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The yeast Rgd1p is a GTPase activating protein of the Rho3 and Rho4 proteins

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Abstract The *RGD1* gene, identified during sequencing of the *Saccharomyces cerevisiae* genome, encodes a protein with a Rho-GTPase activating protein (GAP) domain at the carboxy-terminal end. The Rgd1 protein showed two-hybrid interactions with the activated forms of Rho2p, Rho3p and Rho4p. Using in vitro assays, we demonstrated that Rgd1p stimulated the GTPase activity of both Rho3p and Rho4p; no stimulation was observed on Rho2p. In addition, the *rho3*Δ*rgd1*Δ double mutant exhibited a dramatic growth defect compared to the single mutants, suggesting that Rgd1p has a GAP activity in vivo. The present study allowed the identification of the first GAP of Rho3p and Rho4p.

Key words: *RHO3*; *RHO4*; *RGD1*; GTPase activating protein; *Saccharomyces cerevisiae*

1. Introduction

The *RGD1* gene was identified as the ORF YBR260c during the systematic sequencing of the *Saccharomyces cerevisiae* genome [1]. Protein comparison with data banks revealed a significant similarity between the C-terminal part of Rgd1p and the Rho-GTPase activating protein (GAP) domain of human Bcr and Abr and yeast Bem2 and Bem3 proteins [2]. The GAP protein is known to activate GTP hydrolysis by small G proteins belonging to the Rho family. Through their C-terminal region, both Bcr and Abr act as GAPs for Rac1, Rac2 and Cdc42Hs [3]; the yeast Bem2 protein is a GAP for Rho1p while Bem3p acts both on Rho1p and Cdc42p [4,5]. The presence of such a Rho-GAP domain in Rgd1p led us to explore whether this protein displayed a GAP activity on yeast Rho proteins. *S. cerevisiae* possesses six Rho proteins: Cdc42p, Rho1p, Rho2p, Rho3p, Rho4p and Rho5p. Cdc42p is an essential protein and plays a role in bud site selection [6]. Rho1p is another essential protein acting on glucan synthase [7] and on Pkc1 kinase [8]. Rho2p, involved in control of actin cytoskeleton dynamics, is not an essential protein [9]. Rho3p and Rho4p are reported to be involved in cell morphogenesis and specifically in actin polarization for bud growth [10]. The last Rho was discovered through genome sequencing [11]. We undertook a systematic approach based on the utilization of the two-hybrid system; this revealed interactions between Rgd1p and the GTP constitutive mutant forms of Rho2p, Rho3p and Rho4p. Then these three Rho proteins and Rgd1p tagged with glutathione *S*-transferase (GST) were expressed in *Escherichia coli* and purified using affinity chromatography for glutathione-Sepharose. We used the spectrophotometric assay reported by Webb [12] to show that Rgd1p was able to stimulate the intrinsic GTPase activity of Rho3 and Rho4 proteins in vitro. In addition, by combining the *rho3*Δ and *rgd1*Δ mutations, we observed that the corresponding double mutant presented a severe growth defect. The present results demonstrate that, as predicted in silico, the Rho2, Rho3 and Rho4 proteins possess a GTPase activity and that Rgd1p, a Rho-GAP domain containing protein, is a GAP of the yeast Rho3 and Rho4 proteins.

2. Materials and methods

2.1. Strains and genetic procedures

E. coli BL21 (DE3) was from Novagen. The *S.cerevisiae* strain FY1679 (*MAT***a**/*MAT*α, *ura3-52*/*ura3-52 leu2Δ1*/+ *trp1Δ63*/+ *his3Δ200*/+) is a derivative of S288C [13]. The *RGD1* gene was inactivated by *URA3* replacement in the X2180 background (*MAT***a**, *ura3-52, rgd1::URA3*) [2]. Yeast genetic procedures and medium preparation were described by Sherman et al. [14]. The FY1679 strain was inactivated for either the *RHO3* or *RHO4* gene according to the procedure described by Wach [15]. Gene inactivation was carried out by replacing the coding sequence by the marker *kan*MX4 giving resistance to G418 [16]. Correct gene replacement in the G418 resistant transformants was verified using the PCR strategy described by Wach [15]. The gene disruption removed 84% of the *RHO3* coding sequence and 100% of the *RHO4* sequence. The diploid strains disrupted for *RHO3* or *RHO4* were sporulated and tetrads were dissected on YPD rich medium containing 1 M sorbitol to prevent cell lysis [10]. Both transformants gave four viable spores per tetrad with a 2:2 segregation of the *kan*MX4 marker. The haploid resistant strains were inactivated for *RHO3* or *RHO4*.

2.2. Yeast two-hybrid analysis

Products for molecular biology were obtained from New England Biolabs and Roche Molecular Biochemicals. The coding sequences for the activated forms of *RHO1* to *RHO5* (Rho1Q68L; Rho2Q65L; Rho3Q74L; Rho4Q131L and Rho5Q91L) generated by PCR mutagenesis were fused to the DNA binding GAL4 domain of plasmid pODB80 [17]. The C-terminal CAAX box was deleted to prevent mislocalization of Rho proteins in the two-hybrid assay. These constructions (pODB80-RHO1 to pODB80-RHO5) were verified by DNA sequencing. The activated *CDC42* allele (Cdc42G12V) cloned in pAS2 (pAS2-CDC42) was a gift from M.N. Simon [18]. The coding sequence for Rgd1p was fused to the DNA activating GAL4 domain by cloning into the *Nco*I-*Xho*I sites of the pACT2 vector (Clontech, California) to give pACT2-RGD1. The yeast HY strain [17] containing pACT2-RGD1 was transformed using the protocol of Gietz et al. [19] with pODB80-RHO1 to pODB80-RHO5 and pAS2-CDC42. These latter plasmids, expressing active forms of the Rho proteins,

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Abbreviations: GAP, GTPase activating protein; GTP, guanosine 5′-triphosphate; GST, glutathione *S*-transferase; MESG, 7-methyl 6-thioguanosine; PCR, polymerase chain reaction; Pi, inorganic phosphate

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were tested in parallel against pACT2 without insert to evaluate aspecific interactions. Cells were plated on SD medium lacking tryptophan and leucine. Protein interactions were tested by picking out and streaking transformants on SD lacking tryptophan, leucine and histidine, with 250 μM of 3-amino-1,2,4-triazole. To confirm interactions between Rgd1p and Rho proteins, β-galactosidase assays were done as previously described [2].

2.3. Preparation of Rho and Rgd1 proteins tagged to GST

The plasmid p783 (gift from B. Daignan Fornier, IBGC, Bordeaux), which contains the HA epitope placed before the *Bam*HI site of pGEX-2T (Amersham-Pharmacia Biotech), was used to express the recombinant proteins in *E. coli*. The entire coding sequences for Rho2, Rho3, Rho4 and Rgd1 proteins were PCR amplified and fused in phase to the carboxy-terminus of *Schistosoma japonicum* GST between the *Bam*HI and *Eco*RI, *Sma*I and *Eco*RI, *Bam*HI and *Eco*RI, and *Bam*HI and *Sma*I sites respectively. These constructions were used to transform *E. coli* BL21 (DE3) and expression of GST-Rho and GST-Rgd1 proteins was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside followed by 3 h incubation at 37°C. GST tagged proteins were purified by glutathione-Sepharose affinity chromatography following the manufacturer's protocol (Amersham-Pharmacia Biotech). Briefly, to minimize proteolysis, the following stages were carried out at 4°C and the buffers maintained in ice. The induced bacterial cultures were centrifuged for 10 min at $1700\times g$ and the pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 7.6; 50 mM NaCl; 5 mM $MgCl_2$; 1 mM DTT; 1 mM PMSF). After disruption of the cells by sonication (MSE 150 W ultrasonic disintegrator, probe ∅ 9.5 mm) and centrifugation for 10 min at $6700\times g$, the supernatant was incubated for 40 min with glutathione-Sepharose 4B (Amersham-Pharmacia Biotech). After centrifugation for 10 min at $6700\times g$, the beads were washed twice with lysis buffer without DTT and PMSF and resuspended with the elution buffer (10 mM reduced glutathione; 50 mM Tris-HCl pH 7.6; 5 mM $MgCl_2$; 1 mM DTT; 150 mM NaCl). After a 10 min incubation and centrifugation for 5 min at $1700\times g$, the supernatant was dialyzed overnight against 10 mM Tris-HCl pH 7.6; 2 mM $MgCl_2$; 0.1 mM DTT. The protein purification was checked by SDS gel electrophoresis. The concentration of recombinant proteins was determined by the Bradford method and the preparations were stored at −80°C.

2.4. Spectroscopic measurements of GTPase activity

The chemicals used to synthesize MESG (7-methyl-6-thioguanosine) and the bacterial purine nucleoside phosphorylase were purchased from Sigma. The rates of GTP hydrolysis by the small GTPases were measured by the MESG/phosphorylase system described by Webb and Hunter [20]. The measurement uses a continuous spectroscopic assay for inorganic phosphate (Pi), based on the guanosine analogue MESG, as a substrate for purine nucleoside phosphorylase. This phosphorolysis gives an absorbance increase at 360 nm due to methylthioguanine production; this absorbance wavelength specific for methylthioguanine is well away from the absorbance regions of guanine nucleotides and proteins. So when the phosphorolysis is coupled to GTP hydrolysis, the change in absorbance gives the total amount of Pi released from the G protein and the GTPase activity. The extinction coefficient $\varepsilon_{360\,nm} = 11\,000\ M^{-1}\ cm^{-1}$ at pH 7.6 was used to calculate the Pi concentration. MESG was synthesized in our laboratory according to the procedure described by Broom and Milne [21]. The synthesized MESG purity was checked by monitoring its absorbance spectrum, between 220 and 420 nm [12]. This compound solubilized in 20 mM Tris-HCl pH 7.6 presented a maximal absorbance at 330 nm. The spectrum which was superposable with the one described in the literature showed that the synthesized product was essentially pure. MESG was stored desiccated at −20°C.

GTP binding to the recombinant GST-Rho proteins was carried out in 40 μl solution (25 mM Tris-HCl pH 7.6; 5 mM DTT; 1.2 mM EDTA; 250 μM GTP) containing about 3 μM final for GST-Rho3p and GST-Rho4p, 6 μM final for GST-Rho2p [20]. The reactional mixture was incubated for 10 min at 30°C. The binding reaction was then blocked by adding 5 mM $MgCl_2$; the addition of $MgCl_2$ established single turn over GTP hydrolysis conditions [20]. GTP hydrolysis was then monitored by adding to the reaction mixture 5 μl MESG solubilized in 20 mM Tris-HCl pH 7.6, at 200 μM final concentration and 1.25 unit of purine nucleoside phosphorylase. The GAP activity was measured by adding different amounts of GST-Rgd1p to the mixture. The final volume was adjusted to 120 μl with 10 mM Tris-HCl pH 7.6; 2 mM $MgCl_2$; 0.1 mM DTT. The reaction mixture was immediately transferred into a 10 mm pathlength quartz cuvette at 30°C. GTP hydrolysis by Rho proteins was directly related to the appearance of methylthioguanine; this compound was quantified by measuring the absorbance at 360 nm with a spectrophotometer Uvikon 922 (Kontron) equipped with a data processing system.

3. Results and discussion

3.1. Determination of GAP activity of Rgd1p

To identify interacting Rho proteins, we systematically tested Rgd1p against the GTP constitutive form of the Rho proteins by two hybrid analysis (Table 1). The use of the activated forms exacerbated interactions between small G proteins and their GAP. Growth examination of the cotransformed HY strains on selective medium revealed two-hybrid interactions between Rgd1p and the Rho2, Rho3 and Rho4 proteins. However, concerning Rho2p, the control strain grew slightly on this medium and the growth difference with the assay was less pronounced compared to the clear difference observed for Rho3p and Rho4p. These two-hybrid interactions between the Rgd1 protein and Rho2p, Rho3p and Rho4p were confirmed using a β-galactosidase test. Then, the recombinant proteins Rgd1, Rho2, Rho3 and Rho4 tagged with GST were expressed in *E. coli* and purified using glutathione-Sepharose affinity chromatography.

The GTP hydrolysis performed by the Rho proteins was analyzed using the MESG/phosphorylase assay developed by Webb and Hunter [20]. Briefly, the Pi released during the nucleotide hydrolysis was collected by the phosphorylase enzyme and then reacted with the MESG to produce methylthioguanine. Measurements of the absorbance at 360 nm were taken to detect methylthioguanine levels. The phosphorolysis reaction was essentially irreversible and the system MESG/phosphorylase acted consequently like an inorganic phosphate sensor [12]. Using various Pi amounts from 0.5 μM to 20 μM, we verified that the appearance of the methylthioguanine correlated well with the Pi concentration. Thus, the MESG/phosphorylase system made it possible to measure the GTP hydrolysis activity. The GTP hydrolysis for Rho2, Rho3 and Rho4 proteins after nucleotide binding was assayed in four experimental conditions: (i) GST alone, (ii) GTP bound GST-Rhop, (iii) GTP bound GST-Rhop with GST, (iv) GTP bound GST-Rhop with GST-Rgd1p. The GTP hydrolysis kinetics by the Rho proteins were monitored for 5 min under single turn-over conditions [12]. The kinetics obtained for the

Table 1
Two-hybrid test between Rgd1p and Rho yeast proteins

	$Gal4_{BD}$-CDC42	$Gal4_{BD}$-RHO1	$Gal4_{BD}$-RHO2	$Gal4_{BD}$-RHO3	$Gal4_{BD}$-RHO4	$Gal4_{BD}$-RHO5	$Gal4_{BD}$
$Gal4_{AD}$-RGD1	−	−	+	++	++	−	−
$Gal4_{AD}$	−	−	+/−	−	−	−	−

Assay conditions were described in Section 2. The table gives the results obtained on the selective medium. −: no growth; +/−, +, ++: different growth levels from minimal to maximal growth.

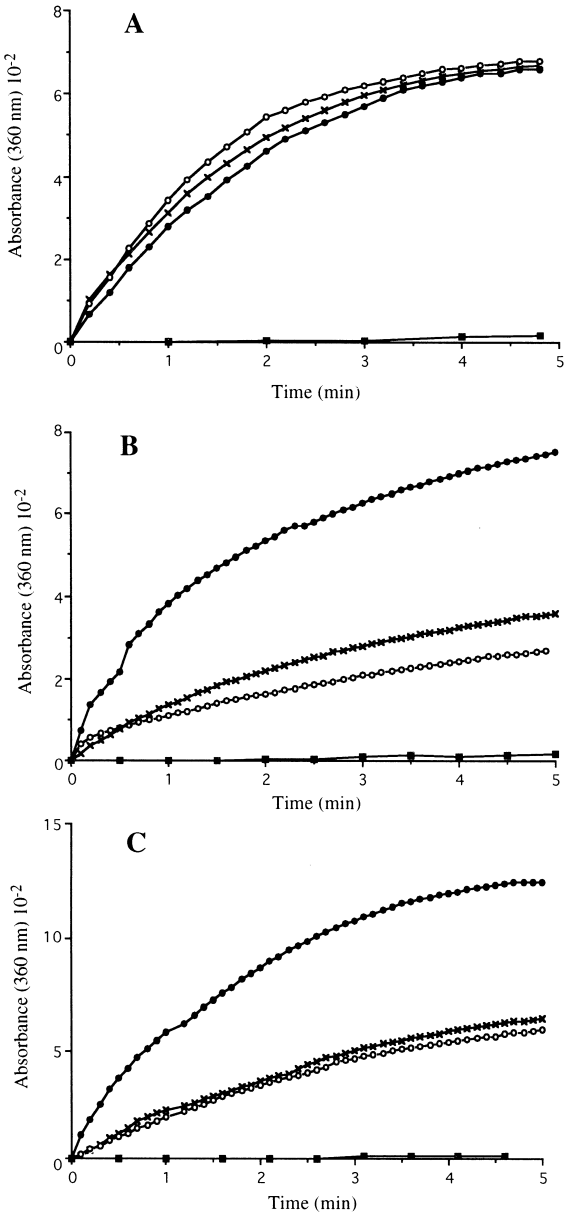

Fig. 1. Activation by Rgd1p of GTP hydrolysis performed by the Rho proteins. γPi released from GTP-bound Rho proteins was measured by the continuous spectroscopic assay of MESG/phosphorylase system as described in Section 2. A: 2.1 μM GST-Rho2p. B: 1.3 μM GST-Rho3p. C: 1.13 μM GST-Rho4p. GST and GST-Rgd1p were added at 50 nM final. The absorbance was monitored during the time course at 360 nm in four experimental conditions: ■: GST alone; ○: GTP bound GST-Rho; ×: GTP bound GST-Rho with GST; ●: GTP bound GST-Rho with GST-Rgd1p.

three GST-Rho proteins were reproducible and the results are shown in Fig. 1. The curves fitted to a single exponential function represented a pseudo first-order reaction. The Rho protein concentrations, which were around 1 μM, may be considered very low compared to the K_m of the phosphorylase for Pi (26 μM). Moreover, MESG was in excess compared to the amount of GTP bound Rho proteins and its concentration was thus considered to be invariable during the reaction time course. Therefore it should be possible to characterize the kinetics of hydrolysis by their constant rate from the slope after linearization of the experimental curves. However, linearization of curves by logarithmic regression was not possible under our conditions. The plateau obtained after 10 min reaction at 30°C did not correspond to the end of the hydrolysis reaction; this could be due to thermal instability of the MESG in the spectrophotometer cuvette [12]. Therefore we used the initial rates of reactions as the kinetic data to determine the GTP hydrolysis level. This initial rate was only related to the Pi concentration. The initial rate was first calculated from the absorbance variation as a function of time. This value was fitted to Pi and Rho protein concentrations according to the equation: initial rate = $(\Delta A/\text{min}) \times 1/\varepsilon_{360} \times 1/[\text{Rho}]$. Whereas no GTP hydrolysis was observed in the absence of Rho proteins, the initial rates of the intrinsic GTP hydrolysis by GST-Rho2p, GST-Rho3p and GST-Rho4p were 1.06 μM, 0.88 μM and 1.09 μM of Pi released/min for 1 μM of GST-Rhop respectively. These results showed that the recombinant Rho2, Rho3 and Rho4 proteins had a similar intrinsic GTPase activity in vitro. The GAP activity of the Rgd1 protein was first tested by adding 50 nM of GST-Rgd1p. Whereas the addition of GST in the reaction mixture did not modify the GTP hydrolysis level, the GST-Rgd1p addition induced a significant increase in GTP amount hydrolyzed by Rho3p and Rho4p. The initial rate in the presence of Rgd1p was 2.08 μM of Pi released/min for 1 μM of GST-Rho3p and 4.58 μM of Pi released/min for 1 μM of GST-Rho4p. No such increase was observed with Rho2p; in the

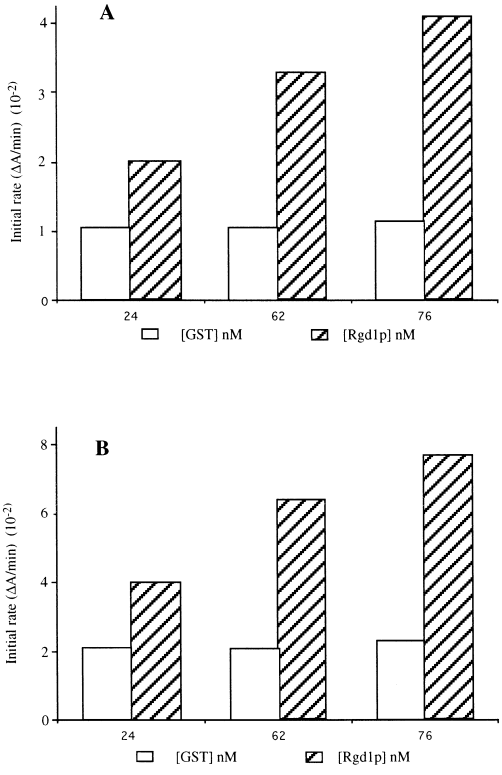

Fig. 2. Effect of increasing concentrations of GST and Rgd1p on the initial rate of GTP hydrolysis by Rho proteins. The experimental conditions were as in Fig. 1 and the initial rate was calculated as the ratio ΔA/min. A: 0.98 μM GST-Rho3p. B: 0.85 μM GST-Rho4p. open bars: initial rate of GTP hydrolysis by GST-Rho proteins with GST; hatched bars: initial rate of GTP hydrolysis by GST-Rho proteins with GST-Rgd1p. The concentrations of Rgd1 used were 24, 62 and 76 nM; equivalent amounts of GST were added in control experiments.

presence of GST-Rgd1p, the initial rate of 0.97 μM of Pi released/min/μM GST-Rho2p was very close to that determined without GST-Rgd1p. In addition no GTP hydrolysis was observed when GST-Rgd1p was added alone to the reaction mixture containing GTP (data not shown).

In order to determine the level of GTP hydrolysis in function of increasing Rgd1 protein concentrations, additional kinetics were established with the protein GST-Rho3 and GST-Rho4 (Fig. 2). The hydrolysis reaction was performed in the presence of 24, 62 and 76 nM GST-Rgd1p. The control experiments were done with the same amounts of GST. Whereas the initial rate of GTP hydrolysis remained relatively stable when the GST concentration varied, it increased with GST-Rgd1 concentrations. The values changed from 1.86 to 3.80 μM of Pi released/min/μM GST-Rho3p and from 4.28 to 8.13 μM of Pi released/min/μM GST-Rho4p. These results show that the activation of GTPase activity of Rho3 and Rho4 proteins was dependent on the GAP Rgd1p concentration.

Thus, as predicted by in silico analysis, we demonstrated that the Rho2, Rho3 and Rho4 proteins were functional in terms of GTP hydrolysis. The initial rate of GTP hydrolysis by Rho3 and Rho4 proteins was increased in the presence of the GST-Rgd1 protein and it was dependent on its concentration. These results were in agreement with an in vitro GAP activity of the Rgd1 protein with respect to Rho3p and Rho4p. Concerning the GST-Rho2 protein, there was no activation of GTP hydrolysis by Rho2p in the presence of the Rgd1 protein and this latter is not a GAP for the Rho2 protein.

3.2. Growth study of *rho3Δ rgd1Δ* and *rho4Δ rgd1Δ* double mutants

The highlighting of an in vitro GAP activity of Rgd1p with respect to the yeast Rho3 and Rho4 proteins led us to examine the growth of the *rho3*Δ *rgd1*Δ and *rho4*Δ *rgd1*Δ double mutants in order to see genetic interactions by the appearance of a new phenotype compared to single mutants. Such a study has already been carried out in yeast in the case of the Rho-GAP protein Bem2 and its target the Rho1 protein; it showed a synthetic lethality at 25°C for the double mutant *bem2*Δ *rho1-104*, whereas the single mutant strains are viable [5]. The *RGD1* inactivated strain did not reveal growth defects in YPD rich medium; a small difference in cell viability was observed in minimal medium at the late exponential phase [2]. Matsui and Toh-e [22] reported that the strains inactivated for the *RHO3* gene showed a significant growth defect whereas the *rho4*Δ strain presented a growth comparable to wild type. We found a similar behavior from our inactivated strains for *RHO3* or *RHO4*; nevertheless, in the genetic background of the yeast strain used, the *rho3*Δ growth defect was less dramatic than that reported by Matsui and Toh-e [22]. Double mutants were obtained by crossing the *rgd1*Δ strain with the *rho3*Δ or *rho4*Δ strains. The growth of the double mutants was examined on rich medium by dropping tests (Fig. 3). The *rho4*Δ *rgd1*Δ strain grew as well as the *rho4*Δ and *rgd1*Δ single mutants, whereas the *rho3*Δ *rgd1*Δ double mutant presented a more severe growth defect than did the *rho3*Δ strain indicating the existence of a functional link between *RHO3* and *RGD1*. Similar results were obtained when these different strains were grown on minimal medium (data not shown). The absence of a *rho4*Δ *rgd1*Δ growth defect is not opposed to a genetic interaction between the *RGD1* and *RHO4* genes. It could be envisaged that Rho3p might compensate the *RHO4* inactivation. Indeed, the literature has reported a functional redundancy between Rho3p and Rho4p [10]. These results are in agreement with the GAP activity of Rgd1p on Rho3p as concluded for Bem2 and Rho1p [5] and additional studies will be necessary to integrate these genetic interactions in terms of cellular mechanisms.

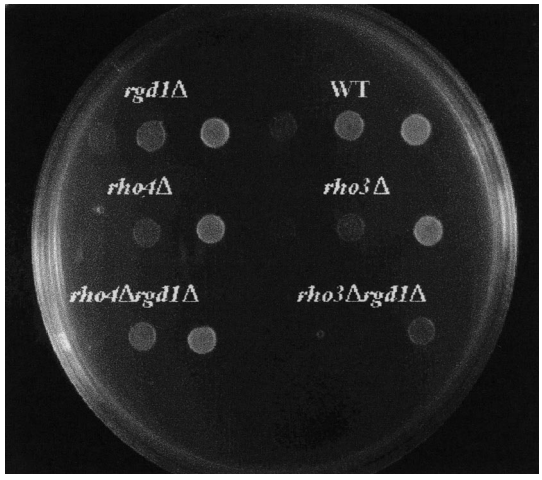

Fig. 3. Growth of single mutant and double mutant strains inactivated for *RHO3*, *RHO4* and *RGD1*. The different yeast cells were dropped on YPD rich medium in 10-fold serial dilutions as indicated on the plate and grown for 1 day at 25°C.

This study demonstrates that the Rho-GAP domain of the yeast Rgd1p is functional. This constitutes the first description of a GAP activity associated with the yeast small GTPase Rho3 and Rho4 proteins. Like these Rho proteins, the *RGD1* gene could be involved in bud enlargement and surface growth in *S. cerevisiae* and could intervene in the control of these cellular aspects.

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