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The *Saccharomyces cerevisiae* RhoGAP Rgd1 is phosphorylated by the Aurora B like kinase Ipl1

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ABSTRACT

Polarized growth of the yeast *Saccharomyces cerevisiae* depends on different biological processes and requires several signaling pathways. Signaling is mediated through a set of proteins, which include Rho3p and Rho4p GTPases. Although these two proteins are involved in the control of distinct aspects of polarized growth in yeast, they have a common regulator: the Rgd1 RhoGAP protein. Here we demonstrate that Rgd1p is phosphorylated by the Aurora B like kinase Ipl1 and we observe that loss of Ipl1 function leads to a new Rgd1p distribution in a small part of the cell population.

1. Introduction

The small GTPases from the Rho family are involved in a wide variety of cellular functions, including cell polarity, morphogenesis or cell dynamics [1]. GTP-bound Rho proteins can interact with multiple effectors to transduce signals leading to different biological responses, i.e. cell cycle regulation, rearrangements of the actin cytoskeleton, regulation of gene transcription, control of apoptosis or cell trafficking [2–4]. In the yeast *Saccharomyces cerevisiae* six Rho GTPases (Cdc42p and Rho1p-Rho5p) were described to be mainly involved in cell polarity. Among them, Rho3p and Rho4p are involved in the establishment of cell polarity in yeast at the bud tip and bud neck respectively [5–8]. Biological activity of Rho proteins is negatively controlled by RhoGTPase-Activating Proteins (RhoGAPs) [9]. Our previous studies have shown that the RhoGAP Rgd1p is the only protein which activates GTP hydrolysis by Rho3p and Rho4p in *S. cerevisiae* [10]. The Rgd1 protein contains a RhoGAP domain at its C-terminal part (aa 486–666) and an F-BAR domain at its N-terminus (aa 1–300). We have previously reported that Rgd1 protein interacts specifically with phospholipids and that this interaction regulates Rgd1p function both by influencing Rgd1p intracellular localization and RhoGAP activity [11].

Post-translational modifications of RhoGAP proteins are another way to control their function. For example, RhoGAP proteins can be modified post-translationally by phosphorylation [9]. It is the case of the mammalian protein MgcRacGAP ("Male germ cell RacGAP"), essential for cytokinesis and which regulates three distinct Rho proteins: Cdc42, Rac1, and RhoA [12–14]. Phosphorylation of MgcRacGAP by Aurora B kinase prevents the binding of MgcRacGAP to PRC1 protein, thus inhibiting the activity of MgcRacGAP towards Cdc42 [15]. In addition, phosphorylation of MgcRacGAP by Aurora B confers RhoGAP activity towards RhoA which is required for cytokinesis [14]. In the present study we report the phosphorylation of the yeast RhoGAP Rgd1p. Using a screening procedure based on the phosphorylation status of the RhoGAP protein, we have been able to identify one of the kinases involved in Rgd1p phosphorylation.

2. Materials and methods

2.1. Preparation of yeast extract, dephosphorylation and Rgd1p follow-up

Unless otherwise indicated, BY4742 strain (*MATα*, *his3Δ*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*) and derivatives were used throughout this study. Cells grown in YPD medium were harvested during exponential growth phase (OD_{600} of 0.5), washed with distilled water and frozen at −80 °C until use. Frozen cells (3 OD_{600} units) were disrupted with glass beads in lysis buffer (50 mM Tris–HCl pH

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7.4, 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% Igepal, and 1 mM PMSF) supplemented with a protease inhibitor mixture (Sigma) at 4 °C, using a Mini-beadbeater (Biospec Products). Cell lysates were centrifuged at 500*g* for 5 min at 4 °C. The supernatants were collected and kept frozen at −80 °C. Protein concentration was determined with Bradford protein assay (Pierce) using BSA as standard. Dephosphorylation of the protein extract was done during 2 h at 30 °C with 300 units of λ-phosphatase in a buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, and 0.01% Brij 35, as recommended by supplier (New England-Biolabs).

The phosphorylation status of Rgd1p contained in protein extracts was determined based on Rgd1p electrophoretic mobility revealed by using specific antibodies. Protein extract from 10^7 cells was separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose membrane for Western blotting. The Rgd1 protein was detected using polyclonal anti-Rgd1p rabbit antibodies and revealed by chimioluminescence (Lumi-light, Roche). The membrane was scanned using a Fluorchem Camera (Alpha Innotech) and analyzed using Alphaease FCTM software (Alpha-Innotech) to evaluate the relative quantities of phosphorylated and non-phosphorylated forms of Rgd1p.

2.2. Plasmid and strain constructs

The *RGD1* coding sequence was amplified from genomic DNA of the *S. cerevisiae* BY4742 strain, and inserted at the *Nde*I and *Xho*I sites of the pET21a plasmid (Novagen, Madison, WI). The resulting construct, designated pET21a-RGD1, allows the expression of a fusion protein that contains, at the Rdg1p C-terminal end one additional leucine, one glutamic acid and 6 histidine residues. The molecular construction was verified by sequencing. The pGEX-2T-IPL1 allowing expression of a GST-Ipl1p fusion protein in *Escherichia coli*, was kindly provided by Sue Biggins [16].

For the localization of Rgd1p *in vivo*, we constructed a BY4742 strain carrying the *ipl1-321* allele. This allele was amplified from the original mutant in the W303 background [16], and integrated at the *IPL1* locus of BY4742. Then three tandem copies of the GFP gene were integrated at the *RGD1* locus using the linearized yeast vector pRS305-3x GFP carrying the last 800 bp of the *RGD1* coding sequence, and fluorescence microscopy was performed as described [11].

2.3. Production and purification of Rgd1p-6x His and GST-Ipl1p

Escherichia coli strain BL21 (DE3) was transformed with the pET21a-RGD1 plasmid, and Rgd1p-6x His expression was induced with 0.5 mM isopropyl-β-D-galactopyranoside [17] during 3 h at 37 °C. Cells were harvested, washed with distilled water and stored at −80 °C until use. For purification of Rgd1p-6x His, the cell pellet was thawed, suspended in lysis buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 1 mM DTT, 0.1% Igepal, 20 mM imidazole, 1 mM PMSF) and sonicated 4 times for 30 s on ice (ultra sonic processor, 130 W, 20 kHz, Vibra Cell) at 80% amplitude; the tubes were kept on ice for 30 s between each sonication step. The crude lysate was centrifuged for 10 min at 4000*g* at 4 °C and using a batch procedure, the supernatant was incubated for one hour at 4 °C with 1 mL Ni-NTA resin (Qiagen) previously equilibrated with wash buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 20 mM imidazole). The Ni-NTA resin was washed four times with the wash buffer (4 mL) and the retained proteins were eluted by incubating the resin twice with the elution buffer (50 mM Tris–HCl pH 7.4, 50 mM NaCl, 200 mM imidazole) during 10 min. Ni-NTA resin was removed by centrifugation. Purity of the material was checked by SDS–PAGE electrophoresis (Fig. S1) and protein concentration was determined using Bradford protein assay (Pierce). GST-Ipl1p fusion protein expression and purification were as described previously [10].

2.4. Ipl1p kinase assay

Ipl1p kinase assay was adapted from Biggins et al. [16]. Kinase activity was assayed for 15 min at 30 °C in 25 µl kinase reaction buffer containing 50 mM Tris–HCl (pH 7.4), 25 mM β-glycerophosphate, 1 mM DTT, 10 µM ATP, 5 mM $MgCl_2$, 1 µCi (37 kBq) of [γ-^{32}P] ATP (Perkin Elmer), with purified GST-Ipl1 or GST proteins at 0.7 µM final concentration. Myelin basic protein (Sigma), dephosphorylated casein (Sigma) or Rgd1-6x His was added to the reaction mixture at 2 µM final concentration. Reactions were stopped with 25 µl of 2× Laemmli buffer (200 mM Tris–HCl pH 6.8, 5% β-mercaptoethanol, 3% SDS, 30% glycerol, 0.001% bromophenol blue) and the samples boiled for 5 min. Samples were loaded on a denaturing 10% polyacrylamide gel and proteins were stained with Coomassie blue. Gels were exposed to "Imaging plate" (Fujifilm) for several days and radioactivity detection was achieved with a FUJI-fla 5000 apparatus.

3. Results

3.1. Rgd1p is a phosphorylated protein

Our aim was to investigate some of the mechanisms by which RhoGAP Rgd1p controls the activity of Rho3p and Rho4p. Previous studies had shown that levels of *RGD1* transcripts remained about constant during exponential growth in standard growth conditions [18]. Also, steady-state levels of Rgd1p did not vary significantly during cell cycle (data not shown). Therefore, we hypothesized that Rgd1p might be regulated at the post-translational level. Given that phosphorylation can regulate RhoGAP in mammals, we analyzed the phosphorylation status of Rgd1p. Anti-Rgd1p antibodies revealed at least two bands (Fig. 1 lane 1) after Western blot analysis of crude protein extracts, suggesting that a fraction of Rgd1p might be phosphorylated. To verify this hypothesis, yeast cell extracts were dephosphorylated with λ-phosphatase. As shown in lane 2, incubation of cell extract with λ-phosphatase led to a shift in Rgd1p electrophoretic mobility, resulting in the disappearance of the highest band. In contrast, incubation of cell extract with λ-phosphatase in the presence of a cocktail of phosphatase inhibitors (Sigma) did not lead to any change in Rgd1p electrophoretic profile (lane 3). Therefore, these results show that the Rgd1p highest band corresponds to a phosphorylated form of Rgd1p and indicate that at least part of Rgd1p is phosphorylated *in vivo*.

3.2. Ipl1p: a kinase involved in Rgd1p phosphorylation

To identify kinases involved in Rgd1p phosphorylation, Rgd1p electrophoretic behavior was examined in mutant strains for genes encoding proteins that belong to the yeast kinome. Signals corresponding to the upper band (highest phosphorylated form of Rgd1p) and the lower band (less or not phosphorylated form) were

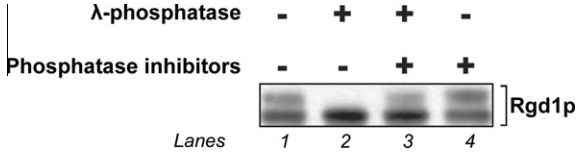

Fig. 1. Rgd1p is a phosphorylated protein. Protein extracts from BY4742 strain were treated or not with λ-phosphatase (300 units), and with or without phosphatase inhibitors. Extracts were separated by SDS–PAGE and Rgd1p was detected by Western blot using α-Rgd1p antibodies.

quantified and the intensity ratio (upper/lower bands) was calculated. This ratio showed only very little variations in BY4742 strain ($n = 5$). However, it was found to vary depending on strain background. Therefore, we normalized the ratio observed in a given mutant by dividing ratio in mutant by ratio in respective parent strain. If Rgd1p phosphorylation is not affected in a mutant, then this normalized ratio will be equal or close to one. For non-essential kinases, we used 123 mutant strains (in BY4742 background) from the Euroscarf library (http://www.euroscarf.de), to which were added 9 strains carrying each a heat sensitive mutation for an essential kinase. Experiments with these heat sensitive strains were carried out at restrictive temperature. Out of 132 mutants tested, 12 showed Rgd1p ratios significantly lower than control, suggesting an influence of the kinase mutation on the phosphorylation status of Rgd1p. We focused our efforts on a mutant impaired for Ipl1 kinase. The *ipl1-321* heat-sensitive mutant exhibited a strong decrease in the normalized ratio (0.4) (Fig. 2) when grown at 37 °C. A slight effect (ratio 0.75) was also noted when the strain was grown at 30 °C. Thus, these results suggest that Ipl1p is likely involved in Rgd1p phosphorylation. The Ipl1p kinase belongs to the Aurora kinase family, which are Serine/Threonine kinases involved in mitotic regulation of various aspects of mitosis and cell cycle, such as segregation of chromosomes and chromatin condensation [19–24]. Ipl1p is the only member of this family in the yeast *S. cerevisiae*. The involvement of Ipl1p in cell cycle is consistent with the role of target proteins of Rgd1p: the Rho3p and Rho4p GTPases which are known to act at different spatial locations during cell cycle.

3.3. Ipl1p phosphorylates Rgd1p in vitro

Ipl1p might phosphorylate either Rgd1p or another kinase that would in turn phosphorylate Rgd1p. The human Aurora B kinase, homolog of Ipl1p, phosphorylates the RhoGAP domain of MgcRacGAP. The GAP domain of MgcRacGAP protein presents a significant similarity (43%) with the RhoGAP domain of Rgd1p (aa 486–666). This suggested that lpl1p kinase might act similarly on Rgd1p. Therefore, GST-Ipl1p kinase activity was assayed using Rgd1p-6x His as substrate in presence of radiolabeled [γ-^{32}P] ATP. Myelin basic protein, which is known to be a substrate of Ipl1 kinase was used as positive control (Fig. 3 lane 3) and casein (Fig. 3 lane 4) as negative control [16,25]. As expected, GST-Ipl1p phosphorylated Myelin basic protein but not casein showing the kinase activity of Ipl1p (Fig. 3 lanes 3, 4). In addition, we observed the autophosphorylation of Ipl1p (lanes 1, 3, 4) already described in the literature [16]. When Rgd1p-6x His was included in the assay, an additional radioactive signal co-migrating with unlabeled Rgd1p-6x His was observed (lane 1). Consistently, when GST was substituted for GST-Ipl1p, no signal was detected (lane 2). Thus, Ipl1p kinase can phosphorylate Rgd1p *in vitro*, suggesting that it might act directly on Rgd1p *in vivo*.

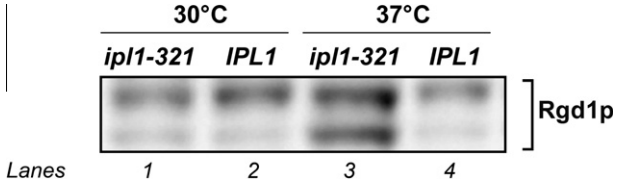

Fig. 2. Involvement of the Ipl1p kinase in Rgd1p phosphorylation. Protein extracts from the *ipl1-321* heat-sensitive mutant and the *IPL1* reference strain, grown at permissive temperature (30 °C) or restrictive temperature (37 °C) were separated by SDS–PAGE. Rgd1p was detected by Western blot.

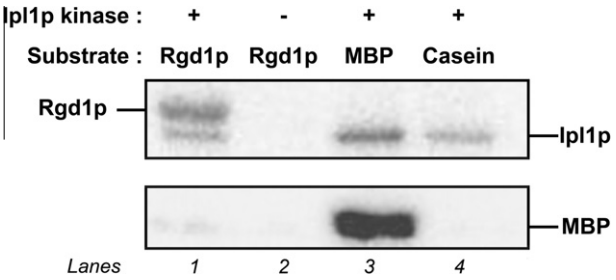

Fig. 3. Ipl1p phosphorylates Rgd1p. Kinase assays were performed using the GST-Ipl1p kinase (Ipl1p, molecular mass = 62 kDa) (lanes 1, 3, 4) or GST (25 kDa) as a control (lane 2). The substrates were Rgd1p-6x His (Rgd1p, 75 kDa), Myelin basic protein (MBP, 19 kDa) and Casein (25 kDa) at 2 μM final concentration. Autophosphorylation of GST-Ipl1p and phosphorylation of Rgd1p-6x His and MBP by GST-Ipl1p are indicated on the side.

3.4. Inactivation of Ipl1p kinase affects Rgd1p localization

The distribution of Rgd1p-3x GFP was analyzed by fluorescence microscopy in the *ipl1-321* heat-sensitive strain as well as in the *IPL1* reference strain, both being in BY4742 background as this background is more appropriate than that of W303 for fluorescence microscopy observation. Strains were grown at permissive temperature (26 °C) and shifted for three hours to the restrictive temperature (37 °C). After three hours at restrictive temperature, cell mortality of heat-sensitive mutant was low and similar to that observed for the reference strain. In the *IPL1* reference strain, at both 26 and 37 °C, Rgd1p was localized at the sites of polarized

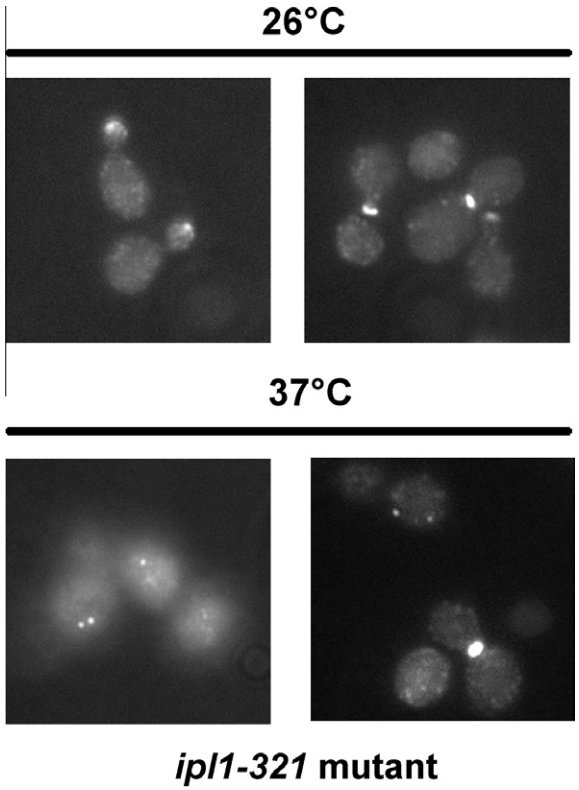

Fig. 4. Involvement of the Ipl1p kinase in Rgd1p localization. Rgd1-3x GFP was observed by fluorescence microscopy in *ipl1-321* strain in BY4742 background at permissive (26 °C) and after three hours at restrictive temperature (37 °C). The images show Rgd1p mislocalization for one part of *ipl1-321* cells at 37 °C.

growth (data not shown) as previously described [11]: during isotropic bud growth (G1 and S phases), Rgd1p was detected at the bud tip, forming intensely fluorescent patches below the cortex; during cytokinesis, Rgd1p was undetectable at the bud tip and was localized at the bud neck only. Next, we investigated the effect of Ipl1p inactivation on the subcellular distribution of Rgd1p. The heat-sensitive mutant displayed normal Rgd1p localization at permissive temperature (Fig. 4). Shifting *ipl1-321* mutant cells to restrictive temperature resulted in changes of Rgd1p distribution in about 5% of the cell population (Fig. 4): one or two intense Rgd1p spots were observed and they were not localized at growth areas. These changes could be reproducibly observed in 2–300 cell samples each obtained from 3 independent clones. This observation was consistent with a functional relationship between Ipl1p and Rgd1p. Rgd1p localization defect may result from the lack of Rgd1p phosphorylation by the Ipl1 kinase.

4. Discussion

In this work, we showed that the yeast Rgd1 protein is phosphorylated and that the Ipl1p kinase is involved in this phosphorylation process *in vivo*. Moreover we demonstrated that Ipl1p directly phosphorylates Rgd1p *in vitro*, suggesting that it does so *in vivo* also. In the future, mass spectrometry analysis should allow us to identify the sites of Rgd1p that are phosphorylated by Ipl1p. The yeast Ipl1 kinase/Rgd1 system is reminiscent of that of human Aurora B kinase/MgcRacGAP. The GAP domain of Rgd1p shows best homology to that of MgcRacGAP and Aurora B kinase phosphorylates MgcRacGAP which is involved in polarized growth and cytokinesis [13]. MgcRacGAP regulates several Rho proteins: Cdc42, Rac1, and RhoA [12–14] and its specificity for Rho proteins comes in part from the MgcRacGAP phosphorylation by the kinase Aurora B [14,15]. By analogy, it might be hypothesized that Ipl1p regulates the activity of Rho3p and Rho4p *via* Rgd1p phosphorylation. *In vitro* measurement of GAP activity of both Ipl1p-phosphorylated and non phosphorylated forms of Rgd1p should allow us to test this hypothesis.

We also observed a change of Rgd1p localization in some *ipl1-321* mutant cells. Interestingly, the same mislocalization was also observed in a *fyv8* mutant (unpublished data). The Fyv8 protein which seems implicated in chromosome segregation and stability [26,27] was shown to interact with Rgd1p using several approaches (http://thebiogrid.org). We hypothesize that the Rgd1p mislocalization observed in the *ipl1-321* mutant could correspond to a transient and cell cycle-dependent Rgd1p localization in wild type, in connection with the Ipl1p kinase activity. A detailed analysis of the Rgd1p localization during the cell cycle performed from synchronous cell culture of both *ipl-321* mutant and wild type strain should help us to investigate this hypothesis.

Since Rgd1p appears to be regulated by phosphorylation, it might as well being subject to dephosphorylation. Bud14p, a regulatory subunit of the type I phosphatase Glc7p, physically interacts with Rgd1p [28,29]. The Glc7 phosphatase is known to regulate several targets of the Ipl1p kinase antagonistically [30]. One function of Bud14p is to locate the Glc7 phosphatase, allowing Glc7p to interact with its targets, and thereby dephosphorylate those [28]. Preliminary results indicate that Bud14 and Glc7 proteins are required for Rgd1p localization: Rgd1p was not properly localized in the absence of Bud14p or of a functional Glc7p (unpublished data). Therefore it might be that the Bud14/Glc7 complex dephosphorylates the sites of Rgd1p phosphorylated by Ipl1p. Thus, Rgd1p might be regulated through a dynamics of phosphorylation/dephosphorylation which might control in part Rho3 and Rho4 GTPase activities. Further investigations are needed to explore this regulation model of Rgd1p action.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.081.

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