Functional characterization of the Bag7, Lrg1 and Rgd2 RhoGAP proteins from *Saccharomyces cerevisiae*

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Abstract Rho proteins are down-regulated in vivo by specific GTPase activating proteins (RhoGAP). We have functionally studied three *Saccharomyces cerevisiae* putative RhoGAP. By first identifying Rho partners with a systematic two-hybrid approach and then using an in vitro assay, we have demonstrated that the Bag7 protein stimulated the GTPase activity of the Rho1 protein, Lrg1p acted on the Cdc42 and Rho2 GTPases and we showed that Rgd2p has a GAP activity on both Cdc42p and Rho5p. In addition, we brought the first evidence for the existence of a sixth functional Rho in yeast, the Cdc42/Rac-like GTPase Rho5.

Key words: Rho protein; GTPase activating protein; *BAG7*; *LRG1*; *RGD2*; *Saccharomyces cerevisiae*

1. Introduction

Monomeric GTPases of the Ras superfamily are key regulators of a wide variety of eukaryotic cellular functions [1]. They act as biological relays in signal transduction pathways by cycling between a GDP-bound inactive form and a GTP-bound active form. Switch to the inactive form is achieved by the acceleration of their intrinsic slow GTPase activity by a class of regulatory factors named GTPase activating proteins (GAP) [2]. In this, GAP are key regulators allowing the attenuation of specific signalling pathways. The Rho GTPases form a subgroup of small G proteins whose best characterized function is the regulation of actin cytoskeleton dynamics and functioning. Their various cellular activities are down-regulated in vivo by RhoGAP factors [3].

In the yeast *Saccharomyces cerevisiae*, five functional Rho proteins, named Cdc42p and Rho1p–Rho4p, have been described [4]. During the cell cycle, yeast cells assume alternative states of polarized growth, which range from tightly focused apical growth to non-focused isotropic growth. Rho GTPases have been shown to be essential in the establishment and maintenance of cell polarity. More specifically, the essential Cdc42 protein is involved in bud site assembly and polarized growth [5]; Rho1p is an essential protein for actin cytoskeleton dynamics, cell wall synthesis and Pkc1 kinase activity [6,7]; Rho2p function is partially redundant with that of Rho1p [8]; Rho3 and Rho4 proteins are involved in the regulation of exocytosis as well as in actin polarization [9]. In silico analysis of the yeast genome predicted another *RHO* gene [4], termed *RHO5*, but no experimental data have yet been associated with the Rho5 protein. One way to understand how a specific Rho GTPase is able to regulate actin cytoskeleton dynamics and cell polarity is to identify its related GAP proteins. Several GAP activities have already been assigned to *S. cerevisiae* Rho proteins: Bem2p, Bem3p, Rga1p and Sac7p have been shown to act as GAP on Rho1p, Sac7p on Rho2p and Bem3p, Rga1p and Rga2p on Cdc42p [10–13]. Besides, it has been demonstrated in our laboratory that the Rgd1 protein acted as a GAP on both Rho3p and Rho4p [14].

In a computer analysis of the yeast proteome, Bag7p, Lrg1p and Rgd2p (product of the open reading frame YFL047w) were reported to have sequence segments related to a RhoGAP domain. The presence of such a domain in these three proteins led us to explore whether they interact or not with Rho proteins and, if so, if they displayed a GAP activity. We used a two-hybrid approach to investigate the interactions between GTP constitutive forms of yeast Rho proteins and Bag7p, Lrg1p or Rgd2p. The positive interactions were functionally tested by an in vitro spectrophotometric GAP assay. We report here that Bag7p stimulates the intrinsic GTPase activity of Rho1p, that Lrg1p acts in the same way on Cdc42p and Rho2p and that Rgd2p is a GAP for both Rho5p and Cdc42p. In addition, we present the first functional data concerning the Rho5 protein from *S. cerevisiae*.

2. Materials and methods

2.1. Strains and genetic procedures

Escherichia coli BL21 (DE3) (*E. coli* B, F^-, *ompT*, $hsdS_B(r_{B^-}m_{B^-})$, *gal*, *dcm*, (DE3) pLysS) was from Novagen. Yeast genetic procedures and medium preparation were described by Sherman et al. [15]. *S. cerevisiae* strains HY (HF7c/Y187) [16] and PJ69-4A (*MAT***a**, *leu2-3*, *112*, *ura3-52*, *trp1-901*, *his3-200*, *gal4*Δ, *gal80*Δ, *GAL2-ADE2*, *lys2::GAL1-HIS3*, *met2::GAL7-lacZ*) [17] were used for the two-hybrid tests. WCG4-11/22a (*MAT*α, *ura3*, *leu2-3*, *112*, *his3-11*, *15*, *pre1-1*, *pre2-2*) [18] was used to produce glutathione *S*-transferase (GST)-tagged proteins in yeast. The *rho5*Δ strain (*MAT*α, *yn1180c::KanMX4*, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, *ura3*Δ*0*) was from the EUROSCARF consortium and the *cdc42-1* thermosensitive strain was from Barthe et al. [19].

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Abbreviations: ATA, 3-amino-1,2,4-triazole; DEP domain, dishevelled-Egl-10-pleckstrin domain; FCH domain, Fes/CIP4 homology domain; LIM domain, Lin-11-Isl-1-Mec-3 domain; GAP, GTPase activating protein; GST, glutathione *S*-transferase; MESG, 7-methyl-6-thioguanosine; PCR, polymerase chain reaction; Pi, inorganic phosphate; RGS, regulator of G protein signalling

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2.2. Protein sequence analyses

Putative functional domains within proteins were detected using the SMART program [20]. ClustalW 1.8, with a Blosum 30 protein matrix [21], was used to perform multiple sequence alignments between RhoGAP domains of the Bag7, Lrg1 and Rgd2 proteins, and to align Rho5p with representative homologous proteins. The identification of PEST motifs within protein sequences was realized using the PEST-FIND algorithm (PC/Gene, Intelligenetics Inc.); the minimal length of the PEST region was 10 residues and a score greater or equal to 5 was considered significant.

2.3. Yeast two-hybrid analysis

Products for molecular biology were obtained from Promega Corporation and Roche Molecular Biochemicals. The coding sequences for the activated forms of Rho1p–Rho5p (Rho1Q68L, Rho2Q65L, Rho3Q74L, Rho4Q131L and Rho5Q91L) were generated by polymerase chain reaction (PCR) mutagenesis and fused to the Gal4p DNA binding domain of plasmid pODB80 [16]. The activated form of Cdc42p (Cdc42G12V) was generated by PCR mutagenesis and fused to the Gal4p DNA binding domain of pODB8 [16]. The C-terminal CAAX box was deleted to prevent mislocalization of Rho proteins during two-hybrid assays. These constructions were verified by DNA sequencing. Coding sequences for Rgd2p and Lrg1p were fused to the Gal4p activating domain by cloning within the *Nco*I/*Xho*I sites of vector pACT2 (Clontech, Palo Alto, CA, USA). The Bag7p coding sequence was inserted within the *Nco*I/*Bam*HI sites of pACT2. The yeast HY and PJ69-4A strains were co-transformed using the Gietz protocol [22] in order to obtain all the Rho and GAP combinations. The plasmids producing activated forms of Rho proteins were tested against the pACT2 without insert to evaluate aspecific interactions. Reciprocally, plasmids producing putative GAP were tested against plasmids pODB8 or pODB80. Transformed cells were plated on synthetic dextrose (SD) medium lacking tryptophan and leucine. Protein interactions were tested by picking out and streaking transformants on SD lacking tryptophan, leucine and histidine. When using the HY strain, 3-amino-1,2,4-triazole (ATA) was added to the medium at molarities of 0.25, 1, 2, 3.5 or 5 mM. The interaction between Rho1p and Bag7p and the interaction between Cdc42p and Lrg1p were best detected using 2 mM ATA. The interaction between Rho2p and Lrg1p was revealed without ATA. Rho5p and Rgd2p interaction was detected using 3.5 mM ATA. When performing two-hybrid tests with the PJ69-4A strain, interactions were tested in parallel by streaking transformants on SD medium lacking tryptophan, leucine and adenine. In order to confirm detected interactions between RhoGAP and Rho proteins, β-galactosidase assays were performed as previously described [19]. Two-hybrid interactions were confirmed by monitoring growth and β-galactosidase activity on at least 10 independent clones.

2.4. Preparation of Rho and RhoGAP proteins tagged with GST

The plasmid p783 (gift from B. Daignan Fornier, IBGC-CNRS, Bordeaux, France), which contains the hemagglutinin A epitope placed before the *Bam*HI site of pGEX-2T (Amersham Pharmacia Biotech), was used to produce recombinant proteins in *E. coli*. The whole coding sequences of Rho1, Rho2, Rho4, Rho5, Cdc42 and Bag7 proteins were PCR amplified and fused in phase to the carboxyl extremity of the *Schistosoma japonicum* GST between the *Bam*HI and *Eco*RI sites. The entire coding sequences of Rho3p and Rgd2p obtained by PCR were inserted in frame with GST between the *Sma*I and *Eco*RI sites and *Bam*HI and *Sma*I sites respectively. All these constructions were used to transform *E. coli* BL21 (DE3), then GST-Rho and GST-RhoGAP protein production was induced by adding 0.4 mM of isopropyl-β-D-thiogalactopyranoside followed by 3 h incubation at 37°C. GST-tagged proteins were then purified by glutathione-Sepharose affinity chromatography following the protocol we have already described elsewhere [14]. The concentration of recombinant proteins was determined by the Bradford method and the preparations were stored at −80°C.

The plasmid pEG-KT [23] was used to fuse the carboxy-terminal part of the Lrg1 protein (amino acids 651–1017) to GST in *S. cerevisiae*. The coding sequence was inserted between the *Xba*I and the *Hin*dIII sites of the plasmid. WCG4-11/22A cells containing this construction were grown in 1 l of raffinose medium lacking uracil at 30°C to early logarithmic phase. Production of the GST-Lrg1(651–1017) fusion protein was induced by addition of 2% final galactose for 16 h. Cells were harvested, resuspended in ice-cold lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 1% Triton X-100, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, and lysed using a 'Cell Disruptor'. Cell debris was removed by centrifugation at $500\times g$, 4°C, for 10 min. GST-Lrg1(651–1017) protein was precipitated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C with gentle shaking, and centrifuged at $1600\times g$, 4°C for 5 min. After washing five times with lysis buffer and five times with 50 mM Tris–HCl, pH 7.5 [24], the beads were stored at −80°C until use. GST protein used as negative control for GAP assays was produced in yeast and purified using an equivalent protocol.

2.5. 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 [25] that was already used to demonstrate the GAP activity of Rgd1p [14]. Briefly, the measurement uses a continuous spectroscopic assay for inorganic phosphate (Pi) appearance, based on the guanosine analogue MESG, as a substrate for purine nucleoside phosphorylase. This phosphorolysis generates an absorbance increase at 360 nm due to methylthioguanine production. Therefore, phosphorolysis is coupled to GTP hydrolysis and the change in absorbance (ΔA) 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 Pi concentration. MESG was synthesized in our laboratory according to the procedure described by Broom and Milne [26], its purity was checked by monitoring its absorbance spectrum between 220 and 420 nm and it was quantified by measuring maximal absorption at 330 nm ($\varepsilon_{330\ nm} = 32\,500\ M^{-1}\ cm^{-1}$ at pH 7.6).

GTP binding to the recombinant GST-Rho proteins was carried out in 40 μl solutions (25 mM Tris–HCl pH 7.6, 5 mM dithiothreitol, 1.2 mM EDTA, 250 μM GTP) containing 10.24 μM of GST-Rho1p, 4 μM of GST-Rho2p, 10.8 μM of GST-Rho5p and 21.2 μM of GST-Cdc42p. The reaction 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 turnover GTP hydrolysis conditions [25]. GTP hydrolysis was then started 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 U of purine nucleoside phosphorylase. The GAP activity was measured by adding to the mixture various concentrations of purified GST-Bag7p, GST-Rgd2p, or of GST-Lrg1(651–1017) bound to glutathione beads. The final volume was adjusted to 160 μl with 10 mM Tris–HCl pH 7.6, 2 mM $MgCl_2$, 0.1 mM dithiothreitol. 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. Initial rate values were fitted to Pi and Rho protein concentrations according to the equation: $(\Delta A/\text{min})\times(1/\varepsilon_{360\ nm})\times(1/[\text{Rho}])$.

3. Results

3.1. In silico analysis

Computer analysis of the entire yeast proteome has revealed the presence of nine proteins containing a typical RhoGAP signature [20]. Nevertheless, a RhoGAP property has not yet been assigned to all these proteins. Indeed, no biochemical data concerning the GAP activity of the proteins encoded by the *BAG7*, *LRG1* and *YFL047w* genes have been reported. A schematic representation of domains displayed by Bag7p, Lrg1p and Yfl047p is presented in Fig. 1. No domain other than the RhoGAP was detected in the Bag7 protein. Concerning Lrg1p, three Lin-11-Isl-1-Mec-3 (LIM) domains were detected along the sequence. The LIM domain is a zinc finger structure that mediates furtive protein–protein interactions in a variety of fundamental biological processes [27]. In *S. cerevisiae*, except for Lrg1p, only three proteins contain LIM do-

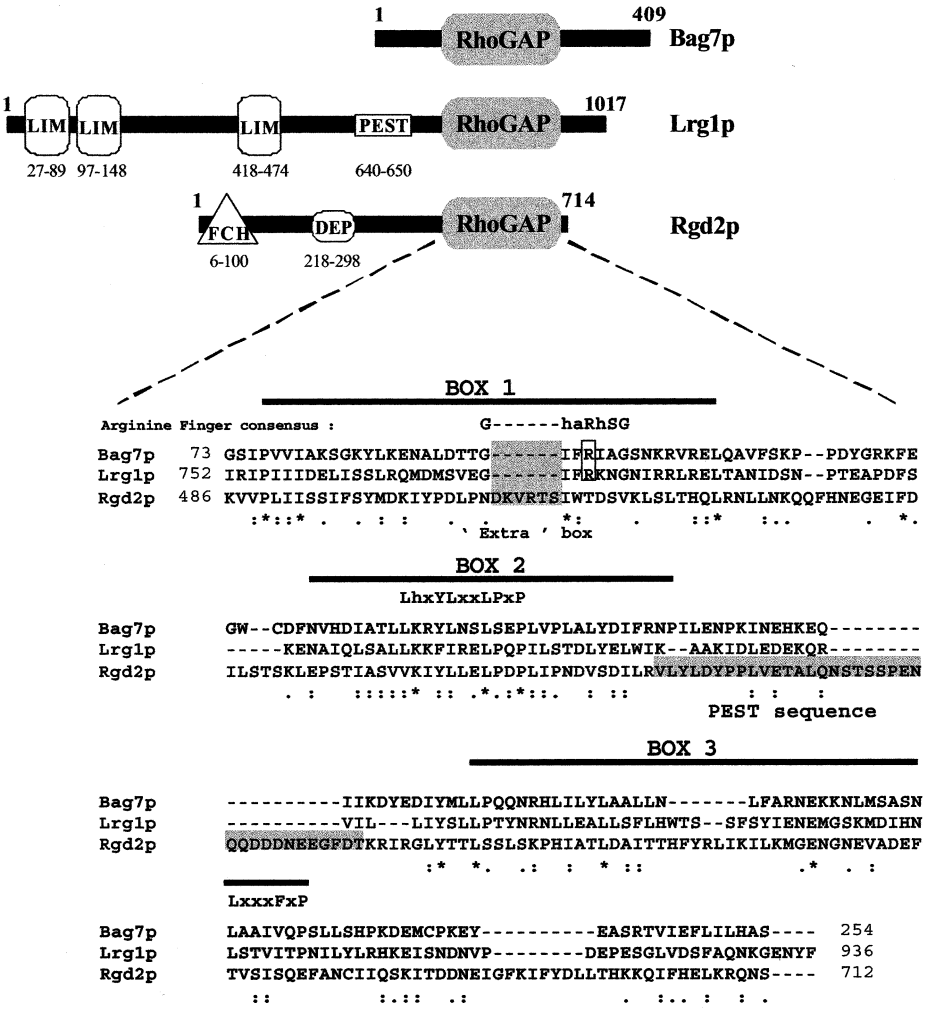

Fig. 1. Schematic representations of domains displayed by the Bag7, Lrg1 and Rgd2/Yfl047 proteins from the yeast *S. cerevisiae*. Protein sequence analyses were performed as described in Section 2. Numbers indicate amino acid positions within proteins. Similar or identical residues from the multiple alignments of RhoGAP sequences are indicated by dots and asterisks respectively. 'Extra' box and PEST sequence detected in the Rgd2p GAP domain are boxed in gray. Consensual boxes are indicated and the typical arginine residue from arginine fingers is surrounded.

mains: one is the Ykr090 protein, whose function is unknown, and the two others are the RhoGAP proteins Rga1 and Rga2. The role of LIM domains in yeast cell signalling has not yet been studied. Moreover, a PEST motif, with a significant score of 9.78, was detected in Lrg1p, suggesting that this protein may be degraded in vivo by a specific mechanism [28]. The Yfl047 protein displayed a Fes/CIP4 homology (FCH) domain at the N-terminal extremity, followed by a dishevelled-Egl-10-pleckstrin (DEP) domain and by a RhoGAP domain at the C-terminal end. The FCH domain is supposed to be involved in interactions with microtubules [29]. The DEP domain [20] is specific for G protein signalling, and is supposed to play a role similar to coiled coil domains in dimerization. The yeast Rgd1 protein, which we have shown to possess a RhoGAP activity [14], displayed an overall organization similar to that observed in Yfl047p [30]. For this reason, we named the gene *YFL047w:RGD2*, for RhoGAP domain 2.

The RhoGAP domain sequences of the three yeast proteins Bag7, Lrg1 and Rgd2 were aligned using the ClustalW program [21]. The multiple alignment revealed the presence of the three typical conserved boxes usually encountered in RhoGAP domains (Fig. 1). Box 1, which contains the arginine finger, has been shown to interact with the P-loop of Rho proteins to allow activation of GTP hydrolysis [31]. Interestingly, the Rgd2p arginine finger shows some discrepancies from the well known consensus sequence GhaRhSG (where h is a hydrophobic amino acid residue and a an aromatic amino acid residue). The Rgd2 protein displays an insertion of six amino acids; this 'extra' box contains an arginine residue that could be involved in GTPase activity acceleration. Nevertheless, an aromatic residue, normally dedicated to the adjacent hydrophobic core stabilization for GTP hydrolysis activation [31], is not found in the proximity of the arginine. Searches in the data banks revealed that such a non-conventional Box 1 is also encountered in the YA2I protein from *Schizosaccharomyces pombe*. YA2I presents the same FCH-DEP-RhoGAP organization as Rgd2p, but no GAP activity has yet been associated with this protein. The regulator of G protein signalling (RGS) proteins, which are negative regulators of heterotrimeric G proteins, have been shown to activate GTP hydrolysis by the Gα subunit while lacking a

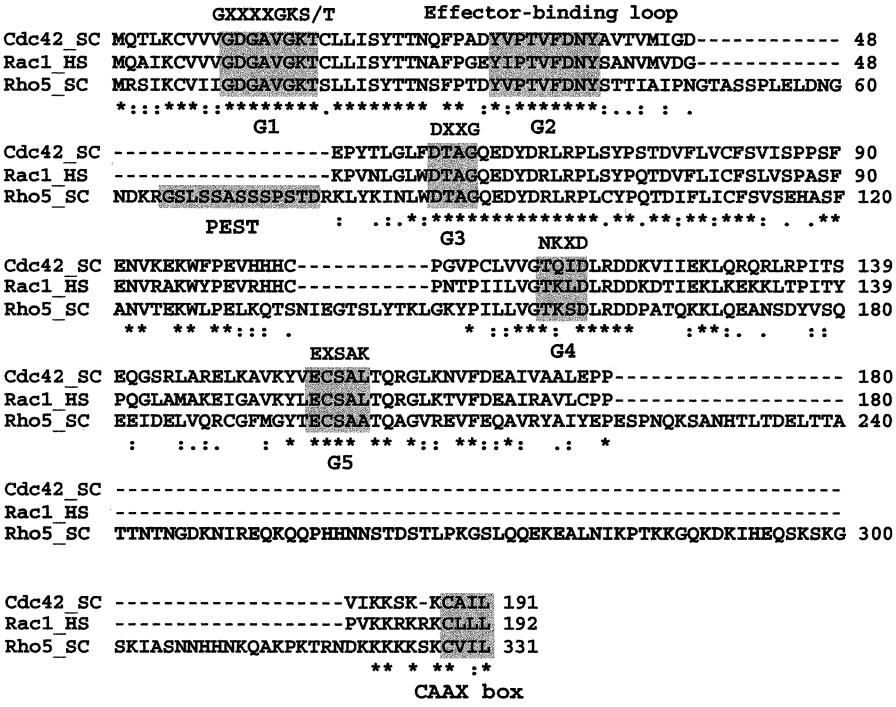

Fig. 2. Rho5p appears as a Cdc42/Rac-like GTPase of *S. cerevisiae*. ClustalW 1.8 was used to align Rho5p (Rho5_SC, SwissProt accession number P53879) with the yeast Cdc42 (Cdc42_SC, SwissProt accession number P19073) and human Rac1 (Rac1_HS, SwissProt accession number P15154) proteins. The P-loop forming domains (G1, G3, G4 and G5), the G2 effector domain, Rho5p PEST sequence and C-terminal CAAX boxes are shaded in gray. Consensus sequences are shown at the top of shaded boxes. Similar or identical residues are indicated by dots and asterisks respectively. Numbers indicate amino acid positions in proteins.

typical arginine finger. We observed that Rgd2p and YA2I did not present any sequence similarity with the RGS proteins, nor any of the typical RGS domains (data not shown). So the biochemical study of Rgd2 protein will be of great interest to appreciate the ability of such an unusual RhoGAP domain to accelerate GTPase activity. Another Rgd2p particularity is the presence of a PEST motif (score 6.06) within the RhoGAP domain itself, between Box 2 and Box 3. PEST motifs are also encountered in some of the other yeast RhoGAP proteins, but they are always located outside the RhoGAP domain (data not shown).

Concerning the six Rho proteins that were detected in the *S. cerevisiae* proteome (i.e. Cdc42p and Rho1p–Rho5p), no experimental data concerning the Rho5 protein have been reported to date. Systematic expression analyses have shown that *RHO5* is expressed in yeast (*Saccharomyces* Genome Database). We observed that Rho5p shows strong sequence similarities with members of the Cdc42 and Rac families (Fig. 2). Indeed, Rho5p presented 46% identity with the Cdc42 protein from the yeast *S. cerevisiae*, *S. pombe*, *Yarrowia lipolytica*, as well as from human. It is interesting to note that, in contrast to *S. cerevisiae* Cdc42 protein, Rho5p shares around 45% identity with different members of the Rac family. In this, Rho5p appears as a unique Cdc42/Rac-like protein in *S. cerevisiae*. Besides, the G2 effector regions present in *S. cerevisiae* Rho5p and Cdc42p are identical (Fig. 2) suggesting that these two proteins might be in part functionally related. We next observed that Rho5p is the only one of the six yeast Rho proteins to contain a PEST motif (score 8.13) (Fig. 2). The presence of a PEST sequence in a yeast small G protein has already been reported for the Ypt10 Rab protein. It has been shown that the Ypt10p PEST sequence played a role in protein stability, and that Ypt10p degradation is dependent on proteasome activity [32]. In a similar way, we suppose that the PEST sequence is implicated in vivo in regulating Rho5 protein abundance.

3.2. *Bag7p, Lrg1p and Rgd2p physically interact with specific yeast Rho proteins*

Following in silico analysis, we looked for yeast Rho proteins displaying physical interactions with Bag7p, Lrg1p and Rgd2p. It has already been reported that although some proteins exhibit GAP activities for several RhoGTPases in cell-free assays, their substrate specificities in vivo are more restricted [33]. The use of an in vivo two-hybrid approach appeared to be the best way to identify Rho proteins specifically interacting with our putative GAP. Moreover, such an approach has already revealed itself as suitable to detect specific Rho–RhoGAP interactions using the yeast Rgd1 protein as a bait [14]. In order to stabilize interactions between Rho proteins and the three putative GAP, we used GTP blocked forms of the six yeast Rho GTPases. Interactions were then tested using two different yeast two-hybrid strains: strain HY, carrying the *HIS3* and *LacZ* reporter genes, and strain PJ69-4A, carrying the *HIS3*, *ADE2* and *LacZ* genes (Table 1). Growth examination of co-transformed cells on selective medium lacking histidine revealed that Bag7p interacts only with Rho1p, and Lrg1p with both Rho2p and Cdc42p. In the same way, we observed that Rgd2p interacts with Rho5p, and an interaction with Cdc42p was unravelled by the use of the PJ69-4A strain. These positive two-hybrid interactions were confirmed owing to the *LacZ* reporter gene, using β-galactosidase assays; in each case, a significant β-galactosidase activity was observed. We noticed that in our experimental con-

ditions, the ADE2 reporter gene from the PJ69-4A strain showed itself inefficient to put into the light any interactions. The physical interaction detected between Bag7p and Rho1p seems physiologically significant. Indeed, computer analysis showed a 40% identity over 369 amino acids of Bag7p with the yeast protein Sac7, which has been shown to be a GAP for Rho1p. Moreover, it has been shown that Bag7p overproduction suppresses the cold sensitivity of a *sac7* strain [13]. The Lrg1 protein seems to play a role during the mating process in yeast [34], and Cdc42p has also been shown to act during mating projection formation [35]; these data indicate that the interaction between Lrg1p and Cdc42p is significant. Rgd2p interacts with the Cdc42p and Rho5p. These results are in good agreement with the close sequence similarities we have described above between these two GTPases. In spite of the limitations of the two-hybrid system which might have hindered the detection of all the existing interactions, new meaningful protein interactions were revealed.

3.3. In vitro GAP assays

It has been described that in some cases proteins containing a GAP domain are able to bind to a given small G protein without accelerating its intrinsic GTPase activity [36]. In order to link the physical interactions demonstrated by two-hybrid tests with biochemical activities, we have carried out in vitro GAP assays. For this, the six different *S. cerevisiae* Rho proteins as well as Bag7p and Rgd2p were produced in *E. coli* as fusion proteins with GST, and then purified using glutathione-Sepharose affinity chromatography. Concerning Lrg1p, we did not succeed in producing this protein tagged with GST, using plasmid p783, or tagged with the $(His)_6$ epitope, based on the well regulated pET system (Novagen). Even truncated forms of Lrg1p fused to GST or to poly(His) could not be produced in bacteria. As Lrg1p seems detrimental to *E. coli*, we decided to produce this protein fused to GST in the yeast *S. cerevisiae*. Unfortunately, the GST-Lrg1 recombinant protein revealed itself insoluble within the cell. To overcome this problem, we fused to GST a shortened form of Lrg1 (residues 651–1017) lacking the LIM and PEST domains. In these conditions, a fraction of GST-Lrg1(651–1017) was recovered in the soluble fraction and was purified in order to be used in GAP assays.

The GTP hydrolysis performed by the Rho proteins was monitored using the MESG/phosphorylase assay developed by Webb and Hunter [25] and already used in our laboratory to demonstrate Rgd1p GAP activity [14]. The MESG/phosphorylase system acts as an inorganic phosphate sensor, and measurements of the absorbance at 360 nm made it possible to detect methylthioguanine levels which correspond to GTP hydrolysis activity (see Section 2). In our tests, no detectable GTP hydrolysis was recorded without any Rho proteins in the reaction mixtures or in the presence of GST alone (data not shown). The GTP hydrolysis for Cdc42, Rho1, Rho2 and Rho5 proteins after nucleotide binding was assayed in three experimental conditions: (i) GTP-bound GST-Rho, (ii) GTP-bound GST-Rho in the presence of GST, (iii) GTP-bound GST-Rho in the presence of GST-RhoGAP. The GTP hydrolysis kinetics by the Rho proteins were monitored for 2 min under single turnover conditions [37]. The kinetics obtained were reproducible and fitted to a single exponential function representing a pseudo first-order reaction. This is in agreement with the low concentration of Rho proteins compared to the K_m of phosphorylase for Pi (26 μM) and to the large excess of MESG whose concentration is considered to be invariable. We used initial rates of reactions in the kinetic data to determine GTP hydrolysis level as already described [14]. Whereas addition of GST in reaction mixtures did not modify the GTP hydrolysis level, we observed that Bag7p induced a significant increase in GTP hydrolysis by Rho1p (Fig. 3A), and that Rgd2p increased Cdc42p and Rho5p GTPase activities (Fig. 3B). By fitting initial rate values to Pi and Rho protein concentrations, we noticed a variation from 0.25 to 1.18 μM of Pi released/min/μM Rho1p, from 0.25 to 0.59 μM of Pi released/min/μM Cdc42p and from 0.33 to 1.31 μM of Pi released/min/μM Rho5p when corresponding GAP concentrations were increased. Since Rgd2p shows a domain organization similar to that of Rgd1p [30], the Rho3p and Rho4p GAP [14], Rgd2p GAP activity was also tested on these two Rho proteins. No GTP hydrolysis activation was detected either on Rho3p or on Rho4p (data not shown), in agreement with the absence of physical interaction by two-hybrid tests. To determine the level of GTP hydrolysis by Cdc42p and Rho2p in the presence of the yeast-produced Lrg1 protein, initial rates of reactions were established using increasing amounts of GST-Lrg1(651–1017) and GST as negative control. Whereas the initial rate of GTP hydrolysis by Cdc42p or Rho2p remained stable when GST concentration varied, it increased with rising GST-Lrg1(651–1017) concentrations (Fig. 3C). Initial rates fitted to Pi and Rho concentrations changed from 0.34 to 1.37 μM of Pi released/min/μM Cdc42p and from 1.64 to 15.5 μM of Pi released/min/μM Rho2p. These results showed that the carboxy-terminal part of Lrg1p containing the RhoGAP domain displayed GAP activity on both Cdc42p and Rho2p, as predicted by the two-hybrid screening results. GTPase activities enhancement revealed in our tests (four- to nine-fold) may seem low compared with what has been described elsewhere with other RhoGAP. For instance, mammalian RhoGAP shows around 50-fold enhancement with Cdc42 using the MESG/phosphorylase activity [38]. However, differences in functional protein production and preparation may explain the variations of GTPase and RhoGAP activities. Concerning the Dbl-like proteins, difficulties in producing functional proteins in *E. coli* have already been mentioned because of insolubility and improper folding [39]. Furthermore, we have not observed GTP hydrolysis activation of Rho3p or Rho4p in the presence of Rgd2p and no activation of Rho4p GTPase activity in the presence of either Bag7p or Lrg1p(651–1017), in agreement with two-hybrid results (data not shown). In addition, where-

Table 1
Yeast Rho proteins specifically interacting with Bag7p, Lrg1p and Rgd2p in two-hybrid assays

	Bag7p		Lrg1p		Rgd2p	
	HY	PJ69-4A	HY	PJ69-4A	HY	PJ69-4A
Rho1p	++	+	−	−	−	−
Rho2p	−	−	+	++	−	−
Rho3p	−	−	−	−	−	−
Rho4p	−	−	−	−	−	−
Rho5p	−	−	−	−	+	++
Cdc42p	−	−	++	++	−	+

Assay conditions were as described in Section 2 for the two strains HY and PJ69-4A. The table summarizes results obtained by tests on selective medium and by β-gal assays. −: no interaction; +, ++: significant interaction detected.

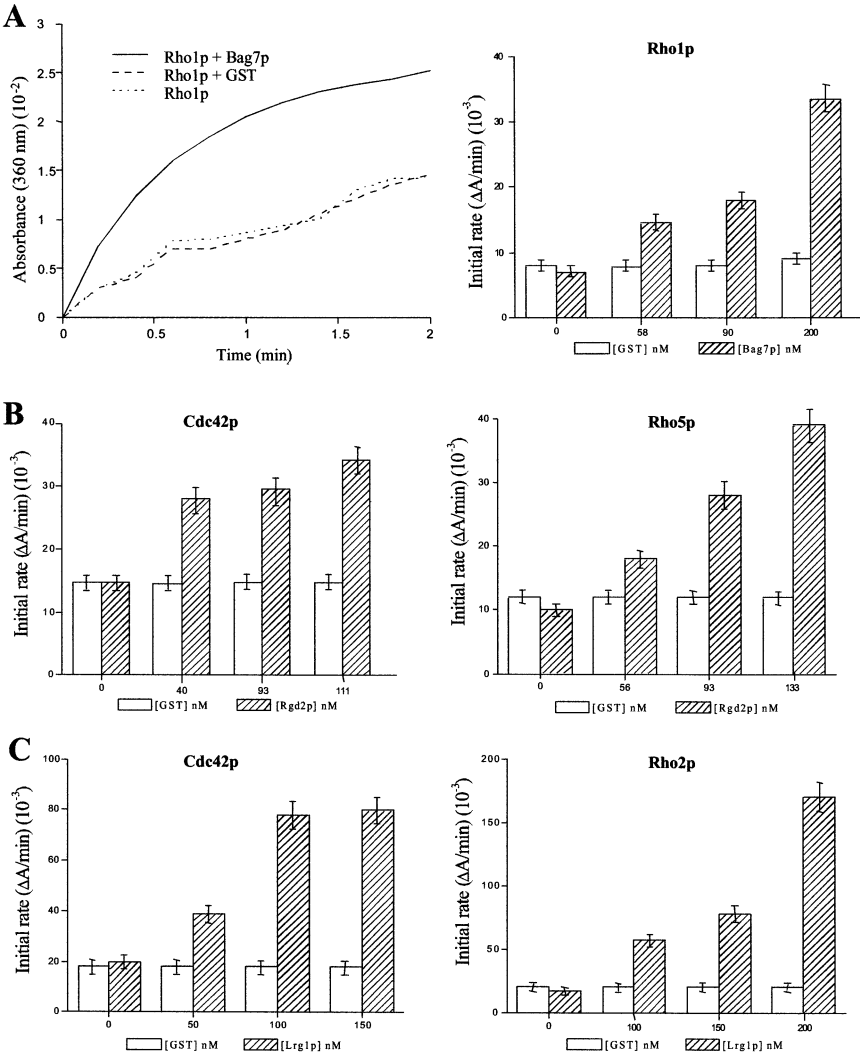

Fig. 3. The Bag7, Lrg1 and Rgd2 proteins display GAP properties in vitro. Pi released from GTP-bound Rho proteins in the presence of GST or RhoGAP was measured by the continuous MESG/phosphorylase spectroscopic assay as described in Section 2, and then initial rates were calculated as the ratio $\Delta A_{360\ nm}$/min. Open bars: initial rates of GTP hydrolysis by Rho proteins in presence of GST; hatched bars: initial rates of GTP hydrolysis by Rho proteins in presence of GST-RhoGAP. A: Example of Rho1p (2.56 μM) GTPase activity acceleration by 200 nM Bag7p as observed by continuous spectroscopic detection (left side); effect of increasing concentrations of Bag7p on the initial rate of GTP hydrolysis by Rho1p (2.56 μM) (right side). B: Initial rates of GTP hydrolysis obtained in the presence of 5.3 μM Cdc42p or 2.7 μM Rho5p and indicated quantities of Rgd2p. C: Effect of increasing concentrations of Lrg1(651–1017) on the initial rate of GTP hydrolysis by Cdc42p (5.3 μM) and Rho2p (1 μM). The results represent the average of three experiments and for each value bars represent the S.D. of all three measurements.

as in vitro GAP assays revealed only a three-fold GTPase activity acceleration [14], in vivo experiments have shown that Rgd1p is indeed a Rho3p/Rho4p GAP [30]. Taken together, all these data allow us to consider that RhoGAp activities reported here are physiologically relevant.

4. Discussion

A fundamental aspect of cell regulation is the ability to modulate protein activity. Rho proteins form a family of well characterized small G proteins that cycle between a GDP-bound inactive form and a GTP-bound active form in the cell. The activity of these proteins is down-regulated by their GTP hydrolysis capacity. Currently, it is well known that specific GAP factors are necessary to accelerate nucleotide hydrolysis in order to inactivate Rho proteins [33]. Here we report data on the characterization of three RhoGAP activities in *S. cerevisiae*. Bag7p appeared to be a GAP for Rho1p and Lrg1p a GAP for Rho2p. Besides, we identified two new RhoGAP for the essential Cdc42 protein, Lrg1p and Rgd2p. The latter GAP also proved to be efficient in activating GTP hydrolysis by the Cdc42/Rac-like Rho5p GTPase. Our results concerning Bag7p are consistent with the fact that this protein shared optimum sequence identity with the Rho1p GAP Sac7p. Moreover, Bag7p overproduction suppresses the cold sensitivity of a *sac7* strain, whereas the other known Rho1p GAP, i.e. Bem2p, Bem3p and Rga1p, could not suppress this phenotype [13]. In addition, we showed by our in vivo two-hybrid tests that Bag7p does not physically interact with any of the other five yeast Rho proteins. All these results indicate that Sac7p and Bag7p GAP activities are partially redundant in the cell. An induction of *LRG1* gene expression in sporu-

lating yeast and a reduced mating efficiency in cells lacking Lrg1p have already been reported [34]. These data and ours indicate that Lrg1p could be required to regulate Cdc42p and/or Rho2p during mating. In particular, it is well known that the Cdc42 GTPase, by inducing actin cytoskeleton reorganization, is essential for the establishment of cell polarity in order to perform cell fusion [35]. Thus, Lrg1 protein, through its GAP activity, may be responsible for Cdc42p down-regulation during mating. Remarkably, the existence of a physical interaction between Lrp1p and Rho1p has recently been reported in a two-hybrid study using the *LexA* detection system [40]. Thus, this and our results strongly support the fact that Lrg1p is a GAP for Rho2p and the two essential yeast GTPases, Cdc42p and Rho1p. Concerning Rgd2p, we demonstrated that this protein, although lacking a consensual arginine finger within its GAP domain, is able to act as a Rho-GAP. The typical Box 1 arginine residue is known to be supplied *in trans* to the catalytic core of Rho proteins in order to participate in the GTP hydrolysis reaction [31]. Some RGS proteins, which are GAP for heterotrimeric G proteins, are known to lack a catalytic arginine finger: in this case, the arginine residue used to stabilize the transition state is supplied *in cis* by a helical domain of the GTPase Gα subunit. According to the literature, GTP hydrolysis mechanisms by Rho and Gα subunits are completely distinct [31]. Our results suggest that the Rgd2p unconventional Box 1, also encountered in the *S. pombe* protein YA2I, represents a new protein sequence enabling GAP activity, for which the structural basis of action remains to be understood.

The *RHO5* gene was originally revealed by in silico analysis of the entire yeast genome [4]. We observed in the laboratory that *RHO5* is not an essential gene, since its inactivation has no specific effect on growth or cell morphology at 30°C or 37°C (data not shown). In this study, we showed that Rho5p is actually a GTPase, with the ability of the bacterially produced protein to hydrolyze GTP. In addition to the presence of a PEST motif, Rho5p sequence analysis also revealed high sequence similarities with Cdc42p, with an effector region which is identical in the two proteins. We also identified Rgd2p as a common RhoGAP regulator for Rho5 and Cdc42 GTPases in yeast. By studying some of the links existing between the *RHO5* gene and the thermosensitive *cdc42-1* allele, we found that the growth characteristics of the *cdc42-1rho5*Δ double mutant strain and the *cdc42-1* strain were identical (data not shown); both strains were able to grow at 30°C and at 37°C in the presence of 1.3 M sorbitol in the medium. Restrictive temperatures were the same for the two strains. In the same way, we observed that overexpressing the *RHO5* gene from a single-copy or a multicopy plasmid did not suppress the thermosensitive growth phenotype of the *cdc42-1* strain (data not shown). These results indicate that, despite their high sequence similarity, Cdc42p and Rho5p should intervene in different cellular aspects. We suppose that although the two GTPases have a common GAP regulator, they interact with distinct effectors within the cell. A challenge for the future is to address if Rho5p physically interacts or not with some of the known Cdc42p effectors, such as Pkc1p or p21 activated kinase.

Table 2
Summary of known GAP regulators for *S. cerevisiae* Rho proteins

Rho GTPase	Regulators with RhoGAP activity
Rho1p	Bag7p (this work), Bem2p [12], Bem3p [10], Lrg1p [38], Rga1p [11], Sac7p [13]
Rho2p	Lrg1p (this work), Sac7p [13]
Rho3p	Rgd1p [14]
Rho4p	Rgd1p [14]
Rho5p	Rgd2p (this work)
Cdc42p	Bem3p [10], Lrg1p (this work), Rga1p [11], Rga2p[a], Rgd2p (this work)

[a]GAP activity deduced from sequence similarities with Rga1p.

Table 2 presents a summary of currently known GAP acting on yeast Rho proteins, based on biochemical and physiological analysis. It is very interesting to note that five to six different GAP activities have been shown to activate GTP hydrolysis by Cdc42p and Rho1p, whereas other Rho are known to be down-regulated by a maximum of two. From the six genes encoding Rho GTPases, *RHO1* and *CDC42* are the two that are essential for yeast survival. They are implicated in a large variety of cellular processes, such as budding, actin cytoskeleton organization or cell wall synthesis [5–7,35]. One can imagine that each GAP is dedicated to negatively regulate a specific Cdc42p- or Rho1p-dependent pathway, and that specificity is achieved through temporal and spatial regulation of RhoGAP. It has been observed that some RGS proteins are regulated by proteolytic processes [41]. For example, the PEST motif-containing protein RGS7 is rapidly degraded through the proteasome pathway [42]. We observed that the yeast RhoGAP Bem2p, Bem3p, Sac7p, Lrg1p and Rgd2p display PEST motifs within their sequences (our unpublished data). Thus, one of the future challenges will be to address if regulation of RhoGAP in yeast uses protein degradation.

It is remarkable that during the course of this study, an enhanced version of the SMART program allowed the identification of two new putative RhoGAP in the yeast proteome, named Ecm25p and Yhr182p (our unpublished data). Although these proteins remain to be studied, it is now obvious that in yeast RhoGAP form a large family of GTPase regulators. The systematic identification of Rho protein regulators and effectors is one of the most promising ways to understand the multiple roles of these small G proteins in *S. cerevisiae*.

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