The Rho3 and Rho4 small GTPases interact functionally with Wsc1p, a cell surface sensor of the protein kinase C cell-integrity pathway in *Saccharomyces cerevisiae*

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Rgd1, a GTPase-activating protein, is the only known negative regulator of the Rho3 and Rho4 small GTPases in the yeast *Saccharomyces cerevisiae*. Rho3p and Rho4p are involved in regulating cell polarity by controlling polarized exocytosis. Co-inactivation of **RGD1** and **WSC1**, which is a cell wall sensor-encoding gene, is lethal. Another plasma membrane sensor, Mid2p, is known to rescue the **rgd1Δwsc1Δ** synthetic lethality. It has been proposed that Wsc1p and Mid2p act upstream of the protein kinase C (PKC) pathway to function as mechanosensors of cell wall stress. Analysis of the synthetic lethal phenomenon revealed that production of activated Rho3p and Rho4p leads to lethality in **wsc1Δ** cells. Inactivation of **RHO3** or **RHO4** was able to rescue the **rgd1Δwsc1Δ** synthetic lethality, supporting the idea that the accumulation of GTP-bound Rho proteins, following loss of Rgd1p, is detrimental if the Wsc1 sensor is absent. In contrast, the genetic interaction between **RGD1** and **MID2** was not due to an accumulation of GTP-bound Rho proteins. It was proposed that simultaneous inactivation of **RGD1** and **WSC1** constitutively activates the PKC–mitogen-activated protein kinase (MAP kinase) pathway. Moreover, it was shown that the activity of this pathway was not involved in the synthetic lethal interaction, which suggests the existence of another mechanism. Consistent with this idea, it was found that perturbations in Rho3-mediated polarized exocytosis specifically impair the abundance and processing of Wsc1 and Mid2 proteins. Hence, it is proposed that Wsc1p participates in the regulation of a Rho3/4-dependent cellular mechanism, and that this is distinct from the role of Wsc1p in the PKC–MAP kinase pathway.

INTRODUCTION

Monomeric GTPases of the Ras superfamily are key regulators of numerous physiological processes. They are classified into five families: Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf and Ran (Van Aelst & D’Souza-Schorey, 1997; Etienne-Manneville & Hall, 2002). In the yeast *Saccharomyces cerevisiae*, six functional Rho proteins, namely Cdc42 and Rho1–Rho5, have been described (Garcia-Ranea & Valencia, 1998; Roumanie et al., 2001; Schmitz et al., 2002). Like most members of the Ras superfamily, Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form to control cell polarity by regulating secretion, actin cytoskeleton organization, and cell wall modelling (Johnson & Pringle, 1990; Matsui & Toh-e, 1992a; Robinson et al., 1999; Adamo et al., 2001; Roumanie et al., 2005). The balance between GDP- and GTP-bound forms is regulated by guanine nucleotide exchange factors, which promote GDP release and binding of GTP, and by GTPase-activating proteins (GAPs), which accelerate GTP hydrolysis by Rho proteins (Van Aelst and D’Souza-Schorey, 1997).

The yeast Rgd1 protein is known to possess GAP activity towards both Rho3 and Rho4 small G proteins (Doignon...
et al., 1999). By activating GTP hydrolysis, Rgd1p negatively regulates Rho3/4 GTPases, and lack of Rgd1p leads to an accumulation of the GTP-bound forms of these two small G proteins in the cell (Roumanie et al., 2000). Inactivation of the RGD1 gene is not associated with a defect in cell polarity (de Bettignies et al., 1999); however, it results in an increase in cell mortality in the late-exponential growth phase (Barthe et al., 1998). Rho3p and Rho4p functions are partially redundant, and are involved in cell polarity by regulating polarized exocytosis and actin cytoskeleton organization (Matsui & Toh-e, 1992b; Roumanie et al., 2005). While no defect is associated with RHO4 deletion, inactivation of both RHO3 and RHO4 genes results in a severe growth phenotype, and lysis of cells with small buds. The Rho3 and Rho4 GTPases have been shown to bind the exocyst complex subunit Exo70, and to interact with formins to activate actin cable formation (Robinson et al., 1999; Dong et al., 2003). Moreover, Rho3p has been shown to have a direct role in post-Golgi secretion by enabling polarized fusion of secretory vesicles with the plasma membrane (Adamo et al., 1999).

A recent study has demonstrated that regulation of cell polarity by Rho3p is independent of GTP hydrolysis (Roumanie et al., 2005), suggesting that, in yeast, Rgd1p GAP activity functions with other proteins to regulate Rho3p and Rho4p function in cell growth.

Indeed, studies from our laboratory have implicated Rgd1p in the regulation of cell polarity. We have reported the existence of functional interactions between Rgd1p and the Arp2/3 complex activators Vrp1p and Las17p (Roumanie et al., 2000). Genetic interactions between RGD1 and WSC1 genes have also been demonstrated previously (de Bettignies et al., 1999). WSC1 encodes a highly O-glycosylated integral membrane protein that acts as a parietal stress sensor once anchored to the plasma membrane (Verna et al., 1997). It has been found that although lack of Rgd1p or Wsc1p under standard growth conditions does not have any effect on yeast, the combination of both rgd1Δ and wsc1Δ mutations leads to cell mortality. At the same time, the Mid2p cell wall sensor, which has functions partially overlapping with those of Wsc1p (Ketela et al., 1999), was characterized as a suppressor of the rgd1Δwsc1Δ synthetic lethality. Nevertheless, co-deletion of RGD1 and MID2 is not lethal under regular growth conditions, but leads to conditional lethality in the late-exponential growth phase (de Bettignies et al., 1999). At the plasma membrane, both the Wsc1 and Mid2 proteins monitor the integrity of yeast cell wall, and can activate the protein kinase C (PKC) pathway (Gray et al., 1997; Verna et al., 1997; Jacoby et al., 1998; Ketela et al., 1999; Rajavel et al., 1999; Martin et al., 2000). The PKC pathway is activated in response to various external stresses, including high temperature, low osmolarity, cell wall perturbation, and mating (Heinisch et al., 1999). Pkc1 kinase activation is dependent on the interaction with stress-activated Rho1 GTPase (Nonaka et al., 1995; Kamada et al., 1996). The PKC pathway is composed of a MAP kinase module involving the Bck1, Mkk1/Mkk2 and Slt2 kinases (Gustin et al., 1998). It is known that activated Wsc1p regulates both the PKC pathway and 1,3-β-glucan synthesis, while Mid2p acts mainly on the PKC pathway (Sekiya-Kawasaki et al., 2002).

To gain insight into the cellular mechanism by which Rgd1p interacts with the cell wall sensor Wsc1, we investigated the role of Rho3 and Rho4 GTPases in the rgd1Δwsc1Δ synthetic lethality. In this study, we report that the lethality involves an accumulation of activated Rho proteins, and that this is detrimental to the survival of wsc1Δ cells; however, the synthetic lethality is not linked to a defect in the MAP kinase module of the PKC pathway. Moreover, functional interactions involving RGD1, and WSC1 or MID2, are distinct. Disturbance in the Golgi-to-plasma-membrane secretion leads to a specific defect in cell wall sensor abundance. Our results support the involvement of Wsc1 in a cellular pathway connected to Rho3p-regulated trafficking.

**METHODS**

**Yeast strains and genetic procedures.** The Saccharomyces cerevisiae strains used in this study, and their genetic backgrounds, are described in Table 1. All the strains, except rho3-V51, are isogenic to the S288C strain. Standard yeast genetic methods and media preparation were performed as described by Sherman et al. (1986). Unless otherwise indicated, yeast strains were grown at 30 °C. Yeast transformation was performed according to Gietz et al. (1995). Dead cells were visualized by Methylene Blue staining, and enumerated by microscopic examination of at least 300 cells (Rose, 1975). The standard deviation on determination of the percentage of lethality was calculated to be 2:5%.

**Production of Rho3 and Rho4 GTPases.** Production of wild-type (WT), GDP- and GTP-blocked Rho3 and Rho4 proteins was carried out using the pCM plasmid-based system, as described previously (Roumanie et al., 2000). GDP-blocked mutant forms were obtained by substitution of threonine with asparagine at position 30 in Rho3p, and at position 86 in Rho4p. GTP-blocked mutants were obtained by replacing glutamates 74 and 131 with leucine in Rho3 and Rho4, respectively. Repression of protein production from the pCM system was obtained by adding 1 μg doxycycline ml⁻¹ to the medium. Turn-on was induced by putting cells in doxycycline-free medium. Complete activation of production was obtained after about 8 h growth (Gari et al., 1997; Roumanie et al., 2000).

**Heat-shock experiments.** The heat-shock experiments were performed essentially as described by Martin et al. (2000). In brief, cells were grown overnight at 21 °C to mid-exponential phase (OD600 0-5), and then either shifted to 39 °C, or kept at 21 °C. After 2 h incubation, proteins were extracted, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with x-phospho-Sh2p antibodies to monitor PKC pathway activation.

**Immunoblot analysis.** Protein extraction from yeast for Western blot analysis was performed according to Riezman et al. (1983). The YEp352-MID2HA and YEp352-WSC1HA plasmids were used to produce Mid2 and Wsc1 proteins tagged at their C-terminals with a 3×his (HA) tag, as described by Rajavel et al. (1999). Blots were probed for phosphorylated Sh2p with x-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody from Cell Signalling Technology (2000). The Blots were probed for phosphorylated Sh2p with x-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody from Cell Signalling Technology (2000).

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was used at 1:10,000 dilution. Primary antibodies were detected with z-mouse or z-rabbit antibodies coupled to horseradish peroxidase (Pierce) at 1:10,000 dilution. Protein–antibody complexes were visualized with the Lumi-LightPLUS system (Roche). Quantification of bands was done using the ImageJ program (National Institutes of Health).

**RESULTS**

**Accumulation of activated Rho3p in WT cells leads to the *rgd1Δ*-like phenotype**

In a previous study, we reported that about 20% of *rgd1Δ* cells die at the end of the exponential growth phase, when grown in minimal medium (Barthe et al., 1998; de Bettignies et al., 1999). As the GAP domain of Rgd1p negatively regulates Rho3p and Rho4p GTPase activities (Doignon et al., 1999), we decided to explore the involvement of these two small G proteins in the *rgd1Δ* phenotype. We hypothesized that, as a consequence of *Rgd1* inactivation, there was an accumulation of activated forms of Rho3p and Rho4p, and that this was detrimental to the survival of the yeast. Constitutively activated (GTP-blocked), inactivated (GDP-blocked) and WT forms of Rho3p and Rho4p were produced in the WT strain grown in YNB minimal medium, in order to examine the effects on cell morphology and lethality. We observed that the constitutively active Rho3 led to a cell growth defect, as compared with cells containing endogenous Rho3p (control vector) only. Expression of the other mutant forms did not show any detectable growth defect (data not shown). Moreover, microscopic examination of cells producing activated Rho3p or Rho4p revealed heterogeneous morphological defects (Fig. 1A). The morphological alterations were found to increase proportionally with an increase in cell-culture density. For example, at an OD600 of 1.5, half of the yeast cells producing GTP-blocked Rho3p demonstrated abnormally elongated cells, with 10% of the cells also exhibiting an elongated bud. These observations suggest that cells overproducing activated Rho3p have defects in polarized growth, in agreement with that reported for the constitutively active *rho3ΔAla-131* allele; indeed, *rho3ΔAla-131* cells have been shown to become elongated and bent (Imai et al., 1996). Upon production of GTP-blocked Rho4p, we observed that about 30% of cells were larger and rounder, with an undefined bud neck. These results indicate impairment of the cytokinesis process.

Regarding the propensity of cells to die upon production of activated Rho proteins, we noted that the percentage lethality was relatively constant and low in the exponential growth phase, but slightly increased in the stationary phase. Importantly, production of GTP-blocked Rho3p led to an increase in cell mortality in the late-exponential phase. The importances suggest that cells overproducing activated Rho3p have defects in polarized growth, in agreement with that reported for the constitutively active *rho3ΔAla-131* allele; indeed, *rho3ΔAla-131* cells have been shown to become elongated and bent (Imai et al., 1996). Upon production of GTP-blocked Rho4p, we observed that about 30% of cells were larger and rounder, with an undefined bud neck. These results indicate impairment of the cytokinesis process.

Table 1. *S. cerevisiae* strains used in this study

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*European *S. cerevisiae* Archive for Functional Analysis, Institute for Microbiology, Frankfurt, Germany.*
activated Rho3 and Rho4 GTPases was found to be similar to that observed in cells producing activated Rho3p only. We also observed that addition of 1 M sorbitol to the medium did not rescue the cell lethality caused by the production of activated Rho3p. We have previously reported that lethality of \( rgd1 \)D cells cannot be suppressed by the addition of an osmotic stabilizer (de Bettignies et al., 1999). Taken together, these data suggest that the \( rgd1 \)D lethality in minimal medium is a consequence of an increase in the amount of activated Rho3p.

**The Rho3 and Rho4 small GTPases functionally interact with Wsc1p**

We have previously demonstrated the existence of a synthetic lethal interaction between the \( rgd1 \)Δ and \( wsc1 \)Δ mutations (de Bettignies et al., 1999). In the present study, we investigated the involvement of Rho3 and Rho4 GTPases in \( rgd1 \)Δ\( wsc1 \)Δ lethality. The different forms of Rho3p and Rho4p were independently or simultaneously produced in the \( wsc1 \)Δ strain, or in the WT strain as a control, to examine the change in cell mortality as a function of growth. Based on the results presented above, and data published previously (de Bettignies et al., 1999), cells were grown in a synthetic complete medium, and not in minimal medium, to prevent an increase in cell mortality due to Rho3p accumulation in the late-exponential phase. Production of the different forms of Rho3p or Rho4p in WT cells had no detectable effect on growth (data not shown). The percentage of cell mortality was found to be low and virtually identical for all WT strains producing the different proteins (Fig. 2A, B). An increase in mortality was observed in late-stationary phase upon co-production of Rho3p and Rho4p (Fig. 2C); we also observed the aforementioned morphological defects that were associated with production of GTP-blocked Rho3p and Rho4p. In contrast to the WT, differences in cell lethality were observed in the \( wsc1 \)Δ cells producing the different forms of Rho proteins. We observed that the \( wsc1 \)Δ cells containing the control vector had an intrinsic lethality of about 10 %, as reported previously (de Bettignies et al., 1999). In addition to this, production of all the Rho3p forms resulted in an increase in cell lethality (Fig. 2D). After 40 h induction, production of the WT and GDP-blocked forms of Rho3p led to similar responses, with around 40 % cell mortality. A pronounced effect, nearly 60 % mortality, was observed upon production of activated Rho3p. These data suggest a predominant role for GTP-bound Rho3p in the synthetic lethality observed in the \( rgd1 \)Δ\( wsc1 \)Δ strain. The results obtained using the different forms of Rho4p strongly suggest that it also plays an important role in \( rgd1 \)Δ\( wsc1 \)Δ lethality (Fig. 2E). However, unlike the results obtained with Rho3p, production of GTP- and GDP-blocked forms of Rho4p led to similar increases in cell mortality. In order to understand why the production of GTP- and GDP-blocked Rho4p led to the production of

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**Fig. 1.** Morphological defects and lethality associated with production of activated Rho3 and Rho4 GTPases. (A) Micrographs of the representative morphology of WT yeast cells producing constitutively activated forms of Rho3p (Rho3-GTP) and Rho4p (Rho4-GTP) from the pCM189 plasmid. An empty plasmid was used as a control. Cells were grown to OD600 1-5 in minimal medium without doxycycline to induce production of Rho3 and Rho4 proteins, and photographs were taken using a phase-contrast microscope. (B) Growth and lethality observed for the \( rgd1 \)Δ strain, and WT cells producing activated Rho3 and Rho4 GTPases, in minimal medium with 3 x excess inositol. Growth of cells containing empty pCM189 vector (Δ), pCM189 producing Rho3-GTP (●) and Rho4-GTP (■), and \( rgd1 \)Δ cells (○), was monitored by OD600. Dead cells were counted as described in Methods.
similar phenotypes, we analysed the effect of different forms of the GTPase in the \textit{wsc1}\textsuperscript{D} strain inactivated for \textit{RHO4}, thus eliminating the possibility that the concomitant synthesis of WT Rho4 protein from the chromosomal \textit{RHO4} gene was affecting cell lethality. Thus, we followed the mortality of the \textit{wsc1\textsuperscript{D} rho4\textsuperscript{D}} cells producing WT, GDP- and GTP-blocked Rho4p from the plasmid (Fig. 2G). We observed that in this genetic background, the production of GTP-blocked Rho4p

**Fig. 2.** Cell lethality results obtained from cultures of WT and \textit{wsc1}\textsuperscript{D} strains producing different forms of Rho3 and Rho4 GTPases. (A, B) WT cells containing empty pCM189 plasmid (▲), and WT cells producing WT Rho3p or Rho4p (■), GDP-blocked Rho3p or Rho4p (×), and GTP-blocked Rho3p or Rho4p (△), from the pCM189 plasmid, were grown in synthetic medium without doxycycline to induce Rho production at \( t=0 \). Cell lethality was determined during growth, as described in Methods. (C) Lethality of WT cells co-producing WT (■), GDP-blocked (×) and GTP-blocked (△) forms of Rho3 and Rho4 proteins from pCM185 and pCM189 plasmids. An empty vector was used as a control (▲). (D, E, F) Experiments similar to those shown in (A, B, C) were performed in the \textit{wsc1}\textsuperscript{D} genetic background. (G) Lethality of \textit{wsc1\textsuperscript{D} rho4\textsuperscript{D}} cells producing WT, inactivated and activated forms of Rho4p; the symbols are the same as for (E).
significantly increased cell mortality, which was approximately 50% after 40 h. Contrary to that observed in the wsc1Δ background, production of WT or GDP-blocked Rho4p in wsc1Δrho4Δ cells did not lead to an increase in cell mortality, with regard to the control vector. Thus, simultaneous synthesis of activated Rho4p from the chromosomal RHO4, and mutant forms from the plasmid, has a notable effect on the growth of the wsc1Δ cells.

We further analyzed the effect of the co-production of activated, inactivated and WT forms of Rho3p and Rho4p in wsc1Δ cells (Fig. 2F). Co-production of the GTP-active forms led to a significant increase in cell mortality, nearly 50% after 40 h, which was almost completely rescued by addition of 1 M sorbitol to the medium (data not shown). Importantly, the synthetic lethality between the wsc1Δ and rgd1Δ mutations is also known to be suppressed by the addition of an osmotic stabilizer (de Bettignies et al., 1999). The lethality observed with the wsc1Δ cells containing control vectors, or co-producing the WT or GDP-blocked forms of Rho3p and Rho4p, was in the order of 20% after 40 h. Thus, Rho3p and Rho4p in their GTP-bound forms have strong cumulative negative effects on the survival of wsc1Δ cells. These results strongly suggest that the accumulation of activated Rho3p and Rho4p following inactivation of RGD1 is detrimental to the survival of cells lacking the WSC1 gene. Notably, it has been shown previously that the synthetic lethality between the rgd1Δ and wsc1Δ mutations is not dependent on Rho3 and Rho4 GTPases (Roumanie et al., 2000).

**Inactivation of the RHO3 or RHO4 gene suppresses the rgd1Δwsc1Δ synthetic lethality**

We next investigated whether the rgd1Δwsc1Δ synthetic lethality could be rescued by inactivating the RHO3 or RHO4 genes. Since activated Rho3p and Rho4p are detrimental to the wsc1Δ cell survival, we hypothesized that reducing the amount of these GTPases would help to keep the cells alive. Accordingly, to assay for suppression, we crossed strains containing the wsc1Δrhd3Δ and rgd1Δ mutations or the rgd1Δrho4Δ and wsc1Δ mutations to evaluate the phenotypes of cells containing the three mutations. Crosses involving the wsc1Δrhd3Δ strain were performed in the presence of 1 M sorbitol to prevent cell lysis (Matsui & Toh-e, 1992b). In agreement with the working hypothesis, the rgd1Δwsc1Δrhd3Δ and rgd1Δwsc1Δrho4Δ triple mutants were found to be viable (Fig. 3A).

Next, we determined the cell mortality by growing the two triple mutant strains in complete medium. The analysis showed that mortality associated with the rgd1Δwsc1Δ mutations was strongly suppressed by inactivation of RHO4 (Fig. 3B). The rho4Δ mutation suppressed more than 85% of cell lethality in the exponential growth phase. Levels of growth of the WT and rgd1Δwsc1Δrho4Δ strains were found to be similar (data not shown). Inactivation of the RHO3 gene also led to a suppressor effect on rgd1Δwsc1Δ cell mortality. The strength of the suppression was not as great as that for the rho4Δ mutation; about 30% of the triple mutant rgd1Δwsc1Δrho3Δ cells still died in the early growth phase (Fig. 3B). However, we observed that addition of 1 M sorbitol to the medium increased rho3Δ suppression to the level observed with the rho4Δ mutation (data not shown). Taken together, these results show that both rho3 and rho4 mutations rescue the growth and lethality defects associated with the rgd1Δwsc1Δ mutations. Hence, reduced levels of activated Rho3 or Rho4 GTPases within the rgd1Δwsc1Δ cells suppress the synthetic lethal phenotype, which is consistent with the mechanism suggested by our analysis of interactions between Wsc1p and the two GTPases.

**The functional interaction between RGD1 and MID2 is not dependent on Rho3 and Rho4 GTPases**

In a previous study, we reported that RGD1 interacts genetically with the cell wall sensor-encoding gene MID2, and it was observed that inactivation of MID2 enhances the rgd1Δ phenotype, and that the double mutant strain has a higher number of dead cells in late-exponential growth.

![Fig. 3. Inactivation of RHO3 or RHO4 suppresses rgd1Δwsc1Δ synthetic lethality. (A) Growth of mutant strains was tested by spotting tenfold serial dilutions of cells onto minimal medium, and the plates were incubated for 2.5 days at 30 °C. (B) WT (○), rgd1Δwsc1Δrho3Δ (▲) and rgd1Δwsc1Δrho4Δ (□) strains were grown in complete medium, and cell lethality was measured during growth, as described in Methods.](https://www.microbiologyresearch.org)
phase in minimal medium (de Bettignies et al., 1999). Based on the data presented above, we investigated whether activated Rho3 and Rho4 GTPases were involved in the rgd1Δmid2Δ genetic interaction. We produced the different forms of Rho3p and Rho4p in mid2Δ cells, and in WT cells as a control. Cells were grown in YNB minimal medium, and the lethality was monitored at different times. As expected, results obtained with WT control cells were similar to those described above. Production of WT, GDP- and GTP-bound blocked Rho GTPases in the mid2Δ cells did not lead to any increase in mortality, with respect to control cells (data not shown). In addition, the lethality of the rgd1Δmid2Δ mutant was not rescued by RHO3 or RHO4 inactivation (data not shown). These results indicate that the Rho3 and Rho4 GTPases are not involved in the interaction between RGD1 and MID2.

The PKC pathway is constitutively active in the rgd1Δwsc1Δrho3Δ and rgd1Δwsc1Δrho4Δ triple mutants

The Wsc1 protein is a major sensor for cell wall integrity signalling that acts upstream of Pkc1p and the Bck1/Mkk1,2/Slt2 MAP kinase cascade (Ketela et al., 1999; Philip & Levin, 2001). We have previously reported that inactivation of RGD1 reduces Rlm1p transcriptional activity, as well as transcription of PST1, which is a target of the PKC pathway; moreover, it has been shown that viability of the rgd1Δ cells in the late-exponential phase is restored by over-expression of the PKC pathway (de Bettignies et al., 2001). Thus, one possibility is that in the rgd1Δwsc1Δ cells, weakening of the cell wall, and a decrease in the activity of the PKC pathway, might be responsible for the synthetic lethality. Consistent with this hypothesis, and as known for mutants defective in the PKC pathway, growth of the rgd1Δwsc1Δ mutant is restored by addition of an osmotic stabilizer to the medium (Cid et al., 1995; de Bettignies et al., 1999). We investigated the activity of the PKC pathway in the rgd1Δwsc1Δrho3Δ and rgd1Δwsc1Δrho4Δ triple mutants described above by monitoring phosphorylation of Slt2p. The Slt2 protein is specifically phosphorylated on residues Thr190 and Tyr192 upon activation of the PKC pathway (Lee et al., 1993). As expected, we found that the control WT strain, as well as the rgd1Δ and wsc1Δ mutants, did not have a detectable amount of activated Slt2p. However, a significantly higher amount of phosphorylated Slt2p was observed in the rgd1Δwsc1Δrho3Δ and rgd1Δwsc1Δrho4Δ triple mutants grown in complete medium at a regular temperature (30 °C) (Fig. 4A). Surprisingly, the abundance of Slt2 protein was increased in the wsc1Δ strain only, even though activity of the PKC pathway was not stimulated (Fig. 4A).

It is known that Slt2p phosphorylation is stimulated upon heat shock of yeast cells (Kamada et al., 1995). As inactivation of RHO4 strongly suppresses the rgd1Δwsc1Δ synthetic lethality in medium without an osmotic stabilizer (Fig. 3), we next determined the Slt2p phosphorylation state in the rgd1Δwsc1Δrho4Δ triple mutant, and in control strains, following heat shock. Although suppression of the synthetic lethality by inactivation of RHO3 is not as effective as with RHO4, the rho3Δ and rgd1Δwsc1Δrho3Δ strains were also included in the experiment to look for common physiological responses between the two triple mutants. The different strains were grown in minimal medium to mid-exponential phase at 21 °C, and then half of the culture was transferred to 39 °C for 2 h, while the other half was kept at 21 °C, and Slt2 phosphorylation was monitored at both temperatures (Fig. 4B). As shown above, the two triple mutant strains showed a constitutive activation of Slt2p phosphorylation at the regular temperature; none of the other strains showed a detectable amount of activated Slt2p at 21 °C. Temperature shift to 39 °C resulted in an overall increase in Slt2 phosphorylation for all the strains tested. However, quantification of the amount of activation showed that, although WT and rho4Δ cells were able to significantly increase phosphorylation of Slt2p, other strains tested showed a partial decrease in the activation of Slt2p phosphorylation (Fig. 4B). Importantly, the two triple mutants showed almost no increase in phosphorylation upon heat shock. Similar defects were observed when we measured the increase in the abundance of Slt2 protein after heat shock (Fig. 4B). Thus, stressing cells inactivated for RGD1 and WSC1 did not induce an additional increase in Slt2p phosphorylation. Altogether, our results suggest that the PKC pathway is constitutively active following inactivation of both the RGD1 and WSC1 genes. Hence, it seems unlikely that the rgd1Δwsc1Δ synthetic lethality could be a consequence of a defect in PKC pathway activity.

The PKC pathway is not involved in the rgd1Δwsc1Δ synthetic lethality

The aforementioned observations led to the suggestion that the synthetic lethality may not be linked to the PKC pathway. To precisely analyse the involvement of PKC pathway activity in the rgd1Δwsc1Δ interaction, we investigated the suppressor effect of genes encoding components of this pathway. We examined whether a single-copy plasmid containing a BCK1-20 activated allele, or multicopy plasmids containing RHO1 or MKK1 (de Bettignies et al., 2001), could suppress the synthetic lethality. The original synthetic lethal strain SLRGD1-1, which already contains a URA3 centromeric plasmid with the RGD1 gene (de Bettignies et al., 1999), was transformed with the different plasmids. The transformants were tested for growth on control and 5-fluoroorotic acid (5-FOA) plates to examine their ability to lose the URA3/RGD1 plasmid. An empty plasmid, and a high-copy plasmid containing RGD1 (de Bettignies et al., 2001), were used as negative and positive controls, respectively. We observed that overexpression of RHO1, BCK1-20 or MKK1 genes did not rescue the growth defect associated with the synthetic lethality (Fig. 5A). This result indicates that overactivation of the PKC–MAP kinase cascade is not sufficient to suppress cellular defects following inactivation of RGD1 and WSC1 genes.

In order to gain additional evidence, we examined the interaction between the rgd1Δ mutation, and BCK1 and
Fig. 4. The PKC–MAP kinase pathway is constitutively activated in rho3Δwsc1Δrgd1Δ and rho4Δwsc1Δrgd1Δ mutants. (A) Detection of Slt2 MAP kinase phosphorylation in WT, rgd1Δ, wsc1Δ and two triple-mutant strains grown in complete medium at 30 °C. Slt2p activation was detected with α-p44/42 antibodies. Total Slt2 protein was visualized with polyclonal α-Slt2 antibodies. A cross-reacting protein was used as a loading control. Results similar to those in (A) were obtained with cells grown in minimal medium at 30 °C. (B) Detection of Slt2p activation following heat shock in WT and mutant strains. The strains were grown in minimal medium at 21 °C to mid-exponential phase, and then half of the cells was quickly transferred to 39 °C for 2 h (with heat shock), and the other half was kept at 21 °C (without heat shock). Phosphorylated and total Slt2 proteins were detected as described for (A). The increase in phosphorylated and total Slt2p upon heat shock was quantified by band densitometry. The fold-increase results are presented as ratios ‘with heat shock’/‘without heat shock’.
SLT2, which are two genes encoding the first and last kinases of the PKC–MAP kinase module, respectively. To perform this analysis, **rgd1Δwsc1Δ** was crossed to a bck1Δ strain, and to a slt2Δ strain, the diploid cells were allowed to sporulate, and the tetrads were dissected on complete medium containing 1 M sorbitol. As a control, the **rgd1Δ** and **wsc1Δ** strains were also crossed. Growth of double-mutant strains was then analysed on complete medium, either with or without an osmotic stabilizer (Fig. 5B). As expected, the **rgd1Δwsc1Δ** double-mutant strain was non-viable on medium lacking sorbitol. Importantly, we observed that the **rgd1Δbck1Δ** and **rgd1Δslt2Δ** double mutants did not exhibit any growth defect on medium lacking sorbitol, indicating the absence of synthetic lethality between **RGD1** and **BCK1**, and between **RGD1** and **SLT2**. Hence, these results are consistent with the idea that a defect in PKC pathway activity is not responsible for the **rgd1Δwsc1Δ** synthetic lethal interaction.

**Mutations in RHO3 specifically impair the abundance of Wsc1 and Mid2 proteins**

The above results suggest that a cellular process distinct from cell integrity signalling is perturbed when both Rgd1p and Wsc1p are absent. Rgd1p acts as a negative regulator of Rho3 and Rho4, which are GTPases that interact with the exocytic apparatus in yeast. It has been demonstrated that activated Rho3p interacts with the exocyst complex, and is important for regulating exocytosis at sites of polarized growth (Robinson et al., 1999; Roumanie et al., 2005). Wsc1 protein is known to be involved not only in sensing cell wall stress, and in activating the PKC pathway, but also in regulating cellular processes connected to secretion. In particular, it has been reported that **WSC1** is a multicopy suppressor of the **sly1** mutant defective for endoplasmic-reticulum–Golgi transport (Kosodo et al., 2001). Moreover, signals from the secretory pathway caused by perturbation in intracellular trafficking have been shown to be transduced by the Wsc family of proteins. It has been observed that Wsc proteins need to be in intracellular compartments along the secretory pathway to induce the arrest of secretion response (ASR) through activation of Pkc1p (Nanduri & Tartakoff, 2001). Interestingly, the Bck1/Mkk1,2/Slt2 MAP kinase cascade activated by Pkc1p does not seem to be required for the ASR, suggesting the existence of a new branch of Pkc1p signalling (Ng, 2001). We decided to explore whether alterations in Rho3-dependent cellular trafficking induced defects in Wsc1 protein abundance. We first made use of the **rho3** mutant (Fig. 6A). Examination of the growth phenotype of the **rgd1Δbck1Δ** and **rgd1Δslt2Δ** double mutants. **rgd1Δ** was crossed to the **bck1Δ** strain, and to the **slt2Δ** strain, and tetrads were dissected on complete medium containing 1 M sorbitol. The **rgd1Δ** and **wsc1Δ** mutations were also combined as a control. Growth of control and double-mutant strains was monitored on complete medium, either with or without 1 M sorbitol.

**Fig. 5.** The PKC–MAP kinase pathway is not involved in the **rgd1Δwsc1Δ** synthetic lethality. (A) Tests of suppression of **rgd1Δwsc1Δ** synthetic lethality by components of the PKC pathway. The **rgd1Δwsc1Δ** synthetic lethal strain containing a low-copy (CEN) **URA3/RGD1** vector was transformed with an empty control vector, a **CEN/TRP1** vector containing the activated **BCK1-20** allele, and a multicopy (2 μ) **LEU2** vector containing **RGD1**, **RHO1** or **MKK1**. Plasmid-shuffle complementation assays were then performed on control **SD** and selective 5-FOA plates. (B) Analysis of the growth phenotype of the **rgd1Δbck1Δ** and **rgd1Δslt2Δ** double mutants. **rgd1Δ** was crossed to the **bck1Δ** strain, and to the **slt2Δ** strain, and tetrads were dissected on complete medium containing 1 M sorbitol. The **rgd1Δ** and **wsc1Δ** mutations were also combined as a control. Growth of control and double-mutant strains was monitored on complete medium, either with or without 1 M sorbitol.
again, no alteration in Ure2p or Fur4p abundance was detected (Fig. 6B). These results point towards a specific defect in the abundance of Wsc1 and Mid2 proteins in the rho3Δ mutant. To investigate stress sensor functionality in the rho3Δ mutant, the effect of the cell wall interfering compound Congo Red was tested on growth; we observed that both the wsc1Δ and rho3Δ strains were unable to grow in the presence of the compound (Fig. 6C). As previously reported, the mid2Δ mutant and, to a lower extent, the rglΔ mutant were resistant, and grew better than the WT and rho4Δ strains on medium containing Congo Red (de Bettignies et al., 1999). These data suggest that the functionality of Wsc1p is impaired in rho3Δ. It has been reported that MID2 is a low-copy suppressor of the rglΔwsc1Δ synthetic lethal interaction, and that the mid2Δwsc1Δ combination is lethal (de Bettignies et al., 1999). Since we observed that the rglΔ mutation does not affect Mid2p abundance, our results lend support to the idea that rglΔwsc1Δ co-lethality is not a consequence of a decrease in the abundance of Mid2p following inactivation of RGD1.

Next, we looked at the behaviour of Wsc1 and Mid2 proteins in the rho3-V51 mutant. The cold-sensitive rho3-V51 allele has a specific secretion defect that does not involve, in contrast to the rho3Δ mutation, detectable effects on the polarized localization of the exocytic machinery or the actin cytoskeleton (Roumanie et al., 2005). Secretion of the periplasmic enzymes invertase and Bgl2 has been shown to be perturbed at both permissive (25 °C) and non-permissive (14 °C) temperatures, and the rho3-V51 cells accumulate

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**Fig. 6.** Abundance of the Wsc1 and Mid2 proteins is strongly reduced in rho3Δ cells. (A) Analysis of Wsc1p abundance in WT, rglΔ, rho4Δ and rho3Δ mutant strains containing the YEp352-WSC13HA plasmid. An empty vector was used as a control. The strains were grown to early exponential phase in synthetic medium at 30 °C, and total proteins were extracted and subjected to SDS-PAGE, and Wsc1-3HA protein was detected by immunoblot analysis using 12CA5 antibodies. The control Ure2 and Fur4 proteins were detected using specific polyclonal antibodies. The positions of molecular mass markers (kDa) are indicated on the left. (B) Analysis of Mid2p abundance in WT, rglΔ, rho4Δ and rho3Δ mutant strains containing the YEp352-MID23HA plasmid. Experiments were performed as described for (A). (C) Analysis of the growth phenotype of the rho3Δ mutant in the presence of the cell-wall-interfering compound Congo Red. WT and mutant strains were grown in complete medium, and tenfold serial dilutions were spotted onto complete medium (YPD), and YPD containing 3 mg Congo Red ml⁻¹. Plates were incubated for 2–3 days at 30 °C.
post-Golgi secretion vesicles in the cytosol (Adamo et al., 1999). Thus, we conducted experiments to follow HA-tagged Wsc1p and Mid2p in rho3-V51 cells and isogenic WT cells at 25 and 14 °C; as shown in Fig. 7(A), we observed a decrease in the abundance of fully O-glycosylated Wsc1p at both temperatures in the rho3-V51 mutant compared with the WT. Similar results were obtained with Mid2 protein (Fig. 7B); the amount of the fully mature form of Mid2p was reduced, and we observed the appearance of a 150 kDa form of the protein in the rho3-V51 mutant at both 25 and 14 °C (Fig. 7B). Thus, a specific block in trafficking between the Golgi apparatus and the plasma membrane led to a negative effect on both Wsc1 and Mid2 proteins. We also examined growth of the rho3-V51 mutant on complete medium containing Congo Red. The rho3-V51 secretion mutant was found to be more sensitive to Congo Red when compared with the WT (Fig. 7C). As the strain background used for studying rho3-V51 has a higher sensitivity to Congo Red than other strains used in this study, we also grew these strains on medium containing 1 M sorbitol as an osmotic stabilizer. Although the addition of sorbitol helped the growth of the WT strain in the presence of Congo Red, rho3-V51 did not show a comparable increase under the same conditions (Fig. 7C). Thus, like rho3A, rho3-V51 is hypersensitive to perturbation of the cell wall, suggesting that cell wall sensors, and in particular Wsc1p, are not functional.

Finally, these results demonstrate that loss of active Rho3p affects Wsc1p and Mid2p behaviour, strongly suggesting that perturbations in polarized exocytosis influence the processing or synthesis of the two proteins. Interestingly,

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**Fig. 7.** The Wsc1 and Mid2 proteins are specifically impaired in the rho3-V51 secretion mutant. (A) Analysis of Wsc1p abundance and maturation in isogenic WT and rho3-V51 strains containing the YEp352-WSC1 3HA plasmid, at 25 and 14 °C. Cells were grown to early exponential phase in synthetic medium, and either shifted to 14 °C for 5 h, or kept at 25 °C. Cells were then processed as described in Fig. 6(A) to detect the Wsc1-3HA protein. An empty vector was used as a control. (B) Analysis of Mid2p abundance and maturation in WT and rho3-V51 strains containing the YEp352-MID2 3HA plasmid at 25 and 14 °C. Experiments were performed as described in (A). (C) Analysis of the growth phenotype of the rho3-V51 mutant in the presence of the cell-wall-interfering compound Congo Red. Isogenic WT and mutant strains were grown in complete medium at 25 °C, and tenfold serial dilutions were spotted onto YPD, and YPD containing 3 mg Congo Red ml⁻¹. Strains were also grown on control and selective media containing 1 M sorbitol. Plates were incubated for 2–3 days at 25 °C.
similar experiments performed in cells overproducing constitutively active Rho3p showed no alteration in Wsc1 and Mid2 protein abundance (data not shown). This might imply that only perturbation of polarized exocytosis due to loss of Rho3p, and not overactivation of exocytosis by Rho3p, has a negative effect on Wsc1 and Mid2 proteins in the cell.

**DISCUSSION**

We have previously reported the existence of synthetic lethality between the *RGDI* and *WSC1* genes in the yeast *S. cerevisiae*. Rgd1 protein is a GAP factor that negatively regulates Rho3p and Rho4p, which are two small GTPases dedicated to the maintenance of cell polarity. Wsc1p and Mid2p are the two major cell surface sensors that activate the PKC–MAP kinase pathway under stress conditions. If one of the two cell surface proteins is absent, the other becomes essential in the response to stress (de Bettignies et al., 1999; Ketela et al., 1999; Rajavel et al., 1999). MID2 overexpression is known to suppress the *rgd1Δwsc1Δ* synthetic lethality, and co-deletion of *RGDI* and *MID2* leads to conditional lethality in the late-exponential growth phase (de Bettignies et al., 1999). Upon analysing the interaction between *RGDI* and *WSC1*, we found that unregulated accumulation of Rho3 and Rho4 proteins was lethal in *wsc1Δ* cells. However, accumulation of activated Rho3p and Rho4p had no effect in the *mid2Δ* genetic background. Thus, Rho3 and Rho4 GTPases act differently on the partially redundant Wsc1 and Mid2 cell wall sensors. This is consistent with previous reports that, despite sharing a function in cell wall sensing, Wsc1p and Mid2p have distinct roles in yeast (Sekiya-Kawasaki et al., 2002; Green et al., 2003). The interaction between *MID2* and *RGDI* must involve an Rgd1p function that is yet to be defined.

We also show that the PKC–MAP kinase pathway is not implicated in the *rgd1Δwsc1Δ* synthetic lethality, and hence the lethality must involve a function of Wsc1p that is different from its role as a cell wall stress sensor. Along the secretory route, distinct signalling pathways monitor ‘traffic jams’ and misfolding of proteins to slow down exocytosis and protein synthesis (Ng, 2001). In particular, it has been shown that the Wsc family of proteins is involved in inter-organellar signal transduction in response to perturbations in exocytosis. The ASR utilizes Wsc proteins trapped along the secretory pathway to initiate relocation of proteins, and then transcriptional changes (Nanduri & Tartakoff, 2001). The Bck1/Mkk1,2/Slt2 MAP kinase cascade activated by Pkc1p is not required for the ASR, suggesting the existence of another branch of Pkc1p signalling (Ng, 2001). We report that disturbances in Rho3p-mediated polarized exocytosis specifically decrease the abundance, and impair the processing, of Wsc1p and Mid2p, although other proteins, such as the plasma membrane protein Fur4, were found to be unaltered. An important difference between these proteins is that Wsc1p and Mid2p are highly glycosylated, whereas the Fur4 uracil permease is not (Silve et al., 1991; Lodder et al., 1999; Philip & Levin, 2001). It has been proposed that O-mannosylation increases the activity of Wsc1p and Mid2p by enhancing their stability (Lommel et al., 2004). Moreover, the defects observed for Wsc1 and Mid2 proteins in the *rho3Δ* mutant are similar to those reported in the protein O-mannosyltransferase *pmt* mutants (Lommel et al., 2004). Based on these results, it is likely that blocking the Rho3-dependent polarized exocytosis impairs Wsc1p and Mid2p glycosylation, thus affecting their stability and function. As a consequence, the Wsc1 protein can no longer function in signal transduction in *rho3* mutants. These data support the idea that Wsc1p, which is normally present on secretory vesicles, or in the plasma membrane, can no longer function when Rho3p-regulated polarized exocytosis is impaired. Together, our data show the existence of a specific functional relationship between Wsc1p and Rho3/4 GTPases that could be linked to the regulation of polarized exocytosis.

Based on our results, the constitutive activation of the PKC pathway observed when both *RGDI* and *WSC1* are inactivated would be an indirect effect of perturbations in Rho3/4p-regulated polarized exocytosis. In agreement with this analysis, we found that phosphorylated and total Slt2p were increased in the *rho3-V51* late-secretion mutant compared with WT cells (see Supplementary Fig. S1). Moreover, we observed that the PKC pathway was specifically activated upon production of GTP-bound Rho3p or Rho4p in WT cells. A strong cumulative effect was obtained when activated Rho3 and Rho4 GTPases were co-produced. No activation was observed when GDP-bound or WT forms of Rho3p and Rho4p were used (see Supplementary Fig. S1). Thus, either blocking or overactivating Rho3p-regulated secretion leads to an increase in the PKC pathway activity. Results obtained from *rho3Δ* and *rho4Δ* showed that Slt2p was not phosphorylated in these mutants, strongly suggesting that the activation is dependent on the presence of GTP-bound Rho3p and Rho4p in the cell. Interestingly, constitutive activation of the cell integrity pathway has also been reported for the O-mannosyltransferase *pmt2Δpmt4Δ* mutant, which is deficient in Wsc1p and Mid2p post-translational processing (Lommel et al., 2004). Taken together, these results demonstrate that the PKC pathway can be activated as a consequence of unbalanced polarized exocytosis, possibly through a decrease in active Wsc1 along the secretory pathway.

Overall, our analyses show that the genetic interaction observed between *RGDI* and *WSC1* may be the consequence of cumulative defects on polarized secretion. One hypothesis is that both Rgd1p and Wsc1p are involved in the pathway down-regulating polarized exocytosis, which is dependent on Rho3 and Rho4 GTPases; the absence of one of the two proteins is partly compensated by the presence of the other under standard growth conditions. Nevertheless, when the mutant cells are subjected to additional stresses, such as unbalanced exocytosis, diauxic shift or high temperatures, down-regulation of polarized exocytosis is not sufficient, and the consequence is cell death. Future work...
will help to elucidate the precise mechanism involving both Rgd1p and Wsc1p in the regulation of Rho3/4p-dependent polarized secretion.

ACKNOWLEDGEMENTS

We thank A. Claveres and M. F. Peypouquet for technical assistance. We are grateful to Dr P. Brennwald, University of North Carolina at Chapel Hill, USA, for providing the rho3-v51 and isogenic control strains, Dr D. Levin, Johns Hopkins Bloomberg School of Public Health, USA, for providing YEp352-Wsc13HA and YEp352-Mid23HA plasmids, and Dr A. Gangar for critical reading of the manuscript. The anti-GST-Sh2p antibodies were a kind gift from Drs H. Martin and M. Molina. The antibodies against Ure2p and Fur4p were provided by Dr C. Cullin, IBGC-Université Bordeaux 2, France, and Dr R. Haguenauser-Tsapis, Institut Jacques Monod, France, respectively. This work was supported by grants from the Université Victor Segalen Bordeaux 2 and the CNRS.

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