RHO2, as TOR2 activates both RHO1 and RHO2 (Schmidt *et al.*, 1997) and ROM2 is a GEF for both RHO1 and RHO2 (Ozaki *et al.*, 1996).

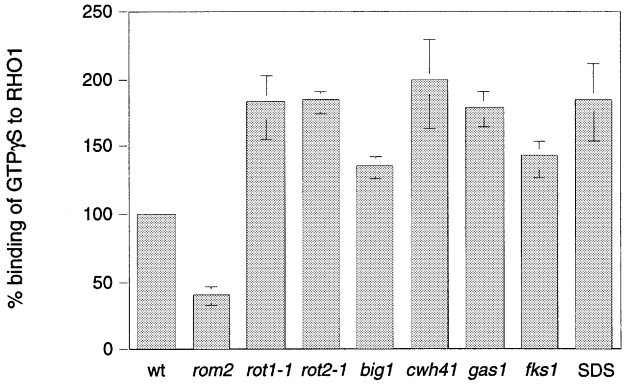

Fig. 6. Cell wall defects cause elevated levels of GEF activity toward RHO1. GEF activity (percentage binding of GTPγS to RHO1) was assayed in extracts from wild-type (wt) (MH272-1d**a**), *rom2* (AS152-1c), *rot1-1* (MB134-1a), *rot2-1* (MB135-4b), *big1 (*PA11-6a), *cwh41* (MB132-2c), *gas1* (MB144-2b) and *fks1* (MB146-3d) cells and from wild-type cells (JK9-3d**a**) grown in the presence of 0.005% SDS (SDS), cultured in YPD at 37°C. GEF activity toward RHO1 in extracts from *rom2*, *rot1-1*, *rot2-1*, *big1*, *cwh41*, *gas1*, *fks1* mutants and SDS-treated wild-type cells was 40, 183, 184, 135, 200, 178, 143 and 185% of the activity from SDS-untreated wild-type cells, respectively. *ROM2* encodes one of two RHO1 GEFs, and the *rom2* mutant was included as a control.

Discussion

We have shown that conditions which alter the cell wall suppress the growth defect of a *TOR2* mutant and activate RHO1. These conditions include 0.005% SDS in the growth medium or loss-of-function mutations in *ROT1*, *ROT2*, *BIG1*, *CWH41*, *GAS1* or *FKS1*. *ROT1* and *BIG1* are novel genes of unknown function. *ROT2* and *CWH41* encode the ER glucosidases II and I, respectively. It is not known why defects in ER glucosidases cause a cell wall defect. *GAS1* encodes a GPI-anchored, plasma membrane protein required for cell wall assembly. *FKS1*

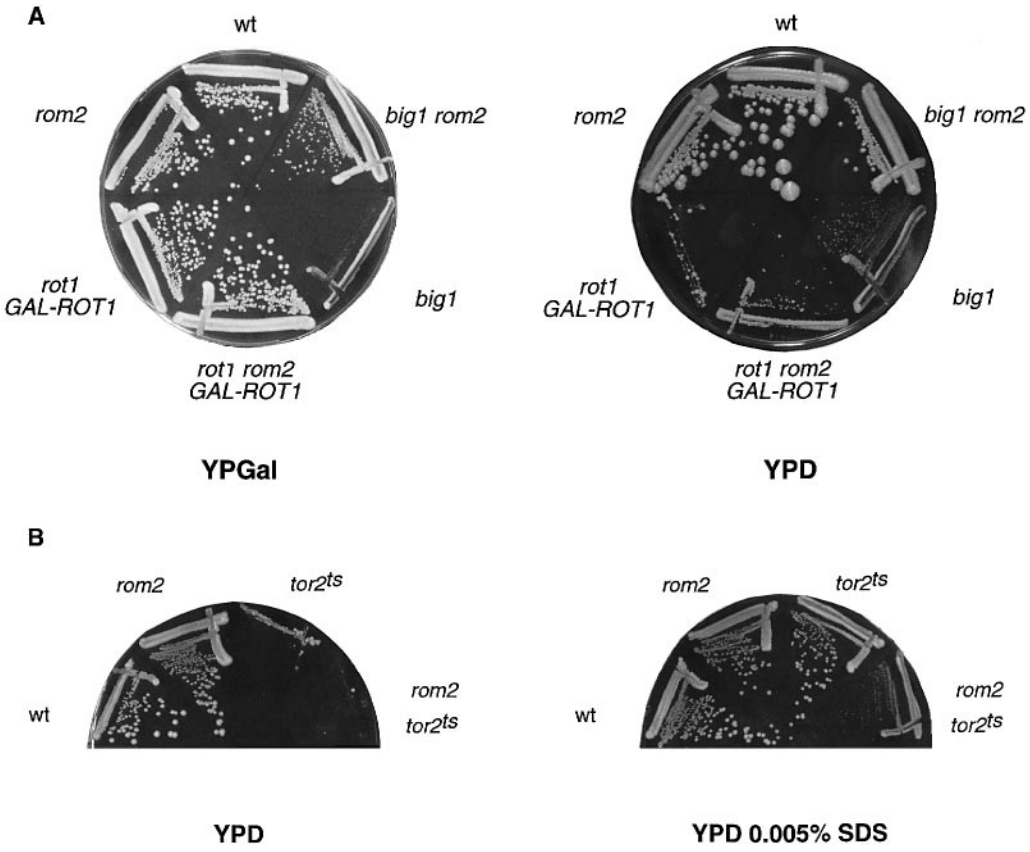

Fig. 7. Cell wall defects activate RHO1 via ROM2. (**A**) Wild-type (wt) (MH272-1d**a**), *rom2* (AS152-1c), *rot1 GAL-ROT1* (MB121-1b), *rot1 rom2 GAL-ROT1* (MB109-1c), *big1* (PA5-7b) and *big1 rom2* (PA34-4a) cells were grown on YPGal for 3 days and on YPD for 8 days, at 30°C. A *ROM2* deletion partly suppresses the loss of *ROT1* or *BIG1* as indicated by growth on YPD. (**B**) Wild-type (wt) (MH272-1d**a**), *rom2* (AS152-1c), $tor2^{ts}$ (MB133-2b) and *rom2* $tor2^{ts}$ (AS153-1a) cells were streaked on YPD and on YPD containing 0.005% SDS and incubated at 37°C. The *rom2* $tor2^{ts}$ mutant grows very poorly compared with the *rom2* and $tor2^{ts}$ mutants.

encodes the catalytic subunit of β-1,3 glucan synthase. Furthermore, we have shown that cell wall defects activate RHO1 via the GEF ROM2.

Why do cell wall defects activate RHO1? As RHO1 controls cell wall synthesis (see Introduction), activation of RHO1 by a cell wall alteration would ensure that cell wall synthesis occurs as needed. The cell wall alterations that activate RHO1 are alterations caused by duress, as shown here, but may also include alterations caused during normal cell growth. The yeast cell wall is remodelled during vegetative growth to allow bud emergence and cell fission, and during mating to allow shmoo formation and cell fusion.

Cell wall defects suppress the growth defect conferred by a *tor2* mutation probably via activation of RHO1. First, *TOR2* mutants are non-viable because they are unable to activate RHO1 (Schmidt *et al.*, 1997). Second, the suppression of a *tor2* mutation by cell wall defects is reversed by overexpression of SAC7, the GAP for RHO1 (data not shown). Thus, cell wall defects most likely suppress by inducing a TOR2-independent pathway for activating RHO1. The activation of RHO1 by cell wall defects is independent of TOR2 (although still dependent on ROM2) because at least *rot1-1* and *rot2-1* suppress a *tor2*-null allele. We therefore propose a cell-wall-sensing pathway that signals to and activates RHO1 when cell integrity is compromised (Figure 8). The observation that a cell wall defect induces a compensating change in cell

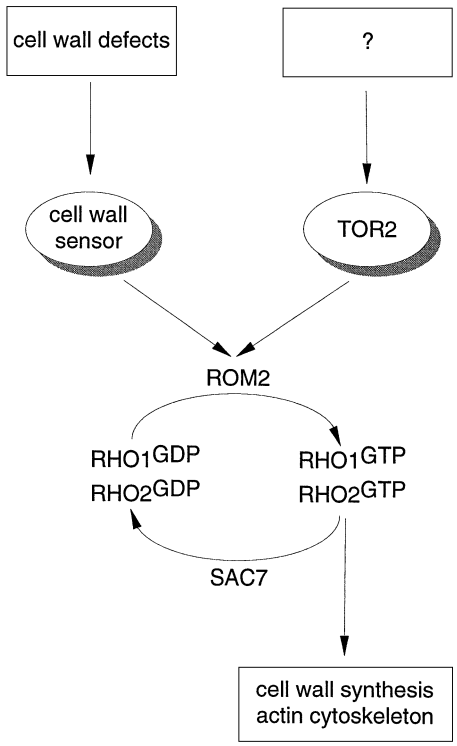

Fig. 8. Model of RHO1 activation via ROM2 by two independent pathways (see Discussion for details).

Table I. Yeast strains used in this study

Strain	Genotype
JK9-3d**a**	*MAT***a** *leu2-3,112 ura3-52 trp1 his4 rme1 HML***a**
JK9-3dα	*MAT*α *leu2-3,112 ura3-52 trp1 his4 rme1 HML***a**
JK9-3d**a**/α	*MAT***a**/*MAT*α *leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1/trp1 his4/his4 rme1/rme1 HML***a**/*HML***a**
MH272-1d**a**	JK9-3d**a** *his3 HIS4*
MH272-1dα	JK9-3dα *his3 HIS4*
MH272-1d**a**/α	JK9-3d**a**/α *his3/his3 HIS4/HIS4*
AS152-1c	MH272-1dα *rom2::HIS3*
AS153-1a	MH272-1dα *ade2 rom2::HIS3 tor2::ADE2-3*/YCplac111::*tor2-21*
MB66	JK9-3d**a**/α *rot1-1/ROT1 rot2-1/ROT2 tor2::ADE2-3/tor2::ADE2-3*/YCplac111::*tor2-21*
MB86-1b	MH272-1dα *rot2::HIS3MX6*
MB97-2a	MH272-1d**a** *rot2::HIS3MX6 ade2*
MB104-2a	MH272-1d**a** *rot2::HIS3MX6 ade2 tor2::ADE2-3*/YCplac111::*tor2-21*
MB109-1c	JK9-3dα *rom2::URA3 rot1::kanMX2*/YCplac111::*GAL1-HA-ROT1*
MB119	JK9-3d**a** *ade2 cwh41::kanMX2 tor2::ADE2-3*/YCplac33::*tor2-21*
MB120	JK9-3d**a** *ade2 gas1::kanMX2 tor2::ADE2-3*/YCplac111::*tor2-21*
MB121-1b	JK9-3dα *rot1::kanMX2*/YCplac111::*GAL1-ROT1*
MB132-2c	MH272-1d**a** *cwh41::kanMX2*
MB133-2b	MH272-1d**a** *ade2 tor2*::*ADE2-3*/YCplac111::*tor2-21*
MB134-1a	MH272-1d**a** *rot1-1*
MB135-4b	MH272-1d**a** *rot2-1*
MB136-2d	MH272-1dα *rot1-1 ade2 tor2::ADE2-3*/YCplac111::*tor2-21*
MB137-1d	MH272-1dα *rot2-1 ade2 tor2*::*ADE2-3*/YCplac111::*tor2-21*
MB140	MH272-1d**a** *ade2 fks1*::*HIS3 tor2*::*ADE2-3*/YCplac111::*tor2-21*
MB144-2b	MH272-1dα *gas1::kanMX2*
MB145-3b	MH272-1d**a** *rot1-1 rot2-1*
MB146-3d	MH272-1d**a** *fks1::HIS3*
MB147	MH272-1d**a** *ade2 chs3::HIS3MX6 tor2::ADE2-3*/YCplac111::*tor2-21*
PA5-7b	MH272-1d**a** *big1::HIS3MX6*
PA11-6a	MH272-1dα *big1::HIS3MX6*/pSEYC68::*GAL1-BIG1-cmyc*
PA31-7b	MH272-1d**a** *big1::HIS3MX6 ade2 tor2::ADE2-3*/YCplac111::*tor2-21*/pSEYC68::*GAL1-BIG1-cmyc*
PA33-6a	MH272-1d**a** *sec18-1*
PA34-4a	MH272-1d**a** *big1::HIS3MX6 rom2::URA3*
PA35-19d	MH272-1d**a** *big1::HIS3MX6 sec18 -1*/pSEYC68::*GAL1-BIG1*-cmyc
PA36-2d	MH272-1d**a** *rot2::HIS3MX6 sec18-1*
PA37-5c	MH272-1d**a** *cwh41::kanMX2 sec18-1*
PA38-2a	MH272-1d**a** *rot1::kanMX2 sec18-1*/YCplac111::*GAL1-HA-ROT1*
SH221	MH272-1d**a** *ade2 tor1*::*HIS3-3 tor2::ADE2-3*/YCplac111::*tor2-21*

wall composition (Kapteyn *et al.*, 1997; Popolo *et al.*, 1997) suggests that a cell-wall-sensing and signalling mechanism exists.

How are cell wall defects sensed? In mammalian cells, integrin receptors interact with the extracellular matrix and, in response to mechanical stress, activate RhoA, which in turn induces formation of focal adhesions and actin stress fibers (Clark and Brugge, 1995; Nobes and Hall, 1995). As RhoA is a structural and functional homologue of RHO1 (Qadota *et al.*, 1994; Yamochi *et al.*, 1994), yeast cells may have an integrin-like receptor that interacts with the cell wall and activates RHO1 in response to a cell wall alteration. The nature of the cell wall alteration sensed by such a receptor would have to be very general, as several different types of cell wall defects activate RHO1. An alternative model is that cell wall defects are sensed indirectly. Kamada *et al.* (1995) have suggested that PKC1, a downstream effector of RHO1, is activated by mechanosensors sensing an outward stretching of the plasma membrane. A cell wall defect could result in a turgor pressure-induced outward stretching of the plasma membrane which would then activate RHO1.

Materials and methods

Strains, plasmids and media

The *S.cerevisiae* strains used in this study are listed in Table I. All strains were isogenic derivatives of JK9-3d. Plasmids used in this study were as follows. pMB2 (YCplac33::*ROT1*) is YCplac33 (*URA3 CEN*) (Gietz and Sugino, 1988) containing a 1.4 kb *Sac*I–*Eco*RI fragment bearing *ROT1*. pMB4 (YCplac111::*GAL1-HA-ROT1*) expresses 2×HA-tagged ROT1 under control of the *GAL1* promoter, in pAS24 (YCplac111::*GAL1-HA*). pAS24 is YCplac111 (*LEU2 CEN*) (Gietz and Sugino, 1988) expressing the 2×HA tag under control of the *GAL1* promoter. pMB6 (YCplac33::*ROT2*) is YCplac33 containing a 4.4 kb *Sac*I–*Xba*I fragment bearing *ROT2*. pMB7 (YEplac195::*SAC7*) is YEplac195 (*URA3* 2μ) (Gietz and Sugino, 1988) containing a 3.5 kb *Eco*RI–*Hin*dIII fragment bearing *SAC7*. pMB8 (YCplac111::*GAL1-ROT1*) expresses *ROT1* under control of the *GAL1* promoter, in pAS23. pAS23 (YCplac111::*GAL1*) is YCplac111 (*LEU2 CEN*) (Gietz and Sugino, 1988) containing the *GAL1* promoter. pPAD24 (YEplac195::*BIG1*) is YEplac195 containing a 1.6 kb *Fsp*I–*Sca*I fragment bearing *BIG1*. pPAD25 (pTB227::*BIG1*-cmyc) is pTB227 (*URA3 CEN GALp CYC1* terminator) expressing C-terminally cmyc-tagged *BIG1* under control of the *GAL* promoter. pAS53 (pGST-RHO1), expressing a *GST-RHO1* fusion under control of the *GAL* promoter, is the yeast GST-fusion vector pEG-KT (*URA3 leu2-d* 2μ) (Mitchell *et al.*, 1993) containing a 1 kb *Bam*HI–*Hin*dIII fragment bearing the entire *RHO1* open reading frame from pGAL-HA-RHO1 (Schmidt *et al.*, 1997). YCplac111::*tor2-21* contains the temperature-sensitive allele *tor2-21* (Helliwell *et al.*, 1998). Rich media (YPD, YPGal) and synthetic minimal media (SD, SGal) complemented with the appropriate nutrients for plasmid maintainance were as described (Guthrie and Fink, 1991).

Construction of GAL-ROT1 and GAL-BIG1

The *ROT1* and *BIG1* open reading frames were placed under the control of the *GAL1* promoter. For *ROT1*, a *Sal*I site was introduced 5′ of the initiation codon by PCR with the following four oligonucleotides: 5′-CACTTTGTCGACATGTGGTCGAAAAAG-3′, 5′-CGACCAGCG-CGCGCCCATAGTAATAAAGTGCAAC-3′, and the #1211 and #1233 sequencing primers (Pharmacia). The 0.9 kb *Sal*I fragment generated by

PCR was ligated into pAS23 and pAS24 to yield pMB8 and pMB4, respectively. pMB4 and pMB8 were transformed into a *rot1::kanMX2/ROT1* diploid, and transformants were sporulated and dissected on galactose-containing medium. The HA-ROT1 construct encoded by pMB4 was functional as determined by the ability of pMB4 to complement the *rot1::kanMX2* disruption. BIG1 was amplified by PCR with the oligonucleotides 5′-GGAGTATCTAGACAAGATGCAAAC-3′ and the cmyc epitope-containing oligonucleotide 5′-CCATCGATTTAGTTCAAGTCTTCTTCAGAAATCAACTTTTGTTCAGATACTTGCTGGGATGG-3′. A PCR-generated 1.1 kb *Xba*I–*Cla*I fragment was cloned into pTB227 (*URA3 GAL1p CEN CYC1* terminator) to yield pPAD25. pPAD25 was transformed into a *big1::HIS3MX6/BIG1* diploid, yielding PA11, which was sporulated and dissected on YPGal. The cmyc-tagged BIG1 was functional as *HIS3 URA3* segregants (PA11-6a) were obtained which grew like the wild type. Surprisingly, on glucose PA11-6a cells grew almost like wild-type cells, suggesting that very low levels of BIG1 were sufficient to confer near wild-type growth.

Selection and scoring of tor2 suppressors

The *tor2^{ts}* mutants used to select the *rot* suppressors contained a chromosomal *TOR2* disruption and a YEplac181-borne (2μ *LEU2*) *tor2^{ts}* allele. The *tor2^{ts}* strains were streaked on rich medium and incubated at 37°C. Approximately 300 colonies were picked. Eight mutants, isolated from three different *tor2^{ts}* alleles (*rot1* alleles: four times from *tor2-21* and twice from *tor2-31*; *rot2* alleles: twice from *tor2-64*) were noted to form two sizes of colonies at 37°C on YPD. The cells from the smaller colonies were *leu2* (had lost the *LEU2 tor2^{ts}* plasmid) and were cold sensitive for growth at 30°C and below, indicating that the *rot* mutation in these strains suppressed a *tor2*-null allele, but only at 37°C. The cells from the larger colonies were *LEU2* (still carried the *tor2^{ts}* gene) and grew at all temperatures. The ability of *rot1* and *rot2* alleles to suppress a *tor2*-null allele at 37°C was confirmed by crossing cells from the small colonies to a wild-type strain and dissecting the resulting diploids at 37°C; *rot1 tor2*-null and *rot2 tor2*-null segregants grew at 37°C, but were unable to grow at 30, 24 and 15°C. Thus, the *rot1* and *rot2* mutations, from the eight mutants, suppressed a *tor2*-null but only at 37°C. The *rot1-1* and *rot2-1* mutants selected for further study were isolated from *tor2-21* and *tor2-64* mutants, respectively.

The *rot1-1* and *rot2-1* mutations in strains with an otherwise wild-type background were scored by back-crossing to a *tor2^{ts}* mutant. Diploids from these crosses were sporulated and dissected at 37°C. Strains that yielded segregants that contained the *tor2^{ts}* allele and were viable at 37°C were inferred to carry a *rot* mutation.

Identification of YMR200w, YBR229c, YHR101c and SAC7

YMR200w, *YBR229c*, *YHR101c* and *SAC7* were isolated by their ability to rescue a *rot1-1 rot2-1* double mutant at 37°C. High- (*LEU2*-based, provided by K.Nasmyth) and low-copy-number libraries (*URA3*-based, provided by A.Koller) were transformed into the double mutant. Transformants were selected at permissive temperature and replica-plated to selective medium at non-permissive temperature. *YMR200w* was isolated 11 times on three independent plasmids from the low-copy-number library. Deletion analysis revealed that a 1.4 kb *Eco*RI–*Sac*I fragment from the insert was both necessary and sufficient for rescue of the *rot1-1 rot2-1* double mutant. *YBR229c* was isolated once from the *LEU2*-based library. Deletion analysis revealed that a 3.9 kb *Sac*I–*Xba*I fragment from the insert was both necessary and sufficient for rescue. *YHR101c* was isolated three times on three independent plasmids from the *LEU2*-based library. Deletion analysis revealed that a 1.6 kb *Fsp*I–*Sca*I fragment was both necessary and sufficient for rescue. *SAC7* was isolated four times on the same plasmid from the *LEU2*-based library. Deletion analysis revealed that a 3.6 kb *Hin*dIII *Eco*RI fragment was both necessary and sufficient for rescue.

YMR200w and *YBR229c* were shown to encode *ROT1* and *ROT2*, respectively, by genetic linkage. Plasmids encoding *YMR200w* and *YBR229c* were integrated at the genomic sites corresponding to the cloned open reading frames and found to segregate opposite *rot1-1* and *rot2-1*. This assignment was further confirmed by transforming the single-copy plasmids containing the open reading frames into the *rot1-1 tor2^{ts}* and *rot2-1 tor2^{ts}* double mutants. Only *YMR200w* was able to complement *rot1-1* and only *YBR229c* was able to complement *rot2-1* (data not shown).

Staining and quantification of chitin

Cells were grown to early logarithmic phase, fixed in formaldehyde and stained with calcofluor white to visualize chitin, as described (Pringle *et al.*, 1989). Chitin was quantified as described (Bulawa *et al.*, 1986). All reagents were purchased from Sigma.

Analysis of ER glycoproteins

ER glycosylation of carboxypeptidase Y (CPY) was analysed as described (Stevens *et al.*, 1982) with the following minor modifications. Cells were labelled with [^{35}S]methionine (Amersham) and were lysed with NaOH/β-mercaptoethanol (Volland *et al.*, 1994). The *GAL-ROT1* mutant PA38-2a was grown in YPraffinose and shifted to YPD for 4 h (before shifting to 37°C) to turn off expression of *ROT1*. The *GAL-BIG1* strain PA35-19d was grown in YPD.

Disruption of ROT1, ROT2, BIG1, CWH41, GAS1, FKS1 and CHS3

The entire open reading frames of *ROT1*, *ROT2*, *BIG1*, *CWH41*, *GAS1* and *CHS3* were replaced by PCR-generated *kanMX2* or *HIS3MX6* cassettes (Wach *et al*., 1994). The oligonucleotide pairs used for generation of the disruption cassettes were as follows, with the oligonucleotide corresponding to the 5′ end of the disrupted gene listed first. *ROT1*: 5′-GGATCGGTATTATAACCATTATTATTTGTTGCACTTTATTACGATATCAAGCTTGCCTCGTCC-3′ and 5′-GGGCCTCTTTCCCCCATTGGTTGAATAAGTATCCCTGTAGTGCGCGGATGGCGGCGTTAGTATCG-3′. *ROT2*: 5′-CGGATATTTTTACGTTTACTTTGTAAGAAAGAGCTAAAAAGATGTAAAGATCTGTTTAGCTTGCCTCGTCC-3′ and 5′-GATTGCTCTGCGTATCTTAAAATAGCGGTCTCGAATCAACCGTATCGAGCTCGTTTAAACTGGATGGCG-3′. *BIG1*: 5′-GTTTTGTAGTTAGGAAACGTAGGCGCATACTAATTAGGAGTAGCAAGGCGCCAGATCTGTTTAGCTTGCC-3′ and 5′-GCGGTAGGTCAAATTTGCTCGAGCAATATATGTAGCTAAGAAACGTTTTAATAGGTACTCTCTGAAGCG-3′. *CWH41*: 5′-CTGTTAATAAGGAAGTAGTGGATAATAACGGTTCAGGTTGCTTCACAGATATCAAGCTTGCCTCGTCC-3′ and 5′-CTGGTTACATATCTGTTTATATGACTGAAAATATCTACATAGATGGATGGCGGCGTTAGTATCG-3′. The disruption cassette for *GAS1* was provided by M.Schönbächler. The disruption construct of *FKS1* was provided by F.Foor (Mazur *et al*., 1995). All disruptions were confirmed by either Southern analysis or PCR.

GEF activity assay

GEF activity was assayed by measuring the binding of [^{35}S]GTPγS to RHO1 as described (Yamamoto *et al.*, 1990), with slight modifications. All procedures were at 4°C, unless stated otherwise. All strains were grown at 37°C (the temperature at which the cell wall defects are known to suppress *tor2^{ts}*) in YPD to an OD_{600} of 0.5–0.8. SDS-treated cells were grown in YPD plus SDS (0.005%) for 1.5 h, at 37 or 30°C, before harvesting. Whole-cell extracts from strains to be assayed for GEF activity 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, 1 mM PMSF), lysing with glass beads in a bead-beater and removing cell debris by centrifugation at 500 *g* for 10 min. GST-RHO1 was prepared as follows. GST-RHO1-expressing yeast cells (JK9-3dα/pAS53) were resuspended in extraction buffer I (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM PMSF) and lysed with glass beads, and cell debris was removed by centrifugation at 500 *g* for 10 min. GST-RHO1 was isolated by incubating the cell extract with GST–Sepharose beads (Pharmacia) for 2 h. Beads were washed with buffer III (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% Triton X-100, 1 mM PMSF). The GEF assay was as follows. GST-RHO1 was washed with and then resuspended in reaction buffer (extraction buffer II), and incubated with 1 mM [^{35}S]GTPγS (Amersham) in the presence of 200 μg of a whole cell extract to be assayed and 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 centrifuged and washed twice with stop buffer. The radioactivity was quantified by scintillation counting. The GEF activity toward RHO1 in a mutant extract was expressed as a percentage of the GEF activity toward RHO1 in a wild-type extract.

Miscellaneous methods

The EMBL and GenBank databases were searched for *ROT1*, *ROT2*, *BIG1* and glucosidase I homologues using the BLAST program (Devereux *et al.*, 1984).

Acknowledgements

We thank Sylvane Desrivières, Thomas Beck and Tobias Schmelzle for helpful discussions and comments on the manuscript. This work was

supported by grants from the Swiss National Science Foundation and the Canton of Basel to M.N.H.

References

Adams,A.E. and Pringle,J.R. (1984) Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.*, **98**, 934–945.

Adams,A.E., Johnson,D.I., Longnecker,R.M., Sloat,B.F. and Pringle,J.R. (1990) CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.*, **111**, 131–142.

Barbet,N., Schneider,U., Helliwell,S.B., Stansfield,I., Tuite,M. and Hall,M. (1996) TOR controls translation initiation and early G_1 progression in yeast. *Mol. Biol. Cell*, **7**, 25–42.

Bender,A. and Pringle,J.R. (1989) Multicopy suppression of the cdc24 budding defect in yeast by CDC42 and three newly identified genes including the ras-related gene RSR1. *Proc. Natl Acad. Sci. USA*, **86**, 9976–9980.

Beretta,L., Gingras,A.C., Svitkin,Y.V., Hall,M.N. and Sonenberg,N. (1996) Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap- dependent initiation of translation. *EMBO J.*, **15**, 658–664.

Bergeron,J.J., Brenner,M.B., Thomas,D.Y. and Williams,D.B. (1994) Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem. Sci.*, **19**, 124–128.

Bourne,H.R., Sanders,D.A. and McCormick,F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature*, **349**, 117–127.

Brown,E.J., Beal,P.A., Keith,C.T., Chen,J., Shin,T.B. and Schreiber,S.L. (1995) Control of p70 s6 kinase by kinase activity of FRAP *in vivo*. *Nature*, **377**, 441–446.

Brunn,G.J., Hudson,C.C., Sekulic,A., Williams,J.M., Hosoi,H., Houghton,P.J., Lawrence,J.C.,Jr and Abraham,R.T. (1997) Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science*, **277**, 99–101.

Bulawa,C.E., Slater,M., Cabib,E., Au-Young,J., Sburlati,A., Adair, W.L.,Jr and Robbins,P.W. (1986) The *S. cerevisiae* structural gene for chitin synthase is not required for chitin synthesis *in vivo*. *Cell*, **46**, 213–225.

Cabib,E., Mol,P.C., Shaw,J.A. and Choi,W.J. (1993) Biosynthesis of cell wall and septum during yeast growth. *Arch. Med. Res.*, **24**, 301–303.

Cafferkey,R., McLaughlin,M.M., Young,P.R., Johnson,R.K. and Livi,G.P. (1994) Yeast TOR (DRR) proteins: amino-acid sequence alignment and identification of structural motifs. *Gene*, **141**, 133–136.

Castro,C., Ribas,J.C., Valdivieso,M.H., Varona,R., del Rey,F. and Duran,A. (1995) Papulacandin B resistance in budding and fission yeasts: isolation and characterization of a gene involved in (1,3)β-D-glucan synthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **177**, 5732–5739.

Clark,E.A. and Brugge,J.S. (1995) Integrins and signal transduction pathways: the road taken. *Science*, **268**, 233–239.

Devereux,J., Haeberli,P. and Smithies,O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.*, **12**, 387–395.

Di Como,C.J. and Arndt,K.T. (1996) Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev.*, **10**, 1904–1916.

Douglas,C.M. *et al.* (1994) The *Saccharomyces cerevisiae* FKS1 (ETG1) gene encodes (an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase). *Proc. Natl Acad. Sci. USA*, **91**, 12907–12911.

Drgonova,J., Drgon,T., Tanaka,K., Kollar,R., Chen,G.C., Ford,R.A., Chan, C.S., Takai,Y. and Cabib,E. (1996) Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science*, **272**, 277–279.

Eng,W.K., Faucette,L., McLaughlin,M.M., Cafferkey,R., Koltin,Y., Morris, R.A., Young,P.R., Johnson,R.K. and Livi,G.P. (1994) The yeast FKS1 gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin- dependent growth. *Gene*, **151**, 61–71.

Esmon,B., Esmon,P.C. and Schekman,R. (1984) Early steps in processing of yeast glycoproteins. *J. Biol. Chem.*, **259**, 10322–10327.

Evangelista,M., Blundell,K., Longtine,M.S., Chow,C.J., Adames,N., Pringle,J.R., Peter,M. and Boone,C. (1997) Bni1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarized morphogenesis. *Science*, **276**, 118–122.

Gietz,R.D. and Sugino,A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, **74**, 527–534.

Guthrie,C. and Fink,G.R. (1991) *Guide to Yeast Genetics and Molecular Biology*. Academic Press, Inc., San Diego, CA.

Hall,A. (1990) The cellular functions of small GTP-binding proteins. *Science*, **249**, 635–640.

Hall,M.N. (1996) The TOR signalling pathway and growth control in yeast. *Biochem. Soc. Trans.*, **24**, 234–239.

Heitman,J., Movva,N.R. and Hall,M.N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, **253**, 905–909.

Helliwell,S.B., Wagner,P., Kunz,J., Deuter-Reinhard,M., Henriquez,R. and Hall,M.N. (1994) TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Biol. Cell*, **5**, 105–18.

Helliwell,S.B., Howald,I., Barbet,N. and Hall,M.N. (1998) TOR2 is part of two related signalling pathways coordinating cell growth in *Saccharomyces cerevisiae*. *Genetics*, **148**, 99–112.

Herscovics,A. and Orlean,P. (1993) Glycoprotein biosynthesis in yeast. *FASEB J.*, **7**, 540–550.

Igual,J.C., Johnson,A.L. and Johnston,L.H. (1996) Coordinated regulation of gene expression by the cell cycle transcription factor Swi4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J.*, **15**, 5001–5013.

Jiang,B., Ram,A.F., Sheraton,J., Klis,F.M. and Bussey,H. (1995) Regulation of cell wall β-glucan assembly: PTC1 negatively affects PBS2 action in a pathway that includes modulation of EXG1 transcription. *Mol. Gen. Genet.*, **248**, 260–269.

Jiang,B., Sheraton,J., Ram,A.F., Dijkgraaf,G.J., Klis,F.M. and Bussey,H. (1996) CWH41 encodes a novel endoplasmic reticulum membrane N-glycoprotein involved in β1,6-glucan assembly. *J. Bacteriol.*, **178**, 1162–1171.

Johnson,D.I. and Pringle,J.R. (1990) Molecular characterization of CDC42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell. Biol.*, **111**, 143–152.

Kamada,Y., Jung,U.S., Piotrowski,J. and Levin,D.E. (1995) The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev.*, **9**, 1559–1571.

Kamada,Y., Qadota,H., Python,C.P., Anraku,Y., Ohya,Y. and Levin,D.E. (1996) Activation of yeast protein kinase C by Rho1 GTPase. *J. Biol. Chem.*, **271**, 9193–9196.

Kapteyn,J.C., Ram,A.F.J., Groos,E.M., Kollar,R., Montijn,R.C., Van Den Ende,H., LLobell,A., Cabib,E. and Klis,F.M. (1997) Altered extent of cross-linking of β1,6-glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall β1,3-glucan content. *J. Bacteriol.*, **179**, 6279–6284.

Kilker,R.D.,Jr., Saunier,B., Tkacz,J.S. and Herscovics,A. (1981) Partial purification from *Saccharomyces cerevisiae* of a soluble glucosidase which removes the terminal glucose from the oligosaccharide Glc3Man9GlcNAc2. *J. Biol. Chem.*, **256**, 5299–5603.

Kilmartin,J.V. and Adams,A.E. (1984) Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. *J. Cell. Biol.*, **98**, 922–933.

Klis,F.M. (1994) Review: cell wall assembly in yeast. *Yeast*, **10**, 851–869.

Kohno,H., Tanaka,K., Mino,A., Umikawa,M., Imamura,H., Fujiwara,T., Fujita,Y., Hotta,K., Qadota,H., Watanabe,T., Ohya,Y. and Takai,Y. (1996) Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *EMBO J.*, **15**, 6060–6068.

Kollar,R., Reinhold,B.B., Petrakova,E., Yeh,H.J., Ashwell,G., Drgonova,J., Kapteyn,J.C., Klis,F.M. and Cabib,E. (1997) Architecture of the yeast cell wall. Beta(1→6)-glucan interconnects mannoprotein, beta(1→)3-glucan, and chitin. *J. Biol. Chem.*, **272**, 17762–17775.

Kunz,J., Henriquez,R., Schneider,U., Deuter-Reinhard,M., Movva,N.R. and Hall,M.N. (1993) Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. *Cell*, **73**, 585–596.

Madaule,P., Axel,R. and Myers,A.M. (1987) Characterization of two members of the rho gene family from the yeast *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **84**, 779–783.

Madden,K., Sheu,Y.J., Baetz,K., Andrews,B. and Snyder,M. (1997) SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science*, **275**, 1781–1784.

Matsui,Y. and Toh-e,A. (1992a) Yeast RHO3 and RHO4 ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. *Mol. Cell Biol.*, **12**, 5690–5699.

Matsui,Y. and Toh-e,A. (1992b) Isolation and characterization of two novel ras superfamily genes in *Saccharomyces cerevisiae*. *Gene*, **114**, 43–49.

Mazur,P., Morin,N., Baginsky,W., el-Sherbeini,M., Clemas,J.A., Nielsen, J.B. and Foor,F. (1995) Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. *Mol. Cell. Biol.*, **15**, 5671–5681.

Mazzoni,C., Zarov,P., Rambourg,A. and Mann,C. (1993) The SLT2 (MPK1) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae*. *J. Cell. Biol.*, **123**, 1821–1833.

Mitchell,D.A., Marshall,T.K. and Deschenes,R.J. (1993) Vectors for the inducible overexpression of glutathione *S*-transferase fusion proteins in yeast. *Yeast*, **9**, 715–723.

Nobes,C.D. and Hall,A. (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*, **81**, 53–62.

Nonaka,H., Tanaka,K., Hirano,H., Fujiwara,T., Kohno,H., Umikawa,M., Mino,A. and Takai,Y. (1995) A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J.*, **14**, 5931–5938.

Orlean,P. (1997) Biogenesis of yeast cell wall and surface components. In Pringle,J., Broach,J. and Jones,E. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces Cerevisiae: Cell Cycle and Cell Biology*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, pp. 229–362.

Ozaki,K., Tanaka,K., Imamura,H., Hihara,T., Kameyama,T., Nonaka,H., Hirano,H., Matsuura,Y. and Takai,Y. (1996) Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *EMBO J.*, **15**, 2196–2207.

Parlati,F., Dominguez,M., Bergeron,J.J. and Thomas,D.Y. (1995) *Saccharomyces cerevisiae* CNE1 encodes an endoplasmic reticulum (ER) membrane protein with sequence similarity to calnexin and calreticulin and functions as a constituent of the ER quality control apparatus. *J. Biol. Chem.*, **270**, 244–253.

Popolo,L., Vai,M., Gatti,E., Porello,S., Bonfante,P., Balestrini,R. and Alberghina,L. (1993) Physiological analysis of mutants indicates involvement of the *Saccharomyces cerevisiae* GPI-anchored protein gp115 in morphogenesis and cell separation. *J. Bacteriol.*, **175**, 1879–1885.

Popolo,L., Gilardelli,D., Bonfante,P. and Vai,M. (1997) Increase in chitin as an essential response to defects in assembly of cell wall polymers in ggp1 mutant of *Saccharomyces cerevisiae*. *J. Bacteriol.*, **179**, 463–469.

Pringle,J.R., Preston,A.E., Adams,A.E.M., Stearns,T. and Drubin,D.G. (1989) Fluorescence microscopy methods for yeast. *Methods Cell. Biol.*, **31**, 357–435.

Qadota,H., Anraku,Y., Botstein,D. and Ohya,Y. (1994) Conditional lethality of a yeast strain expressing human RHOA in place of RHO1. *Proc. Natl Acad. Sci. USA*, **91**, 9317–9321.

Qadota,H., Python,C.P., Inoue,S.B., Arisawa,M., Anraku,Y., Zheng,Y., Watanabe,T., Levin,D.E. and Ohya,Y. (1996) Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. *Science*, **272**, 279–281.

Ram,A.F., Brekelmans,S.S., Oehlen,L.J. and Klis,F.M. (1995) Identification of two cell cycle regulated genes affecting the β1,3-glucan content of cell walls in *Saccharomyces cerevisiae*. *FEBS Lett.*, **358**, 165–170.

Romero,P.A., Dijkgraaf,G.J.P., Shahinian,S., Herscovics,A. and Bussey,H. (1997) The yeast *CWH41* gene encodes glucosidase I. *Glycobiology*, **7**, 997–1004.

Saunier,B., Kilker,R.D.,Jr., Tkacz,J.S., Quaroni,A. and Herscovics,A. (1982) Inhibition of N-linked complex oligosaccharide formation by 1-deoxynojirimycin, an inhibitor of processing glucosidases. *J. Biol. Chem.*, **257**, 14155–14161.

Schmidt,A., Kunz,J. and Hall,M.N. (1996) TOR2 is required for organization of the actin cytoskeleton in yeast. *Proc. Natl Acad. Sci. USA*, **93**, 13780–13785.

Schmidt,A., Bickle,M., Beck,T. and Hall,M.N. (1997) The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell*, **88**, 531–542.

Shaw,J.A., Mol,P.C., Bowers,B., Silverman,S. J., Valdivieso,M.H., Duran,A. and Cabib,E. (1991) The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *J. Cell. Biol.*, **114**, 111–123.

Shimizu,J., Yoda,K. and Yamasaki,M. (1994) The hypo-osmolarity-sensitive phenotype of the *Saccharomyces cerevisiae* hpo2 mutant is due to a mutation in PKC1, which regulates expression of β-glucanase. *Mol. Gen. Genet.*, **242**, 641–648.

Simons,J.F., Ebersold,M. and Helenius,A. (1998) Cell wall 1,6-β-glucan synthesis in *S. cerevisiae* depends on ER glucosidase I and II, and the molecular chaperone BiP/Kar2p. *EMBO J.*, **17**, 396–405.

Stevens,T., Esmon,B. and Schekman,R. (1982) Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell*, **30**, 439–448.

Thomas,G. and Hall,M.N. (1997) TOR signalling and control of cell growth. *Curr. Opin. Cell. Biol.*, **9**, 787–787.

Trombetta,E.S., Simons,J.F. and Helenius,A. (1996) Endoplasmic reticulum glucosidase II is composed of a catalytic subunit, conserved from yeast to mammals, and a tightly bound noncatalytic HDEL-containing subunit. *J. Biol. Chem.*, **271**, 27509–27516.

Volland,C., Urban-Grimal,D., Geraud,G. and Haguenauer-Tsapis,R. (1994) Endocytosis and degradation of the yeast uracil permease under adverse conditions. *J. Biol. Chem.*, **269**, 9833–9841.

von Manteuffel,S., Gingras,A.-C., Ming,X.-F., Sonenberg,N. and Thomas,G. (1996) 4E-BP1 phsophorylation is mediated by the FRAP-p70S6K pathway and is MAP kinase independent. *Proc. Natl Acad. Sci.*, **93**, 4076–4080.

Wach,A., Brachat, A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.

Yamamoto,T., Kaibuchi,K., Mizuno,T., Hiroyoshi,M., Shirataki,H. and Takai,Y. (1990) Purification and characterization from bovine brain cytosol of proteins that regulate the GDP/GTP exchange reaction of smg p21s, ras p21-like GTP-binding proteins. *J. Biol. Chem.*, **265**, 16626-16634.

Yamochi,W., Tanaka,K., Nonaka,H., Maeda,A., Musha,T. and Takai,Y. (1994) Growth site localization of Rho1 small GTP-binding protein and its involvement in bud formation in *Saccharomyces cerevisiae*. *J. Cell. Biol.*, **125**, 1077–1093.

Zheng,X.F., Florentino,D., Chen,J., Crabtree,G.R. and Schreiber,S.L. (1995) TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. *Cell*, **82**, 121–130.

Received November 17, 1997; revised and accepted February 18, 1998