Overactivation of the Protein Kinase C-Signaling Pathway Suppresses the Defects of Cells Lacking the Rho3/Rho4-GAP Rgd1p in *Saccharomyces cerevisiae*

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ABSTRACT

The nonessential *RGD1* gene encodes a Rho-GTPase activating protein for the Rho3 and Rho4 proteins in *Saccharomyces cerevisiae*. Previous studies have revealed genetic interactions between *RGD1* and the *SLG1* and *MID2* genes, encoding two putative sensors for cell integrity signaling, and *VRP1* encoding an actin and myosin interacting protein involved in polarized growth. To better understand the role of Rgd1p, we isolated multicopy suppressor genes of the cell lethality of the double mutant *rgd1*Δ *mid2*Δ. *RHO1* and *RHO2* encoding two small GTPases, *MKK1* encoding one of the MAP-kinase kinases in the protein kinase C (PKC) pathway, and *MTL1*, a *MID2*-homolog, were shown to suppress the *rgd1*Δ defects strengthening the functional links between *RGD1* and the cell integrity pathway. Study of the transcriptional activity of Rlm1p, which is under the control of Mpk1p, the last kinase of the PKC pathway, and follow-up of the *PST1* transcription, which is positively regulated by Rlm1p, indicate that the lack of *RGD1* function diminishes the PKC pathway activity. We hypothesize that the *rgd1*Δ inactivation, at least through the hyperactivation of the small GTPases Rho3p and Rho4p, alters the secretory pathway and/or the actin cytoskeleton and decreases activity of the PKC pathway.

IN *Saccharomyces cerevisiae*, the Rho family of GTPases is thought to have a central role in the polarized growth process (Drubin and Nelson 1996; Pruyne and Bretscher 2000). The main functions assigned to these GTPases involve bud formation and cell surface growth, which might occur through the involvement of the actin cytoskeleton and the secretory pathway (Imai *et al.* 1996; Tanaka and Takai 1998). Although six open reading frames (ORFs) could encode Rho-GTPases in yeast (Garcia-Ranea and Valencia 1998), genetic and functional analyses have allowed the identification of five Rho members: Cdc42 and Rho1 to Rho4. These small GTPases function as binary switches, which are turned on and off by binding to GTP or GDP, respectively. The GTP-bound form interacts with its specific target and performs its cell functions (Tanaka and Takai 1998). Small GTPases are regulated by GAPs (GTPase-activating proteins), GEFs (GDP-GTP exchange factors), and a GDP dissociation inhibitor.

During the sequencing of the genome of *S. cerevisiae*, we identified a new gene encoding a protein with a Rho-GAP homology domain (Doignon *et al.* 1993). This protein, called Rgd1p (for related GAP domain), was shown *in vitro* to be a GTPase activating protein for the Rho3 and Rho4 proteins (Doignon *et al.* 1999). Thus, in activating the hydrolysis of GTP, Rgd1p negatively regulates the action of these two Rho proteins. Rho3p and Rho4p play a role in bud formation and have some partially overlapping functions (Matsui and Toh-e 1992a). Deletion of *RHO4* did not affect cell growth, whereas deletion of *RHO3* caused a severe growth delay and a decrease in cell viability. Overexpression of *RHO4* suppressed the growth defect in *rho3* cells. Depletion of both *RHO3* and *RHO4* gene products resulted in lysis of cells with a small bud, which could be prevented by the presence of osmotic stabilizer in the medium (Matsui and Toh-e 1992b). In this latter condition, Rho3p- and Rho4p-depleted cells lose cell polarity as revealed by chitin delocalization and by random distribution of actin patches.

Analysis of *rho3* suppressors has revealed genetic links with some regulatory elements of the actin cytoskeleton such as *CDC42* and *BEM1* (Matsui and Toh-e 1992b). We have previously shown relationships between *RGD1* and the actin cytoskeleton-linked genes such as *VRP1*, *LAS17*, and *MYO1*; the combinations *rgd1*Δ *vrp1*Δ, *rgd1*Δ *las17*Δ, and *rgd1*Δ *myo1*Δ are synthetic lethal (Roumanie *et al.* 2000). Moreover, when the *VRP1* product is absent, the production of GTP-constitutive forms of Rho3p and Rho4p is detrimental to yeast cells in agreement with an *in vivo* GAP activity of Rgd1p on both GTPases (Roumanie *et al.* 2000). Nevertheless, *RGD1* inactivation does not lead in itself to a defect in actin organization or in budding pattern, at least under standard growth condi-

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tions. *RHO3* also displays genetic interactions with the *SEC4* gene whose product is involved in exocytosis (Imai *et al.* 1996). Moreover, a physical interaction of Rho3p with Exo70p, a component of the exocyst complex, and with Myo2p, the myosin responsible for secretory vesicle movement, has been reported (Robinson *et al.* 1999). Thus, Rho3p regulates cell polarity by simultaneously directing the rearrangements of the actin cytoskeleton and the polarized delivery and fusion of secretory vesicles to specific sites on the cell surface (Adamo *et al.* 1999).

We also reported genetic interactions between *RGD1* and the *SLG1* and *MID2* genes (de Bettignies *et al.* 1999). *SLG1* has also been designated *HCS77* (Gray *et al.* 1997) and *WSC1* (Verna *et al.* 1997), but for simplicity this gene is referred to here as *SLG1*. Slg1p and Mid2p are both plasma membrane proteins with partial overlapping functions (Ketela *et al.* 1999). They act upstream of the protein kinase C (PKC) pathway and are thought to monitor the state of the cell surface and relay the information to Pkc1p (Gray *et al.* 1997; Verna *et al.* 1997; Jacoby *et al.* 1998; de Bettignies *et al.* 1999). The protein kinase C is mostly regulated by the small GTPase Rho1p *in vivo* (Nonaka *et al.* 1995; Kamada *et al.* 1996). The Pkc1p activates a mitogen-activated protein (MAP) kinase cascade, named the PKC pathway, consisting of Bck1p (Costigan *et al.* 1992; Lee and Levin 1992), Mkk1p/Mkk2p (Irie *et al.* 1993), and the MAP kinase Mpk1p (Torres *et al.* 1991; Lee *et al.* 1993). Activation of this pathway is particularly important in response to various external stresses, including high temperature, low osmolarity, and cell wall disruption, as well as being important during mating (Heinisch *et al.* 1999). The protein Slg1 is linked to the PKC pathway by the finding that this MAP kinase cascade is activated by heat stress via Slg1p (Gray *et al.* 1997). A direct interaction of Slg1p with Rom2p, one of the Rho1p-GEFs, has been recently reported and this interaction is responsible for the activation of the PKC pathway through Rho1p (Philip and Levin 2001).

The loss of *RGD1* function amplifies the phenotype due to the *SLG1* deletion and the small-budded double-mutant cells die because of defects in cell wall structure and lysis upon bud growth. In parallel, the inactivation of *MID2*, the other putative sensor for cell integrity signaling in *S. cerevisiae* (Rajavel *et al.* 1999), exacerbates the specific phenotype of the *rgd1*Δ mutant with an increase in dead cells at late exponential phase in minimal medium (de Bettignies *et al.* 1999). Taken together, our results suggest that Rgd1p has a regulatory role in connection with both the PKC pathway and the actin cytoskeleton organization in *S. cerevisiae*.

To further elucidate the function of *RGD1*, we isolated multicopy suppressors of the viability defect of the *rgd1*Δ mutation in minimal medium. Phenotypic and genetic analysis has allowed the identification of several multicopy as well as monocopy suppressors of *rgd1*Δ: the *RHO1* and *RHO2* genes encoding two GTPases (Madaule *et al.* 1987) involved in actin cytoskeleton organization (Yamochi *et al.* 1994; Kohno *et al.* 1996), the *MID2*-homolog *MTL1* (Ketela *et al.* 1999; Rajavel *et al.* 1999), and the *MKK1* gene coding for one of the MAP-kinase kinases of the PKC pathway, (Irie *et al.* 1993). Considering the suppressor effect of additional PKC pathway components, we show that activation of the PKC pathway prevents lethality of *rgd1*Δ cells. Analysis of the transcriptional activity of Rlm1p, one of the targets of the last kinase in the PKC pathway, and study of the *PST1* transcription, which is positively regulated by Rlm1p, showed that the *rgd1*Δ mutation decreases the activity of this MAP-kinase pathway in minimal medium at late exponential phase. This decrease in PKC pathway activity is at least partly responsible for the *rgd1*Δ cell viability loss under particular growth or physiological conditions.

MATERIALS AND METHODS

Strains, media, and transformations: The *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) was used for cloning and propagation of all plasmids. *E. coli* cells were cultured in DYT medium (Bacto tryptone, Bacto yeast extract, NaCl) and transformed by standard $CaCl_2$ method (Sambrook *et al.* 1989). The *S. cerevisiae* experiments were performed mainly in X2180 genetic background and the yeast strains used are listed in Table 1. Yeast cells were grown under standard conditions either in YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) or in synthetic minimal YNB (0.67% YNB without amino acid, 2% glucose) supplemented with the appropriate nutrients. Solid media contained an additional 2% agar. Caffeine growth inhibition was assayed at 3 mg/ml; when indicated, sorbitol was added to a final molarity of 1 M. The YNB inositol-3X medium contains a threefold inositol concentration (6 mg/liter instead of 2 mg/liter). Unless otherwise stated, the growth temperature used was 30°. Yeast transformations were carried out by the lithium acetate method (Agatep *et al.* 1998).

DNA manipulations, plasmids, and yeast genomic DNA library: Standard procedures were used for DNA manipulations (Sambrook *et al.* 1989). DNA sequencing was performed using ALF DNA sequencer (Amersham Pharmacia Biotech).

All the plasmids used in this study are listed in Table 2. The high-copy vector pRS425 and low-copy vector pRS415 carrying *RGD1* and *MID2* were already described (de Bettignies *et al.* 1999). The *RHO1* and *RHO2* genes were subcloned from the library YEp13 plasmids as 2.7-kb and 4.9-kb *Hin*dIII-*Pvu*II DNA fragments, respectively, and inserted between the *Hin*dIII and *Sma*I sites of the centromeric plasmid pRS315. In a similar way, the 2.9-kb *Sal*I-*Hin*dIII fragment carrying the *MTL1* gene was inserted into the corresponding sites of pRS315. The *MKK1* gene was amplified by PCR and cloned between the *Xho*I and *Pst*I sites in pRS315. The high-copy plasmid YEp352 and low-copy plasmid YCp50 containing the *URA3* marker and bearing *PKC1* were kindly provided by D. Levin as was the *BCK1* gene and its hyperactive allele *BCK1-20* in pRS314. DNA fragments bearing the alleles of *BCK1* were removed from the plasmid using *Sac*I and *Xho*I restriction enzymes and cloned in the corresponding sites of pRS426 and pRS316 plasmids, which contain the *URA3* marker. The low-copy plasmid pRS316 containing the *MPK1* gene was a gift from C. Mann. The high-dosage *MPK1* was obtained by removing a *Kpn*I-*Spe*I DNA

TABLE 1

***S. cerevisiae* strains**

Strains	Genotypes	Source
X2180-1A	*MAT***a** *SUC2 mal mel gal2 CUP1*	Y.G.S.C.
LBG37-10C	*MAT***a** *ura3-52*	This work
LBG2-4C	*MAT***a** *leu2*	This work
LBG37-5C	*MAT***a** *leu2 ura3-52*	This work
LBG4-3D	*MAT***a** *his3-11,15 rgd1::HIS3*	de Bettignies *et al.* (1999)
LBG36-8A	*MAT***a** *trp1*Δ*63 mid2::TRP1*	de Bettignies *et al.* (1999)
LBG59-15A	*MAT***a** *trp1*Δ*63 his3-11,15 mid2::TRP1 rgd1::HIS3*	de Bettignies *et al.* (1999)
LBG40-3C	*MAT***a** *leu2 his3-11,15 rgd1::HIS3*	This work
LBG42-2D	*MAT***a** *leu2 trp1*Δ*63 mid2::TRP1*	This work
LBD-A7	*MAT***a** *leu2 trp1*Δ*63 his3-11,15 mid2::TRP1 rgd1::HIS3*	Our laboratory
LBG44-6B	*MAT***a** *ura3-52 his3-11,15 rgd1::HIS3*	This work
LBG92-4B	*MAT***a** *leu2 ura3-52 his3-11,15 rgd1::HIS3*	This work
LBG93-5A	*MAT***a** *leu2 ura3-52 trp1*Δ*63 mid2::TRP1*	This work
LBG94-3C	*MAT***a** *leu2 ura3-52 trp1*Δ*63 his3-11,15 mid2::TRP1 rgd1::HIS3*	This work
FY1679	*MAT***a***/MAT*α *ura3-52/ura3-52 leu2*Δ*1/LEU2 trp1*Δ*63/ TRP1 his3*Δ*200/HIS3 GAL2/GAL2*	Winston *et al.* (1995)
LBOR100	*MAT***a***/ MAT*α *ura3-52/ura3-52 his3*Δ*200/HIS3 rgd1::kanMX4/ rgd1::kanMX4 GAL2/GAL2*	Our laboratory

fragment from this plasmid and inserting it into the corresponding sites of pRS426.

The genomic library was constructed by B. Daignan-Fornier (Daignan-Fornier *et al.* 1994) by digesting yeast DNA partially with *Sau*3A and cloning fragments ranging from 2 to 8 kb into the *Bam*HI site of the YEp13 shuttle vector. This vector, which contains the *S. cerevisiae LEU2* gene as a selection marker, is present at a high copy number in transformed cells (Broach *et al.* 1979).

Screening for multicopy suppressors: The *rgd1*Δ *mid2*Δ *S. cerevisiae* strain LBD-A7 was transformed with the YEp13 genomic DNA library and transformants were grown on YNB solid medium for 7 days. Two milliliters of a liquid methylene blue staining solution (methylene blue 0.1 g/liter; trisodium citrate dihydrate 20 g/liter) was poured gently onto the plate surface. After an overnight storage at 4°, the transformants forming white or paler colonies than *rgd1*Δ *mid2*Δ were selected, and cells picked up from the top of the colony were streaked on YNB to get rid of cross-contamination of the colonies. After this primary screening, the selected transformants were cultured in liquid YNB inositol-3X medium, which gives a *rgd1*Δ *mid2*Δ cell lethality near 100% as determined using methylene blue staining (see Figure 1). The growth and the rate of dead cells were monitored after 45 and 65 hr of culture and transformants displaying a reduced lethality rate were further analyzed. Library plasmid DNA was extracted from these transformants using the Robzyk and Kassir (1992) procedure and transferred into XL1-Blue. Plasmid DNA was then submitted to restriction analysis. To confirm the plasmid dependency of the multicopy suppressor effect, the YEp13-based plasmids were reintroduced into the original double-mutant strain, and lethality rate was determined again by methylene blue staining after 45 and 65 hr of growth. Both ends of the inserts carried by the plasmids that still led to a reduced lethality of the double-mutant strain were sequenced.

Determination of cell lethality: Two different methods were used to determine lethality rate. First we used microscopic examination after staining with methylene blue as described previously (Rose 1975; de Bettignies *et al.* 1999). The lethality rate was then calculated from the counting of at least 400 cells. We also used staining with propidium iodide and analysis by flow cytometry (Deere *et al.* 1998). The FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and the Cellquest Software were used to determine the lethality rate from counting 10,000 events. As a gate was used to discard cell aggregates from analysis, the values determined were slightly lower using flow cytometry than methylene blue staining. The method used was indicated in each experiment.

Detection of the MID phenotype: The mating pheromone induced death (MID) phenotype was revealed, as previously described (Iida *et al.* 1994; de Bettignies *et al.* 1999). Briefly, the lethality rate of shmoos of *MAT***a** cells was measured 5 and 7 hr after exposure to 6 μM α-factor.

Rlm1p transcriptional activity: The plasmids YS116, pBTM116, and pYW71 were given by K. Matsumuto (Watanabe *et al.* 1997). The plasmid YS116 is a YEp-based *URA3* plasmid harboring the *lacZ* reporter gene containing LexA DNA-binding sites in its promoter. The yeast shuttle vector pBTM116 produces the LexA DNA-binding domain alone and the plasmid pYW71 the fusion protein LexA-Rlm1ΔN in which the MADS box DNA-binding domain of Rlm1p has been replaced with the DNA-binding domain of LexA. For convenience, the *TRP1* marker harbored by these plasmids was removed using the *Xba*I and *Nae*I unique sites and replaced by the *Sma*I-*Nhe*I fragment of the YDp-L plasmid carrying *LEU2* (Berben *et al.* 1991) to give pBLM116 and pYL71 plasmids, respectively (Table 2). The transactivation activity of LexA-Rlm1ΔN was measured by using the *lacZ* reporter gene carried by the plasmid pYS116. β-Galactosidase assays were performed as described previously (Kaiser *et al.* 1994). The same cell amount corresponding to an OD_{600} equivalent to a 0.3 unit was used for each assay, which was performed in triplicate. Activities were calculated according to the adapted Miller formula.

Northern blot analysis: Cells cultivated in YNB were collected and washed in 0.9% NaCl before freezing in dry ice. Total RNAs were extracted from 2×10^8 cells as described previously (Aves *et al.* 1985). Five-microgram samples of total RNAs, denatured with glyoxal, were separated by agarose gel electrophoresis and transferred to a GeneScreen nylon membrane (Dupont, Wilmington, DE; New England Nuclear, Boston) as described previously (White *et al.* 1986). *PST1* and

TABLE 2

***S. cerevisiae* plasmids**

Names	Yeast markers	Source
YEp13	*LEU2 2μ*	
YEp13-*RGD1*	*LEU2 2μ RGD1*	This work
YEp13-*MID2*	*LEU2 2μ MID2*	This work
YEp13-*RHO1*	*LEU2 2μ RHO1*	This work
YEp13-*RHO2*	*LEU2 2μ RHO2*	This work
YEp13-*MKK1*	*LEU2 2μ MKK1*	This work
YEp13-*MTL1*	*LEU2 2μ MTL1*	This work
pRS315	*LEU2 CEN6 ARSH4*	
pRS415-*RGD1*	*LEU2 CEN6 ARSH4 RGD1*	de Bettignies *et al.* (1999)
pRS415-*MID2*	*LEU2 CEN6 ARSH4 MID2*	de Bettignies *et al.* (1999)
pRS315-*RHO1*	*LEU2 CEN6 ARSH4 RHO1*	This work
pRS315-*RHO2*	*LEU2 CEN6 ARSH4 RHO2*	This work
pRS315-*MKK1*	*LEU2 CEN6 ARSH4 MKKI*	This work
pRS315-*MTL1*	*LEU2 CEN6 ARSH4 MTL1*	This work
YCp50	*URA3 CEN4 ARS1*	
YCp50-*PKC1*	*URA3 CEN4 ARS1 PKC1*	D. Levin
YEp352	*URA3 2μ*	
YEp352-*PKC1*	*URA3 2μ PKC1*	D. Levin
pRS316	*URA3 CEN6 ARSH4*	
pRS316-*BCK1*	*URA3 CEN6 ARSH4 BCKI*	This work
pRS316-*BCK1-20*	*URA3 CEN6 ARSH4 BCK1-20*	This work
pRS316-*MPK1*	*URA3 CEN6 ARSH4 MPK1*	C. Mann
pRS426	*URA3 2μ*	
pRS426-*BCK1*	*URA3 2μ BCK1*	This work
pRS426-*BCK1-20*	*URA3 2μ BCK1-20*	This work
pRS426-*MPK1*	*URA3 2μ MPK1*	This work
pYS116	*URA3 2μ (LexAop-CYC1)-LacZ*	K. Matsumoto
pBLM116	*LEU2 2μ LexA*	This work
pYL71	*LEU2 2μ LexA-RLM1ΔN*	This work

RPB4 DNA fragments were obtained by PCR using the primers 5′-TGTTGAATGATTGGGCTGGG-3′ and 5′-AAGAAG CAACAACAAGGAGG-3′ for *PST1* and 5′-GAATGTTTCTAC ATCAACC-3′ and 5′-GAGTGTTTCTAGGTTTGAC-3′ for *RPB4*. Probes were labeled with [α-^{32}P]dCTP (Amersham, Buckinghamshire, UK) using a random priming kit (Promega, Madison, WI). After hybridization, blots were washed according to the GeneScreen recommendations and quantification was achieved using a Phosphor-Imager (Storm 860, Molecular Dynamics, Sunnyvale, CA). The *RBP4* gene of which the product is present during all growth phases (Rosenheck and Choder 1998) was used as an internal standard; *PST1* mRNA levels were normalized to *RPB4*, using the first point as an arbitrary unit of 1.

RESULTS

Screening and characterization of *rgd1*Δ *mid2*Δ multicopy suppressor genes: Loss of the Rho-GAP encoding *RGD1* function results only in a slight cell mortality in YNB medium, making genetic screens difficult. The *rgd1*Δ mutant presents an ~15% lethality rate beginning at the late exponential phase, the dead cells being mainly small budded (Barthe *et al.* 1998). We noted an enhancement of the *rgd1*Δ-specific viability loss at the late exponential phase when *MID2* was inactivated. The percentage of dead small-budded cells is ~60% in *rgd1*Δ *mid2*Δ (de Bettignies *et al.* 1999). Thus, to easily screen multicopy suppressors of the *rgd1*Δ mutation, the *rgd1*Δ *mid2*Δ double mutant was used. This strain was transformed with the yeast YEp13 genomic library and screened for restoration of cell viability, selecting the whiter or clear blue-colored colonies after addition of a methylene blue solution onto the plate surface. From ~20,000 yeast transformants, 82 positive clones were retained. To confirm and to better discriminate the suppressor effects, the 82 transformants were cultured in liquid YNB inositol-3X medium, a medium containing threefold the inositol concentration of standard YNB. Indeed, whereas the wild-type and *mid2*Δ strains grew normally under this growth condition, the cell lethality of the *rgd1*Δ and *rgd1*Δ *mid2*Δ strains was higher and that of the double mutant close to 100% at late exponential phase (Figure 1). The cell growth and viability were monitored after 45 and 65 hr of culture when the mutant response was observable. Of the 82 isolated transformants, 22 presented a cell lethality lower than that of the double mutant. To verify that the suppression was plasmid dependent, the plasmids extracted from the 22 yeast transformants were reintroduced in *rgd1*Δ *mid2*Δ. Cell viability was again

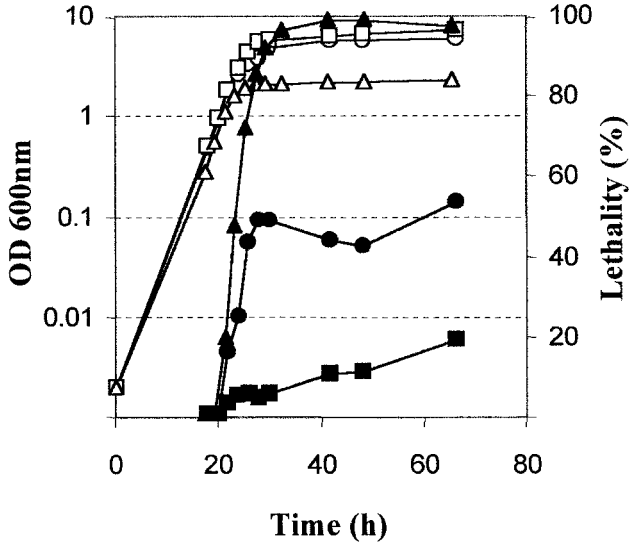

FIGURE 1.—Growth and lethality of mutant strains. The *rgd1*Δ (LBG4-3D, ●), *mid2*Δ (LBG36-8A, ■), and *rgd1*Δ *mid2*Δ (LBG59-15A, ▲) strains were cultivated at 30° in YNB inositol-3X medium. Growth (open symbols) was followed by measuring OD_{600} and dead cells (solid symbols) were visualized 10 min after mixing 30 μl of the culture with 30 μl of methylene blue staining solution. The ratio of blue-stained cells over total cells was determined from countings of at least 400 cells. The wild-type X2180 strain displays the same response as *mid2*Δ in these conditions (not shown).

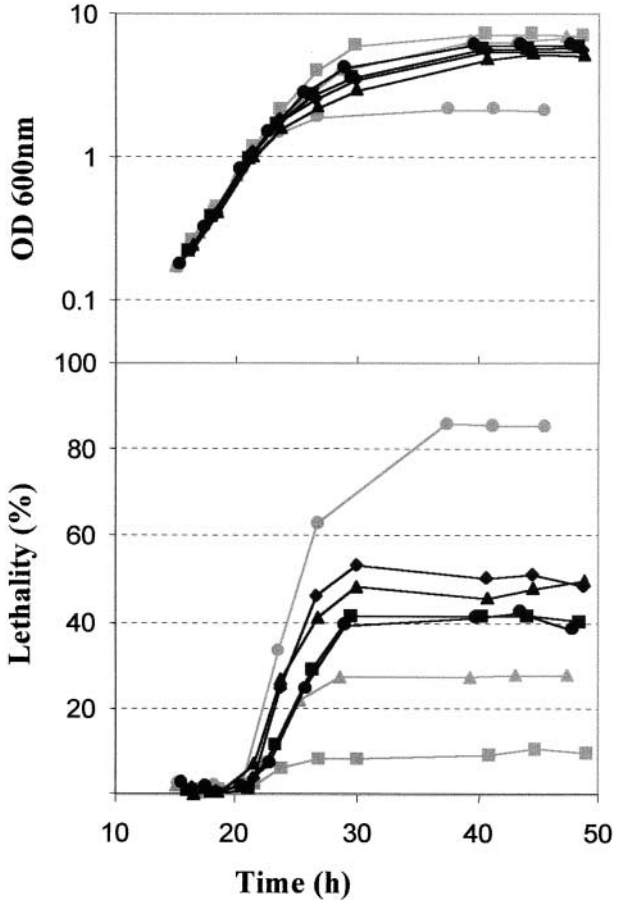

FIGURE 2.—Growth and lethality of the *rgd1*Δ *mid2*Δ (LBD-A7) carrying the different suppressor genes on the high-copy plasmid YEp13 and cultivated in YNB inositol-3X medium. Cell lethality was determined by FACS as described in MATERIALS AND METHODS. *rgd1*Δ *mid2*Δ containing YEp13 (shaded circle), YEp13 carrying *RGD1* (shaded square), *MID2* (shaded triangle), *RHO1* (solid circle), *RHO2* (solid square), *MKK1* (solid triangle), or *MTL1* (solid diamond).

examined from the new transformants after 45 and 65 hr of culture in liquid YNB inositol-3X. After this step, only 12 plasmids partially suppressing the cell mortality of the double mutant at the late exponential phase and in stationary phase were retained. The insert junctions of plasmids were sequenced and different genes were identified comparing the sequences with the *Saccharomyces cerevisiae* Genome Database. The *RGD1* and *MID2* genes were isolated two and four times, respectively, giving an internal screening control. Because the inserts of the remaining plasmids contained just one complete ORF, we were able to directly assign the specific multicopy suppressor effects to the *RHO1*, *RHO2*, *MKK1*, and *MTL1* genes. *RHO1* and *RHO2* genes were found two times each, and the *MKK1* and *MTL1* genes were found only one time each.

Phenotypic study of the multicopy suppressor genes in *rgd1*Δ *mid2*Δ: To specify the effect of the different suppressor genes, a phenotypic study was first undertaken in the double-mutant background. Each double-mutant strain transformed by one of the previously identified suppressor genes was cultivated in liquid YNB inositol-3X medium and the cell viability was monitored during growth by flow cytometry (Figure 2). As expected, the strain containing the *RGD1* gene on YEp13 presented the lowest lethality. The viability value was consistent with the absence of lethality of *mid2*Δ, and in that way this transformant behaved like a *mid2*Δ strain (DE BETTIGNIES *et al.* 1999). In the same way, the strain containing the YEp13 plasmid-borne *MID2* gene displayed the same behavior as a *rgd1*Δ strain. Indeed, after 30 hr of growth this strain exhibited ~25% viability loss. Overexpression of *RHO1*, *RHO2*, *MKK1*, and *MTL1* led to a suppressor effect with an intermediate percentage of cell lethality compared to 85% obtained with the plasmid YEp13. The strains carrying the high-copy plasmid-borne *RHO1* or *RHO2* gave similar results after 30 hr of growth with ~40% of mainly small-budded dead cells; it was ~50% for *MKK1* and *MTL1*. The suppressor effect was also observed in YNB standard medium with the same gradation. The percentages of cell lethality were 60, 5, and 18% for the double-mutant strain containing the YEp13 without insert and carrying *RGD1* or *MID2*, respectively. Overexpression of *RHO1* and *RHO2* gave a value of 20%, whereas it was slightly higher (25%) for the strain overexpressing *MKK1* and *MTL1* (data not shown).

Introduction of the *rgd1*Δ mutation into the *mid2*Δ

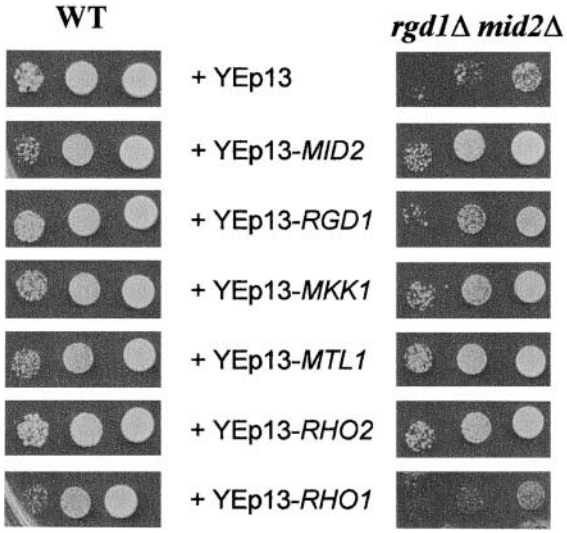

FIGURE 3.—Effect of the multicopy suppressors on the caffeine hypersensitivity of the *rgd1*Δ *mid2*Δ double mutant. Cell suspensions of exponentially growing *rgd1*Δ *mid2*Δ strains containing the different genes were diluted to the same concentration. Five microliters of 10-fold serial dilutions from each strain were dropped onto solid YPD medium containing 3 mg/ml caffeine and incubated for 2 days at 30°. As a control, the effect of high-copy plasmids was also examined in the wild-type strain X2180.

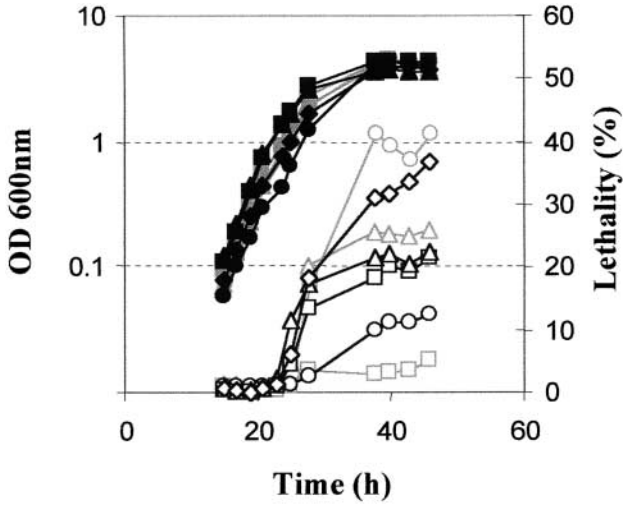

FIGURE 4.—Growth (solid symbols) and lethality (open symbols) of the *rgd1*Δ (LBG40-3C) carrying the different suppressor genes on the high-copy plasmid YEp13 and cultivated in YNB inositol-3X medium. Cell lethality was determined by FACS. *rgd1*Δ containing YEp13 (shaded circle), YEp13 carrying *RGD1* (shaded square), *MID2* (shaded triangle), *RHO1* (solid circle), *RHO2* (solid square), *MKK1* (solid triangle), or *MTL1* (solid diamond).

strain accentuates the *mid2*Δ caffeine sensitivity and the *rgd1*Δ *mid2*Δ double mutant is hypersensitive to caffeine, which can be remedied by addition of 1 M sorbitol (DE BETTIGNIES *et al.* 1999). We wanted to determine whether these suppressor genes could also suppress the caffeine hypersensitivity of the double mutant. Growth of the double mutants transformed with YEp13 and with YEp13 carrying the different genes were examined on YPD plates containing 3 mg/ml caffeine (Figure 3). The different plasmids introduced in the wild-type strain did not modify caffeine sensitivity (Figure 3) and no growth difference was observed when *rgd1*Δ *mid2*Δ carrying these plasmids was cultivated in the presence of caffeine and sorbitol (data not shown). In the presence of caffeine only, introduction of *MID2* in *rgd1*Δ *mid2*Δ resulted in the *rgd1* phenotype, as expected. Since the *rgd1*Δ alone is not sensitive to the drug, *MID2* overexpression completely suppressed the double-mutant hypersensitivity. In the same way, *RGD1* overexpression only partially suppressed this sensitivity, consistent with what we obtained in a *mid2*Δ strain (DE BETTIGNIES *et al.* 1999). In agreement with their suppressor effects on cell viability, overexpression of *MKK1*, *MTL1*, and *RHO2* in the double mutant also decreased the caffeine sensitivity. Surprisingly, *RHO1* overexpression did not show any effect under these test conditions. The lack of response of the *RHO1* gene with respect to caffeine seems to indicate a cellular mechanism for *RHO1* action that is distinct from that occurring in the other suppressor genes.

Analysis of the multicopy suppressor effect in *rgd1*Δ and *mid2*Δ mutants: Considering the results obtained in the double-mutant background, we investigated whether the identified genes were multicopy suppressors of *rgd1*Δ and/or *mid2*Δ mutations. We therefore undertook similar phenotypic studies in the single mutants and we first tested the effect of gene overexpression on cell viability (Figure 4). As *mid2*Δ does not show any cell mortality in minimal medium, this test was applied only to the *rgd1*Δ strain. To exacerbate its defect and thus to better assess the suppressor effect, the *rgd1*Δ strain was cultivated in liquid YNB inositol-3X medium; in this growth condition the cell mortality reached >40% instead of 15% in the standard YNB. First of all, we verified that the YEp13-borne *RGD1* in the *rgd1*Δ strain gave restoration of cell viability as previously obtained with other high-copy plasmids (DE BETTIGNIES *et al.* 1999). For the *RHO1*, *RHO2*, *MTL1*, and *MKK1* genes, we observed again a partial rescue of *rgd1*Δ cell viability with percentages ranging from 15 to 30%. This test also revealed a suppressor effect due to *MID2* overexpression with ~25% cell mortality. These results, based on viability rescue, show that all the genes isolated in our screen, as well as *MID2*, are *rgd1*Δ multicopy suppressors. The caffeine response was not tested in the single mutants because *rgd1*Δ does not exhibit any sensitivity toward caffeine compared to wild type and the *mid2*Δ sensitivity was not easily usable to examine the suppression effects. To discriminate the effects of *RHO1*, *RHO2*, *MTL1*, and *MKK1* in the two single mutants, we next examined the MID phenotype shown by both single mutants. As for the *mid2*Δ strain (ONO *et al.* 1994), *rgd1*Δ shmoos die when exposed to the mating pheromone (DE BETTIG-

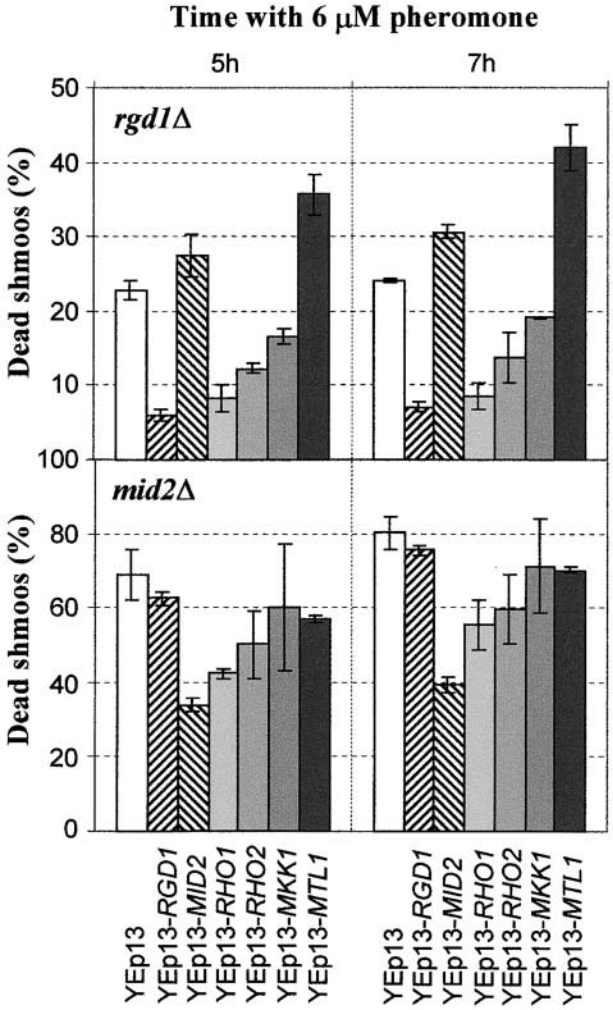

FIGURE 5.—Mating pheromone induced death phenotype of the *rgd1*Δ (LBG40-3C) and *mid2*Δ (LBG42-2D) strains carrying the suppressor genes. *MAT***a** strains were grown in YNB to reach an OD_{600} value ~0.3. Then α-factor was added to a final concentration of 6 μM, and cells were further incubated at 30°. After 5 and 7 hr of pheromone exposure, shmoos lethality was determined by mixing 30 μl of each culture with the same volume of methylene blue staining solution. The ratio of blue over total shmoo cells was determined from countings of at least 400 shmoos. Data are the mean values and standard deviations obtained from three to four independent experiments.

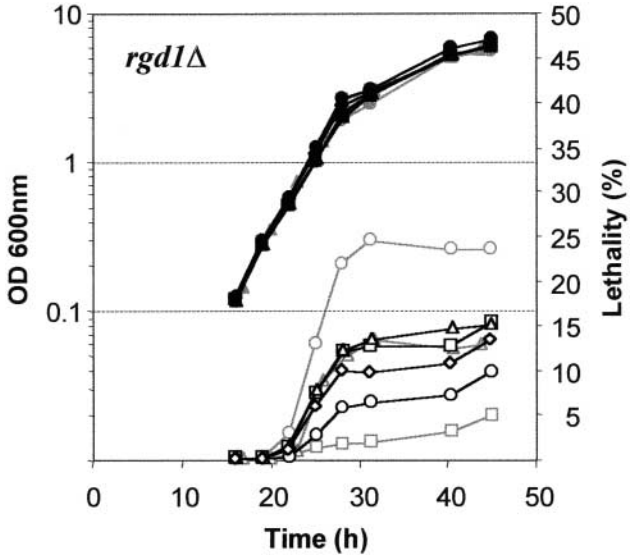

FIGURE 6.—Growth (solid symbols) and lethality (open symbols) of the *rgd1*Δ (LBG40-3C) carrying the different suppressor genes on the low-copy plasmids pRS315 or pRS415 and cultivated in YNB inositol-3X medium. Cell lethality was determined by FACS. *rgd1*Δ containing pRS315 (shaded circle), pRS315 carrying *RHO1* (solid circle), *RHO2* (solid square), *MKK1* (solid triangle), or *MTL1* (solid diamond), and pRS415 carrying *RGD1* (shaded square) or *MID2* (shaded triangle).

NIES *et al.* 1999). Therefore, *MAT***a** *rgd1*Δ and *mid2*Δ cells containing the different high-copy plasmids were treated with a 6 μM α-factor in YNB and the viability of shmoos was examined to determine the suppressor effects of *RHO1*, *RHO2*, *MTL1*, and *MKK1* (Figure 5). As expected, *RGD1* in the *rgd1*Δ background complemented the shmoos lethality in the presence of the pheromone. Unlike the result in the cell viability test, no effect was detected when *MID2* was used to suppress the MID phenotype of *rgd1*Δ. Conversely, the introduction of *RGD1* in high copy did not suppress the MID phenotype of the *mid2*Δ mutant; however, the complementation of MID phenotype was not complete even when the *MID2* gene itself was used. In parallel, it was verified that YEp13-borne *RGD1* and *MID2* did not lead to shmoos lethality in a wild-type strain (data not shown). When we addressed the suppressor effects of *RHO1*, *RHO2*, *MTL1*, and *MKK1* in *mid2*Δ, we observed a partial suppression from these genes. The *RHO* genes were more efficient than *MKK1* and *MTL1*, the homolog of *MID2*. In *rgd1*Δ, the *RHO1*, *RHO2*, and *MKK1* genes clearly suppressed the MID phenotype, and *RHO1* and *RHO2* showed the strongest response with shmoos lethality of 8 and 14%, respectively. On the contrary, *MTL1* overexpression did not suppress the *rgd1*Δ mutation, but rather was detrimental by increasing the shmoos lethality. Except for the *MTL1* overexpression effect on the *rgd1* MID phenotype, these results are consistent with a multicopy suppressor effect of the *RHO1*, *RHO2*, *MTL1*, and *MKK1* genes that is specific to the *rgd1*Δ mutation.

Suppressor effect of the genes carried by low-copy plasmid in *rgd1*Δ: The suppressor effect was also investigated from these genes carried by the low-copy plasmids pRS315 or pRS415. These plasmids were introduced in the *rgd1*Δ strain and cell viability was followed by flow cytometry during growth in YNB inositol-3X medium (Figure 6). For unclear reasons, the *rgd1*Δ strain containing the low-copy plasmid showed a slightly reduced lethality (25%) with respect to what we observed with the high-copy plasmid YEp13. Introduction of *RGD1* complemented its mutation and *MID2* partially sup-

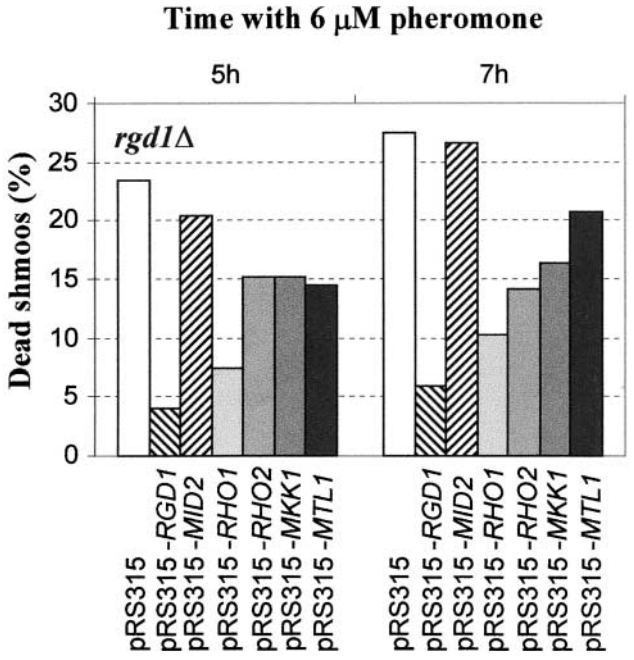

FIGURE 7.—Mating pheromone induced death phenotype of the *rgd1*Δ (LBG40-3C) strains carrying the different suppressor genes in low-copy plasmid. Experiments were done as indicated in Figure 5.

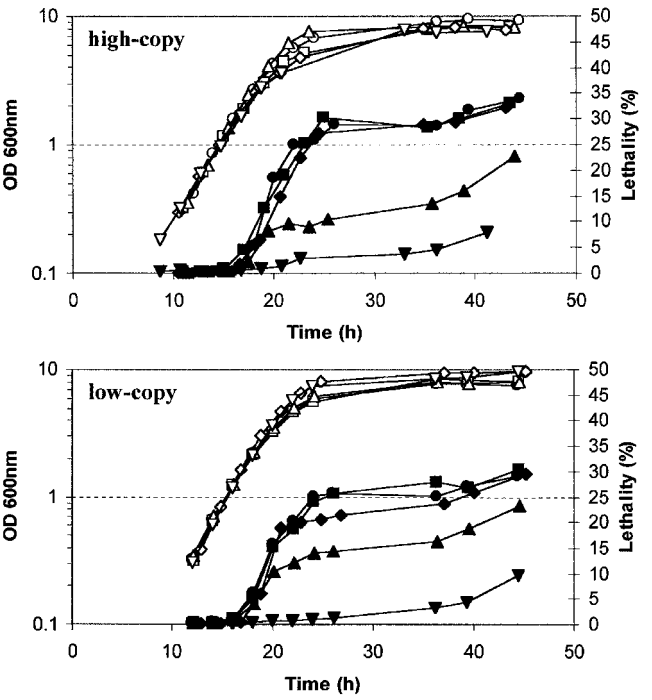

FIGURE 8.—Growth (open symbols) and lethality (solid symbols) of the *rgd1*Δ (LBG44-6B) carrying the *PKC1*, *BCK1*, and *MPK1* genes and the activated allele *BCK1-20* on high- and low-copy plasmids and cultivated in YNB inositol-3X medium. Cell lethality was determined by FACS. *rgd1*Δ containing the high- or low-copy plasmid empty (●), or with the genes *PKC1* (◆), *BCK1* (■), *BCK1-20* (▼), or *MPK1* (▲).

pressed the cell viability loss of *rgd1*Δ strain. Concerning *RHO1*, *RHO2*, *MTL1*, and *MKK1*, we again found a suppressor effect; the *RHO1* gene presented the strongest suppressor with <10% cell lethality after 30 hr of culture. Interestingly, *MTL1* in low copy gave a better suppressor effect than in high copy, with ~15% lethality at 30 hr, similar to *RHO2* and *MKK1* ones. To determine if the suppression by low-copy genes was also relevant to the other phenotype caused by the *RGD1* inactivation, we examined the MID phenotype (Figure 7). As with the high-copy plasmid, *RHO1*, *RHO2*, and *MKK1* partially suppressed the MID phenotype of *rgd1*Δ. The more pronounced suppression was obtained with the *RHO* genes. For *MTL1*, in contrast to what we observed with the high-copy plasmid, its introduction into the low-copy plasmid allowed the detection of a partial suppressor effect in agreement with the results observed from the cell viability test. As before, *MID2* did not modify the *rgd1*Δ shmoos lethality. Thus, even if both inactivations of *RGD1* and *MID2* led to the MID phenotype, they affect different mechanisms. Taken together, the results show the involvement of these four genes in suppression of both *rgd1*Δ phenotypes even when expressed from low-copy plasmids.

Suppression by PKC pathway components in *rgd1*Δ: The identification of *RHO1* and *MKK1*, two genes involved in the PKC pathway in *S. cerevisiae*, as *rgd1*Δ suppressors, led us to examine the suppression effects of other components belonging to this pathway. Thus, high-copy and low-copy plasmids carrying the *PKC1*, *BCK1*, and the *SLT2/MPK1* genes acting upstream and downstream of *MKK1* were transformed into the wild-type and *rgd1*Δ strains. Cell growth and viability of these transformed strains grown in YNB inositol-3X medium were observed (Figure 8). No deleterious effect on cell proliferation was observed as a result of *PKC1*, *BCK1*, and *MPK1* overexpression. When carried by high-copy plasmids, only *MPK1* overexpression allowed a net restoration of the cell viability of the single mutant. A similar response was obtained with *MPK1* carried on a centromeric plasmid. Unlike with the high-copy plasmid, *PKC1* carried on YCp50 presented little suppressor effect. In addition, the activated mutant allele *BCK1-20* isolated as a suppressor of a *pkc1* deletion, whose product probably mimics the phosphorylated active form of Bck1p (LEE and LEVIN 1992), was introduced on high-copy pRS426 or low-copy pRS316 vectors in the LBG44-6B strain. In both cases, a strong suppression effect was observed with <10% of dead cells after 40 hr of culture (Figure 8). Our results show that an increase in the PKC pathway activity suppresses the cell mortality of the *rgd1*Δ mutant.

Rlm1p transcriptional activity in the *rgd1*Δ mutant: The previous results suggest that the *RGD1* inactivation might decrease the signaling activity of the PKC pathway. To test whether the *rgd1*Δ mutation could lower the PKC pathway activity, we monitored the transcriptional activation of Rlm1p in the mutant background. The

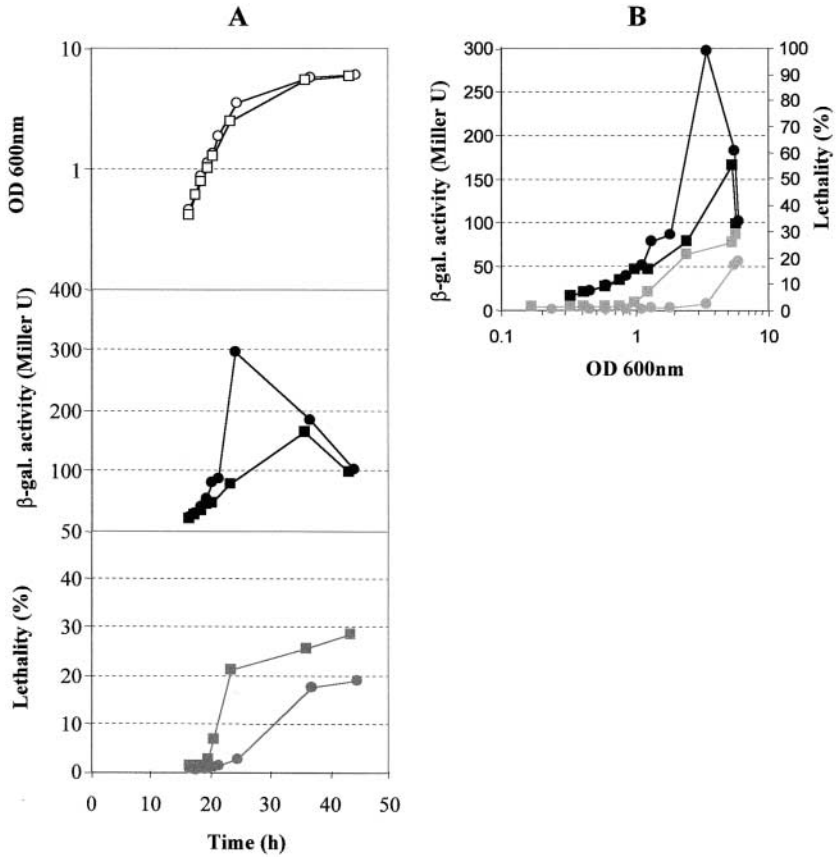

FIGURE 9.—Rlm1p transcriptional activity in *rgd1*Δ (LBG92-4B, ■) and wild-type (LBG37-5C, ●) strains. The strains LBG92-4B and LBG37-5C, containing the two plasmids each expressing the reporter and transactivator genes as described in MATERIALS AND METHODS, were grown in YNB medium. (A) Lethality (shaded symbols) and β-galactosidase activity (solid symbols) during growth (open symbols). (B) β-Galactosidase activity and lethality as functions of growth.

RLM1 gene encodes a member of the MADS-box family of transcription factors, which was identified initially as a loss-of-function mutant that suppresses the lethality associated with a high level of Mkk1p-S386P (WATANABE *et al.* 1995). *RLM1* has a key role in transmitting the cell integrity signal. Recently, it was reported from a genome-wide analysis that the majority of genes whose expression changed following kinase-cascade activation was regulated through the transcription factor Rlm1p (JUNG and LEVIN 1999). To follow the ability of Rlm1p to activate transcription, we quantified expression of the *lacZ* reporter gene directed by the LexA-Rlm1ΔN fusion protein in wild-type and *rgd1*Δ mutant cells. The LexA-Rlm1p chimera is phosphorylated by the MAP kinase Mpk1p and activates, in turn, the transcription of the LexA-operator-controlled *lacZ* reporter gene (WATANABE *et al.* 1997). Thus, to determine whether the *rgd1*Δ mutation could decrease the PKC pathway activity and, subsequently, whether a defect in PKC signaling triggered the cell viability loss in *rgd1*Δ, the β-galactosidase activity and the cell lethality were monitored during growth in YNB (Figure 9). The β-galactosidase activity measured in wild-type cells increased with cultivation time up to the entry into stationary phase. As the activity increases exponentially during the exponential growing phase and since β-galactosidase is relatively stable in *S. cerevisiae*, we suppose that the *RLM1* activation progressively increases during this phase up to the stationary phase. We then observed a net decrease of the reporter activity in stationary phase, suggesting that the *RLM1* activity was then weakened in the wild-type resting cells. In the *rgd1*Δ mutant, the β-galactosidase activity was in the same range as the wild type at the beginning of the culture. However, it was lower when *rgd1*Δ cells died (Figure 9A). To better compare the *RLM1* transcriptional activity with the cell mortality appearance, the data of β-galactosidase activity and lethality rates were analyzed as functions of growth (Figure 9B). In this presentation, we observed that for cell densities up to one OD unit, the β-galactosidase activity increased and was identical in wild-type and *rgd1*Δ cells, indicating that the Rlm1p was similarly activated in both strains at the beginning of the growth. As shown before, from the time when *rgd1*Δ cell mortality appeared, the reporter activity increased less than in wild-type cells. Such a result could be explained by the mortality of part of the yeast culture, but it is difficult to correlate the activity variation with the viability loss. To determine whether a PKC-activation defect could trigger the *rgd1*Δ mortality or, alternatively, whether the lethality might cause the activity change, we tried to examine the timing of both events during growth in the mutant with respect to wild type. Our results showed that the two parameters varied at the same time in *rgd1*Δ and did not permit us to conclude that *RLM1* is involved in activating change in cell mortality appearance.

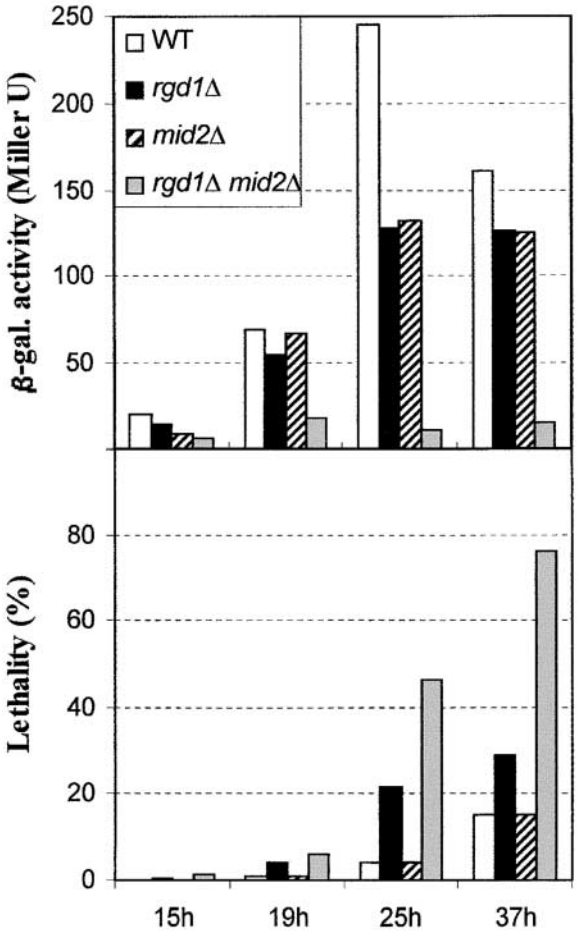

FIGURE 10.—Rlm1p transcriptional activity in *rgd1*Δ (LBG92-4B), *mid2*Δ (LBG93-5A), *rgd1*Δ *mid2*Δ (LBG94-3C), and wild-type (LBG37-5C) strains. The YNB medium was inoculated at the same OD_{600} nm for each strain, and β-galactosidase activity and lethality were determined at the indicated times.

A similar study was initiated with the double-mutant *rgd1*Δ *mid2*Δ; for that, the β-galactosidase activity was measured during growth in YNB medium from the double mutant, the respective single mutants, and the wild-type strain containing the two plasmids each expressing the reporter and transactivator genes. Four time points were chosen and again the reporter activity was compared with the lethality rate (Figure 10). First, a similar β-galactosidase activity defect was observed in *mid2*Δ and *rgd1*Δ, although the *mid2* cells displayed the same viability as wild-type cells. The defect of the *RLM1* transcriptional activity was consistent with the known activator role of Mid2p on the PKC pathway and on Mpk1p phosphorylation under stress conditions (KETELA *et al.* 1999; RAJAVEL *et al.* 1999). Our results also revealed the role of Mid2p on Rlm1p activity through the PKC pathway during vegetative growth at 30°. For *rgd1*Δ *mid2*Δ, while the cell lethality was <5% at 19 hr, the β-galactosidase was very low compared to other strains. In the same way, at 25 and 37 hr, whereas the lethality was not complete, no significant β-galactosidase activity was detected. The lack of reporter activity in the double-mutant background is due to the combined effects of both mutations. It may only partly be explained by *mid2*Δ inactivation and indicates some role for Rgd1p in activating the PKC pathway. Taken together, these results suggest that the decrease of the PKC pathway activity following *rgd1*Δ inactivation is in part responsible for cell lethality, but is not enough in itself to trigger lethality and that another defect due to *RGD1* inactivation and cumulative to the PKC pathway activation defect is involved in lethality appearance.

Transcriptional regulation of the *PST1* gene: To confirm that the changes in β-galactosidase activity in the wild-type and *rgd1*Δ strains reflected changes in PKC pathway activation, we examined the expression of the *PST1* gene that was demonstrated to be dependent on the PKC pathway activity through the transcription factor Rlm1p (JUNG and LEVIN 1999). The *PST1* transcriptional level was determined by Northern analysis from the wild-type and *rgd1*Δ strains grown in YNB; cell lethality of both strains was also measured. Quantification of the *PST1* mRNA levels was achieved through PhosphorImager measurements and normalized to the expression of *RPB4* whose transcript is present at similar levels during all growth phases (ROSENHECK and CHODER 1998). The expression profile of *PST1* along the growth (Figure 11) is strikingly similar to the profile of the β-galactosidase activity described in Figure 9A and allows us to estimate the evolution of the PKC pathway activity. The result confirms that in a wild-type strain the PKC activity rises suddenly at the end of the exponential phase to reach a peak when cells enter the stationary phase and then diminishes in resting cells. This activation of the PKC pathway seems dramatically reduced in *rgd1*Δ and lethality appears in this mutant precisely at the time when induction of the activity should occur. Given that the expression of *PST1* is normalized to that of *RPB4*, differences in expression cannot be explained by the lethality of 15% of the cells. Thus, inactivation of the *RGD1* gene leads to a clear defect in PKC pathway activation, at least under the growth conditions used.

DISCUSSION

The inactivation of the *RGD1* gene encoding a RhoGAP for the small GTPases Rho3p and Rho4p (DOIGNON *et al.* 1999) gives a viability loss at late exponential phase in minimum medium (DE BETTIGNIES *et al.* 1999). Dead cells present a small bud as some mutants of the PKC pathway do (LEVIN *et al.* 1990); however, adding sorbitol to the medium does not compensate for this defect. Likewise, the MID phenotype of *rgd1*Δ is not prevented by osmotic stabilization, contrary to the observations with mutations in *MPK1/SLT2* (ERREDE *et al.* 1995). However, as for mutants altered in the PKC-signaling pathway (COSTIGAN *et al.* 1992; MARTIN *et al.* 1996), the heat-shock sensitivity of the *rgd1*Δ mutant is

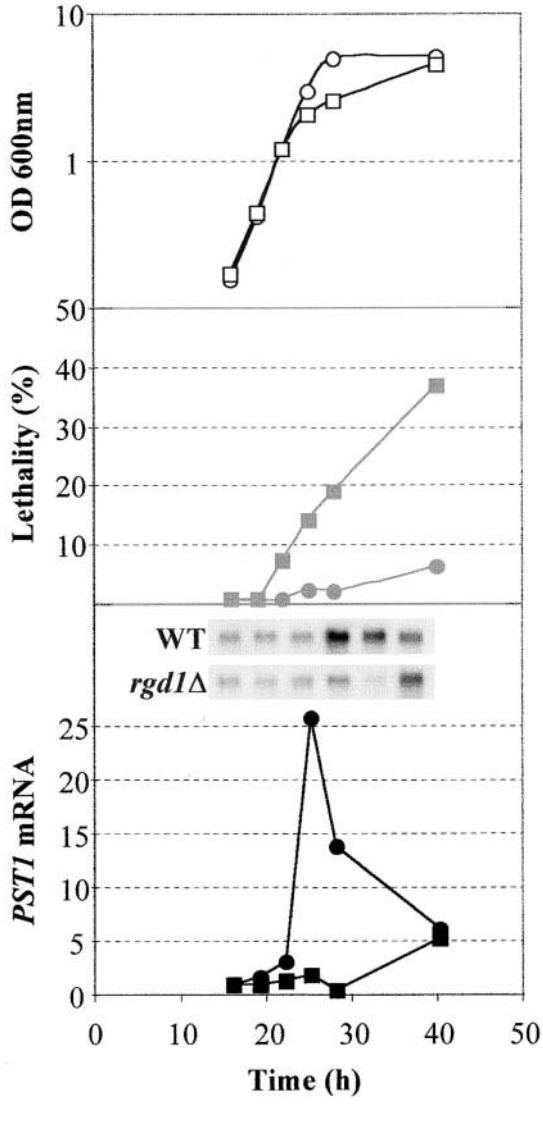

FIGURE 11.—*PST1* Northern analysis from the *rgd1*Δ (LBG40-3C, ■) and wild-type (X2180-1A, ●) strains grown in YNB. (Top and middle) Growth and cell lethality. (Bottom) The variations of *PST1* mRNAs normalized to *RPB4* mRNAs. The scale corresponds to ratios of *PST1* signal intensities with respect to *RPB4* signal intensities detected by Northern analysis and quantified by Phosphor-Imager, using the first point as an arbitrary unit of 1.

suppressed by the addition of 1 M sorbitol (DE BETTIGNIES *et al.* 1999); in addition, the *RGD1* inactivation exacerbates the caffeine sensitivity of *mid2*Δ mutants, which is also rescued by sorbitol. Such phenotypic data suggest that *RGD1* presents some functional links with the cell wall integrity pathway. This proposition is reinforced by the discovery of genetic interactions between *RGD1* and either *SLG1* or *MID2*, two putative sensors for cell wall integrity signaling in *S. cerevisiae* (GRAY *et al.* 1997; KETELA *et al.* 1999; RAJAVEL *et al.* 1999).

In this study, we isolated *RHO1*, *RHO2*, *MKK1*, and *MTL1* as multicopy suppressors of the *rgd1*Δ *mid2* cell lethality. All four suppressor genes are specific to the *rgd1*Δ mutation; they partially suppress both *rgd1*Δ defects, cell lethality and the MID phenotype, and they also work when carried by low-copy plasmids. *RHO1* was the more efficient suppressor with *RHO2*, *MKK1*, and *MTL1* giving a suppression response in the same range. We also found that *MID2* was a low-copy suppressor but only of the *rgd1*Δ cell viability, indicating that the shmoo lethality in *rgd1*Δ and *mid2*Δ should be due to distinct altered mechanisms. *RHO1*, *MKK1*, and *MTL1* were previously shown to be involved in the PKC-signaling transduction pathway. *MKK1* encodes one of the MAP-kinase kinases of the PKC pathway. The Rho1p GTPase mediates bud growth by controlling polarization of the actin cytoskeleton and cell wall synthesis (DRGONOVA *et al.* 1999). It controls the cell integrity signaling pathway through two functions. A first essential function of Rho1p is to bind and activate the protein kinase C (KAMADA *et al.* 1995; NONAKA *et al.* 1995; MARTIN *et al.* 2000). Recently, it was shown by two-hybrid experiments that the cytoplasmic domains of Slg1p and Mid2p interact with the Rho1p-exchange factor Rom2p. The function of the sensor-Rom2p interaction would be to stimulate nucleotide exchange toward the small G-protein, Rho1p (PHILIP and LEVIN 2001). Second, Rho1p serves as an integral regulatory subunit of the 1,3-β-glucan synthase complex and stimulates this activity in a GTP-dependent manner (DRGONOVA *et al.* 1996). In addition, it is postulated that Rho1p controls the actin cytoskeleton via its interaction with Bni1p, which binds to profilin (EVANGELISTA *et al.* 1997; IMAMURA *et al.* 1997). *MTL1* encodes a polypeptide showing 50% identity with Mid2p. Overexpression of *MTL1* partially suppressed the pheromone sensitivity of *mid2*Δ. As for *mid2*Δ, Mpk1p activation is diminished in the *mtl1*Δ mutant in response to heat shock (RAJAVEL *et al.* 1999). Concerning *RHO2*, it is involved, as is *RHO1*, in bud formation and organization of the actin cytoskeleton (MATSUI and TOH-E 1992b). Rho2p was shown to be the only yeast Rho family member that can repolarize the actin cortical patches in the *pfy*Δ strain (MARCOUX *et al.* 2000). It is interesting to note that *MID2* overexpression as well as *ROM1* and *ROM2*, two genes coding for exchange factors of Rho1p (OZAKI *et al.* 1996), also suppress the profilin-deficient phenotype of yeast cells (MARCOUX *et al.* 1998, 2000). It was thus proposed that Mid2p, Rom2p, and Rom1p are in the same signaling pathway, acting upstream of Rho2p to correct the profilin-deficient phenotype (MARCOUX *et al.* 2000). This is consistent with the identification of *ROM2*, *RHO2*, and *MID2* as multicopy suppressors of the *cik1* and *kar3* mutations giving a chromosome instability and a karyogamy defect, respectively (MANNING *et al.* 1997). From these and our data, we can suppose that the *RHO2* suppressor gene is also linked by some means or other to the cell wall integrity pathway. It will be interesting to study the involvement of Rho2p in the PKC pathway activity.

In view of our results and the data from the literature, we can interpret the suppressor effect of *RHO1*, *MKK1*, and *MTL1* as the consequence of the overactivation of the PKC pathway. The effect of inactivation and overexpression of *MID2* on the *rgd1*Δ lethality is in agreement with such a hypothesis. Likewise, the synthetic lethality of *rgd1*Δ *slg1*Δ and the associated phenotypes could be explained by the diminution of PKC pathway activity caused by the action of both mutations. Nevertheless, although Mid2p and Slg1p show overlapping functions and signal through Rho1p (Ketela *et al.* 1999; Rajavel *et al.* 1999), *SLG1* is not a dosage suppressor of the *rgd1*Δ phenotypes (data not shown), revealing a discriminating function in signal transduction between these two proteins. For *RHO2*, even if no direct link were established with the cell wall integrity pathway, we can postulate that the *RHO2* suppressor effect also acts by increasing the PKC pathway activity. Besides the suppressors, we present evidence indicating that activation of the PKC pathway is necessary to compensate for the defects caused by the *RGD1* deletion. Indeed, among the high- and low-dosage kinases of the PKC pathway, *MPK1* encoding the last kinase suppressed the *rgd1*Δ mortality in the same range as *RHO2*, *MKK1*, and *MTL1*; a slight effect was visualized with *PKC1* in low copy. In addition, with the hyperactive Bck1-20p form in low copy number the rescue of cell viability is very close to that obtained when *RGD1* is carried by the same plasmid, showing that the overactivation of the PKC pathway is sufficient to compensate for the *rgd1*Δ defect.

On the basis of the phenotypes of the *RGD1* deleted strain and the genetic relations between *RGD1* and the PKC pathway, *RGD1* was thought to be required for the activation of the PKC pathway during the growth phase in which PKC pathway activity seemed optimal. Indeed, the follow-up of the *RLM1* transcriptional activity and of the *PST1* transcription level indicates that, in our conditions, the PKC pathway activity is not constant during growth and increases particularly in the transition phase and that the inactivation of the *RGD1* gene decreases the level of PKC pathway activation when the mutant expresses the phenotype. Similar analysis revealed that *MID2* inactivation led to a significant reduction in Rlm1p activation without any significant lethality and showed that a decrease in PKC pathway activation in itself is not enough to cause lethality. Moreover, introduction of the *rgd1*Δ mutation in the *mid2*Δ strain led to a dramatic reduction in Rlm1p activation, which seems to precede the very high lethality of the strain. Taken together and considering that overactivation of the PKC pathway suppresses the lethality of the *rgd1*Δ, these results allow us to conclude that the *rgd1*Δ mutation alone weakens the activity of the PKC pathway, but also causes another more subtle defect, which, cumulative with the decrease of the PKC pathway, subsequently results in cell lethality during a particular growth phase in minimal medium, where the activation of this signaling pathway seems particularly important. Hence, the small-budded death phenotype of the *rgd1*Δ mutant at late exponential phase, which is reminiscent of the phenotype of some PKC pathway mutants, is at least partly due to a failure to activate the PKC pathway under this growth phase.

Thus, we show that *RGD1* acts somewhere upstream of the PKC pathway. Given that Rgd1p has been shown to have a Rho-GAP activity toward Rho3p and Rho4p, we have to consider that the defect of PKC pathway activation at late exponential phase observed in the *rgd1*Δ might be mediated by the small GTPases Rho3p and Rho4p. Indeed, a strain carrying another *RGD1* deletion removing the Rho-GAP domain presents a cell lethality like *rgd1*Δ (Barthe *et al.* 1998). In addition, consistent with such a hypothesis, introduction of the constitutively active forms of either Rho3p or Rho4p (Roumanie *et al.* 2000) in the wild-type background and growing in YNB inositol-3X medium triggers a cell mortality at late exponential phase with dead small-budded cells, as for the *rgd1*Δ strain (our unpublished data). Bud growth occurs as the result of two combined events: first, an increased synthesis and assembly of cell wall components and, second, polarization of growth by rearrangement of the cytoskeleton and of the secretory machinery to specifically deliver cell wall constituents to the bud. It was recently reported that, beyond its control in actin organization, Rho3p plays a role in exocytosis (Adamo *et al.* 1999). Thus the *RGD1* inactivation might lead to subtle changes in the cellular mechanisms achieved by Rho3p and Rho4p and to an impairment in polarization and vesicle transport that might indirectly affect cell wall assembly. Such a hypothesis is corroborated by the diminished resistance of the double mutant *rgd1*Δ *mid2*Δ, with regard to that of the single mutant *mid2*Δ, to Calcofluor white and Congo red, two drugs interfering with cell wall assembly (de Bettignies *et al.* 1999).

We can postulate, then, whether the decrease of the PKC pathway activity in the *rgd1*Δ mutant would be the consequence of an altered polarized growth whose effects would be observable in particular physiological states. Such a hypothesis would be consistent with the findings that Pkc1p and Slg1p are essential for the repression of rRNA and ribosomal protein genes in response to a defect in the secretory pathway (Nierras and Warner 1999; Li *et al.* 2000). These authors propose that in a secretory defective cell that can no longer synthesize either the plasma membrane or the cell wall, the continued synthesis of proteins leads to osmotic stress and repression of ribosome synthesis. Likewise, the *mrs6-2* mutation that leads to low levels of Ypt protein prenylation, and causes vesicle polarization defects and thermosensitive growth, can be suppressed by genes involved in cell wall maintenance, such as *SLG1* and *MPK1* (Bialek-Wyrzykowska *et al.* 2000). It was proposed that at high temperature *mrs6-2* cells have a vesi-

cle-polarization alteration that causes defects in the formation of the cell wall. In *rgd1*Δ we can imagine the reciprocal effect with a secretory pathway more active and/or active in the wrong conditions, due to the absence of negative regulation of Rho3p and Rho4p by its GAP, and consequently a structurally modified cell envelope leading to a signaling decrease of PKC pathway. Further experiments will shed some light on this hypothesis.

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