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Evidence for specific interaction between the RhoGAP domain from the yeast Rgd1 protein and phosphoinositides

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ABSTRACT

The Rho GTPase activating protein Rgd1 increases the GTPase activity of Rho3p and Rho4p, which are involved in bud growth and cytokinesis, respectively, in the budding yeast *Saccharomyces cerevisiae*. Rgd1p is a member of the F-BAR family conserved in eukaryotes; indeed, in addition to the C-terminal RhoGAP domain Rgd1p possesses an F-BAR domain at its N-terminus. Phosphoinositides discriminate between the GTPase activities of Rho3p and Rho4p through Rgd1p and specifically stimulate the RhoGAP activity of Rgd1p on Rho4p. Determining specific interactions and resolving the structure of Rgd1p should provide insight into the functioning of this family of protein. We report the preparation of highly pure and functional RhoGAP domain of Rgd1 RhoGAP domain using a high yield expression procedure. By gel filtration and circular dichroïsm we provide the first evidences for a specific interaction between a RhoGAP domain (the RhoGAP domain of Rgd1p) and phosphoinositides.

1. Introduction

The small GTPases of the Rho family trigger a large variety of cellular functions including cell polarity, morphogenesis or cell dynamics [1]. Rho proteins in the GTP-bound active state can interact with several effectors to transduce signals leading to different biological responses including cell cycle regulation, actin cytoskeletal rearrangements, regulation of gene transcription, control of apoptosis and membrane trafficking [2–4]. In *Saccharomyces cerevisiae* six Rho GTPases (Cdc42p and Rho1p–Rho5p) have been described being mainly involved in cell polarity. Rho proteins are regulated by **Rho G**TPase-**A**ctivating **P**roteins (**RhoGAPs**) [5]. Previous work demonstrated that Rgd1p is the only RhoGAP shown to increase GTP hydrolysis by Rho3p and Rho4p in *S. cerevisiae* [6]. Rho3p and Rho4p are involved in the establishment of cell polarity at the bud tip and bud neck in yeast cell, respectively [7–10]. The Rgd1 protein contains a RhoGAP domain at its C-terminal part (aa 486–666) and an F-BAR domain at its N-terminal extremity (aa 1–300). Recently, we reported a specific interaction between phospholipids, Rgd1p and the Rho3p and Rho4p GTPases [11]. We demonstrated that phosphoinositides discriminate between the GTPase activities of Rho3p and Rho4p through Rgd1p and specifically stimulate the RhoGAP activity of Rgd1p on Rho4p. Phosphoinositides were also shown to control the recruitment of Rgd1p to membranes via the F-BAR domain throughout cell cycle [11]. Taken together these data are consistent with functional interplay between lipids, the RhoGAP domain and its related GTPases in yeast growth and suggest subtle interactions between phosphoinositides and the whole Rgd1 protein. Our goal was then to investigate interactions between phospholipids and Rgd1p using chromatographic and liquid NMR approaches. As the size of the entire Rgd1 protein (666 aa) was a strong technical limitation for NMR approach, we first decided to explore interactions between the Rgd1p RhoGAP domain alone and phospholipids. For this purpose, the RhoGAP domain of Rgd1p was produced and purified, and then protein interaction was assayed with phospholipids. Here we describe the strategy used to obtain a biologically active RhoGAP domain of Rgd1p suitable for biological and structural investigations including NMR and show direct interactions between phosphoinositides and the RhoGAP domain of Rgd1p using gel filtration and circular dichroïsm approaches.

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2. Materials and methods

2.1. Obtaining the RhoGAP domain of Rgd1p tagged with 6× His

A truncated form of Rgd1p, ranging from aa 450 to aa 666 (C-terminus) with conserved RhoGAP catalytic activity was previously produced as a GST fusion protein [11]. Because of its size, the GST tag was not appropriate for NMR studies. We then replaced it by a 6× His tag at the C-terminus. To achieve this construction the *RGD1* coding sequence ranging from nucleotides 1348 to 1998 was amplified from genomic DNA of *S. cerevisiae* BY4742 strain using the forward primer F-450-Rgd1-NdeI-(5′CACATATGATTTCTCACATTCAGACTAACAACAATATG-3′) adding a *Nde*I restriction site (underlined) and the reverse primer R2-XhoI-Rgd1 (5′- TGCTCGAGTTCAGGCTCAAAAGCTTG-3′) adding a *Xho*I restriction site (underlined). Amplicon was inserted into *Nde*I and *Xho*I sites of the plasmid pET21a (Novagen, Madison, WI) to create a C-terminus fusion in-frame with additional leucine and glutamic acid followed by a 6× His tag. The molecular construction was verified by sequencing.

2.2. Production and purification of the RhoGAP domain

Escherichia coli strain BL21 (DE3) was transformed with the recombinant plasmid described above allowing expression of the RhoGAP domain fused to 6× His. Transformants were grown overnight at 37 °C in 25 mL of LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with 100 μg/mL ampicillin. The culture (25 mL) was used to inoculate 1 L of M9 medium (KH_2PO_4 3 g/L, Na_2HPO_4 6 g/L, NaCl 5 g/L, $MgSO_4$ 1 mM, NH_4Cl 1 g/L, glucose 2 g/L) supplemented with 100 μg/mL ampicillin. Depending on the kind of isotopic labelling $^{15}NH_4Cl$ 1 g/L and ^{13}C-glucose 2 g/L replaced the corresponding molecules in M9 medium. Bacteria were grown during 2 h until the culture reached an OD_{600} of 0.8, and expression of the RhoGAP domain-6× His was induced by adding 500 μM isopropyl-β-D-galactopyranoside (IPTG). After 3 h of induction at 37 °C, cells were harvested and stored at −80 °C until use. For purification, the cell pellet was thawed, suspended in 10 mL of lysis buffer (Tris–HCl at pH 7.6 50 mM, NaCl 250 mM, Igepal 0.1%, imidazole 20 mM, PMSF 1 mM) and submitted to 3 cycles of 30 s sonication in ice (ultra sonic processor, 130 W, 20 kHz, Vibra Cell) at 80% amplitude, interrupted by 30 s in ice. The crude lysate was centrifuged for 5 min at 8300×*g* and the supernatant incubated for one hour with 1 mL of Ni–NTA resin (Qiagen) previously equilibrated with wash buffer (Tris–HCl at pH 7.6 50 mM, NaCl 250 mM, imidazole 20 mM). The resin was washed four times with 15 mL of wash buffer and the proteins eluted by incubating the resin twice with 500 μL elution buffer (Tris–HCl at pH 7.6 50 mM, NaCl 250 mM, imidazole 300 mM) during 10 min. Eluates were pooled and elution buffer exchanged with 20 mM Tris–HCl at pH 7.4 using a centrifugal filter device (Millipore, MWCO 3000). The protein concentration was brought from 12 mg/mL to 65 mg/mL by membrane ultrafiltration. Protein concentration was determined using Bradford protein assay (Pierce).

2.3. Mass spectrometry analysis

Protein identification was performed on the trypsin-digested sample by LC-MS/MS on a Dionex U-3000 Ultimate nano LC system coupled to a nanospray LTQ-OrbiTrap XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) and data were searched by SEQUEST through Bioworks 3.1.1 interface (ThermoFinnigan). Molecular mass determination was performed on the co-crystallized sample with sinapinic acid matrix by a MALDI-MS in linear and positive modes using a Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker, Bremen, DE) equipped with a Nd:YAG smart beam laser.

2.4. RhoGAP activity assay

The biological activity of the RhoGAP domain was tested using the *S. cerevisiae* Rho3 GTPase fused at N-terminus with GST tag. GST-Rho3p was produced as previously reported [6]. The RhoGAP assay was conducted as elsewhere described [11]. Briefly, assay was achieved in three steps: (i) the GST-Rho3p was loaded with [γ-^{32}P]-GTP and blocked in the GTP-bound state with 36 mM ice-cold $MgCl_2$, (ii) the RhoGTPase activity was assayed for 10 min at room temperature with or without the RhoGAP domain or the full length Rgd1p, (iii) organic compounds were separated with activated charcoal and free inorganic labelled phosphate released during [γ-^{32}P]-GTP hydrolysis was counted in the supernatant. The percentage of hydrolyzed GTP by the Rho GTPase was determined as reported [11].

2.5. Chromatographic analysis of the expressed RhoGAP domain of Rgd1p

To check the purity and the absence of multimerization of the RhoGAP domain produced in *E. coli*, an aliquot of the Ni–NTA purified RhoGAP domain was loaded on a Superdex 75 gel filtration column (gelscare 1 × 30 cm). The column was eluted with PBS (NaCl 137 mM, KCl 2.7 mM, NaH_2PO_4 4.3 mM, KH_2PO_4 1.4 mM) at 0.5 mL/min and elution followed by recording the OD at 280 nm. Calibration of the column was performed with BSA (Mw 67000); ovalbumin (Mw 43000); ribonuclease A (Mw 13700); aprotinin (Mw 6512); vitamin B12 (Mw 1355).

2.6. PI(4, 5)P_2 binding

Gel filtration was performed using 20 μM of the RhoGAP domain of Rgd1p and micelles made with 1 mM PI(4, 5)P_2 as already described [11]. The protein was pre-incubated with PI(4, 5)P_2 micelles diluted at concentrations up to 250 μM for 30 min on ice prior to loading on a Superdex 75 gel filtration column (gelscare 1 × 30 cm) equilibrated with a buffer containing Tris–HCl at pH 7.3 20 mM, DTT 0.1 mM, NaN_3 1 mM. The peak surface of free (eluted at 10.7 ml) and bound (eluted at 8.1 ml) forms of the RhoGAP domain was determined and used to calculate the percentage of protein binding for each phosphoinositide concentration.

2.7. Circular dichroïsm

CD spectra have been recorded on a Jasco J-815 Spectrometer at 40 °C. The sample was 45 μM RhoGAP protein at pH 7.3 in 20 mM Tris–HCl, 0.1 mM DTT, 1 mM NaN_3 with or without PS or PI(4, 5)P_2 micelles at 125 μM. Far-UV spectra were recorded from 180 to 270 nm using a 0.1 mm path length. Near-UV spectra were recorded from 260 to 330 nm using a 1 mm path length.

2.8. NMR spectroscopy

Experiments were carried out on a Bruker Advance III 800 MHz spectrometer equipped with a TXI triple resonances probe. A 0.8 mM sample of $^{13}C/^{15}N$ double-labelled RhoGAP (deuterated Tris–HCl at pH 7.3 10 mM, EDTA 2 mM, DTT 2 mM, NaN_3 0.01%, D_2O 5%, TriMethylSilylPropionate 0.5 mM) was used to record at 40 °C $^1H/^{15}N$ and $^1H/^{13}C$ heteronuclear single quantum correlation (HSQC) spectra. We used a two-dimensional $^1H/X$ correlation sequence via Insensitive Nuclei Enhanced by Polarization Transfer (INEPT) [12]. The spectral width of the $^1H/^{15}N$ HSQC was set to

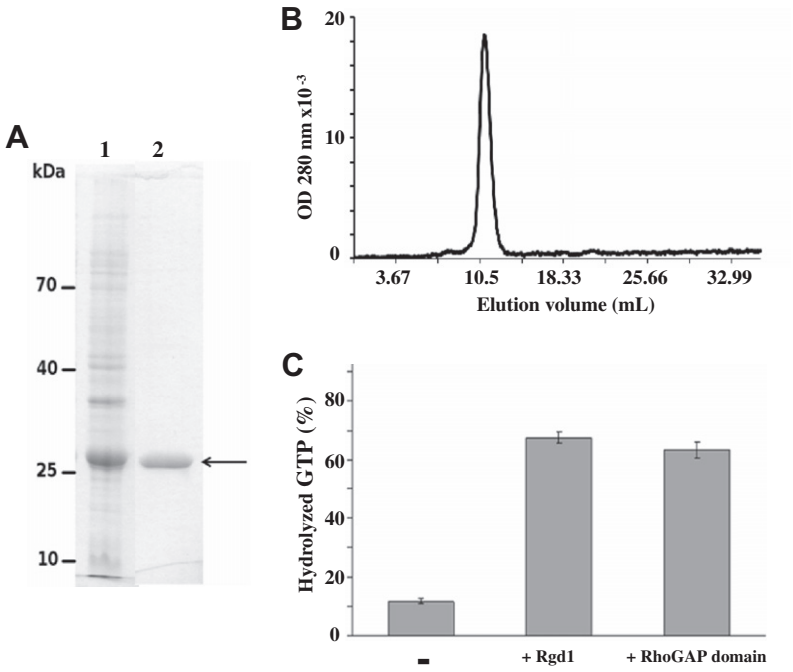

Fig. 1. Quality control of the purified RhoGAP domain. (A): Samples from initial and final steps of purification were analysed on a 12% SDS–PAGE and stained with Coomassie Blue: 2 μL of crude cell extract after centrifugation at 8300×*g* (track 1), 6.5 μg of the protein obtained after imidazole-elution and buffer exchange by membrane ultrafiltration (track 2). Arrow indicates the RhoGAP domain. (B): Elution profile obtained on size exclusion column (Superdex 75, gelscare 1 × 30 cm) of the purified ^{13}C and ^{15}N labelled RhoGAP domain of Rgd1p. The electrophoresis and gel filtration profiles are representative of RhoGAP domain purification for each labelling condition. (C): Rgd1p RhoGAP activity assay on GST-Rho3p: GTP hydrolysis by Rho3 GTPase (0.5 μM) in the absence of Rgd1 protein (−), in the presence of 1 μM Rgd1p (+Rgd1) and in the presence of 1 μM of the Rgd1p RhoGAP domain (+RhoGAP domain). Bars indicate the standard deviations calculated from triplicate measurements.

20 ppm (^{15}N) and 14 ppm (^{1}H). The frequency offset was set to 119.5 ppm and 4.7 ppm, respectively, for ^{15}N and ^{1}H. The spectrum was acquired with 64 complex points in F1 dimension and 2048 points in F2 dimension. The spectral width of the $^{1}H/^{13}C$ HSQC was set to 90 ppm (^{13}C) and 14 ppm (^{1}H). The frequency offset was set to 40 ppm and 4.7 ppm, respectively, for ^{13}C and ^{1}H. The spectrum was acquired with 128 complex points in F1 dimension and 2048 points in F2 dimension. Both HSQC spectra were processed to obtain 2048 × 512 points real matrices. The spectra were processed using the Bruker TOPSPIN 2.0 software and analysed using the Sparky Software (University of California San Francisco, Thomas L. Goddard).

3. Results

3.1. Expression, purification and characterization of the RhoGAP domain of Rgd1p

The RhoGAP domain of Rgd1p was produced in three forms: not-labelled, ^{15}N labelled and $^{15}N/^{13}C$ labelled. In each labelling condition the amount of protein obtained was about 10 mg/L of culture medium. Homogeneity and purity of the material was checked by SDS–PAGE electrophoresis and size exclusion analysis. A unique band was observed on the electrophoresis profile after the single step affinity purification and membrane ultrafiltration (Fig. 1A). Furthermore, on gel filtration chromatography, this material demonstrated a single peak eluted at a retention volume corresponding to the expected size for the monomeric protein (Fig. 1B). The purity of the RhoGAP domain was superior to 95% as assessed by SDS–PAGE and analysis of integration data from gel filtration chromatography (Fig. 1A and B). Moreover, data obtained from gel filtration demonstrated the absence of multimeric forms of the protein (Fig. 1B). The presence of the RhoGAP domain was confirmed by analysing the peak eluted from gel filtration chromatography by MS/MS mass spectrometry. Molecular mass determined by MALDI-TOF analysis (Fig. S1) was in good agreement with data obtained by gel filtration and SDS–PAGE analysis for the monomer of the RhoGAP domain (26 kDa). The biological activity of the RhoGAP domain was tested and compared to the RhoGAP activity obtained for the full-length Rgd1. Results in Fig. 1C showed that the expressed RhoGAP domain of Rgd1p, tagged with 6× His, kept a biological activity similar to the entire Rgd1 protein. The obtained protein was then tested for its ability to be used in NMR analysis. The quality of the recorded ^{13}C and ^{15}N two-dimensional spectra (Fig. S2) indicated that building a three-dimensional model of the RhoGAP domain of Rgd1p by NMR is feasible. It is now possible to easily produce large quantities of the double-labelled RhoGAP domain of Rgd1p and to maintain the 1–2 mM concentrated sample stable for several days at high temperature. These data, taken together, show that the preparation procedure leads to a pure and functional Rgd1 protein certainly folded in the right conformation.

3.2. Interaction between the RhoGAP domain of Rgd1p and phosphoinositides

Size exclusion chromatography was used to assay the ability of the RhoGAP domain of Rgd1p to bind PI(4, 5)P_2 as previously suggested [11]. Upon increasing concentrations of PI(4, 5)P_2 micelles, we noticed a gradual decrease of the intensity of the peak at 10.7 ml (free protein) concomitant with an increase in peak intensity at 8.1 ml corresponding to the bound protein to the PI(4, 5)P_2 micelles (Fig. 2 inset). Full saturation was obtained for 20 μM protein at about 100 μM PI(4, 5)P_2 micelles (Fig. 2). Control experiments where phosphatidylserine replaced PI(4, 5)P_2 were

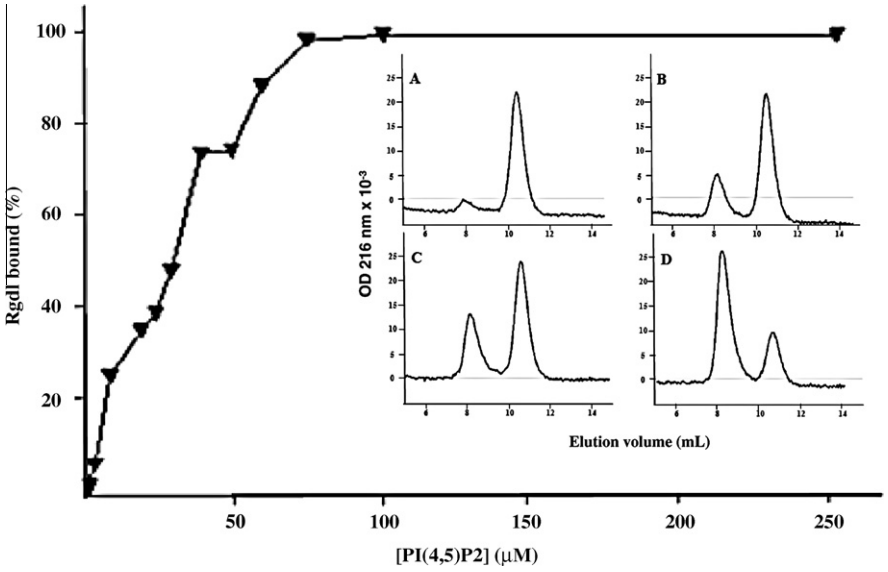

Fig. 2. PI(4, 5)P_2 binding to RhoGAP domain. Percentage of bound RhoGAP domain of Rgd1p as a function of PI(4, 5)P_2 micelles concentration determined from the gel filtration profiles (see inset). Inset: 20 μM of the RhoGAP domain of Rgd1p were pre-incubated with increasing concentrations of PI(4, 5)P_2 micelles and ran over a gelscare (1 × 30 cm) Superdex 75 gel filtration column. Free RhoGAP domain eluted at 10.7 ml, while the protein bound to PI(4, 5)P_2 micelles eluted at 8.1 ml. Elution profiles of the RhoGAP domain with 5 μM (A), 10 μM (B), 25 μM (C) and 40 μM (D) PI(4, 5)P_2 micelles are shown among the 12 chromatograms obtained with PI(4, 5)P_2 micelles concentration ranging from 0 to 250 μM. The concentration of PI(4, 5)P_2 for which 50% of the RhoGAP domain was bound to micelles was derived from the curve.

performed. In this case, no shift of the unbound protein peak was observed during chromatography (data not shown). This interaction was also verified by circular dichroism analysis. Circular dichroïsm of the RhoGAP protein displayed a Far-UV CD spectrum with two well-defined minima at 208 nm and 222 nm, and a maximum at 192 nm (Fig.3A). This demonstrated that the recombinant RhoGAP used here exhibited a defined conformation in solution. From the CD signal at 222 nm, the alpha helical content could be estimated to 26%, suggesting that around 60 residues of the RhoGAP protein (216 residues) are in helical conformation [13]. This is roughly in agreement with the *in silico* calculated alpha helical content (33%) expected for the Rgd1p RhoGAP domain. The RhoGAP protein also displayed a Near-UV CD spectrum with a slight positive ellipticity at 280 and 290 nm (Fig.3B). This showed that aromatic residues are in an asymmetric environment, suggesting the presence of a well-defined tertiary structure. The same analysis was done in the presence of a three-fold excess concentration of PI(4, 5)P_2 with respect to Rgd1p (protein concentration was identical in each assay). Under this condition, a decrease of intensity (Far and Near UV) was observed (Fig.3). Interestingly, no change of ellipticity was observed in both Far and Near-UV region upon the presence of a three-fold excess of PS (125 μM). These observations suggested that the PI(4, 5)P_2, but not the PS, affected the dynamics of the protein, not only at the secondary structural level, but also at the tertiary structural level.

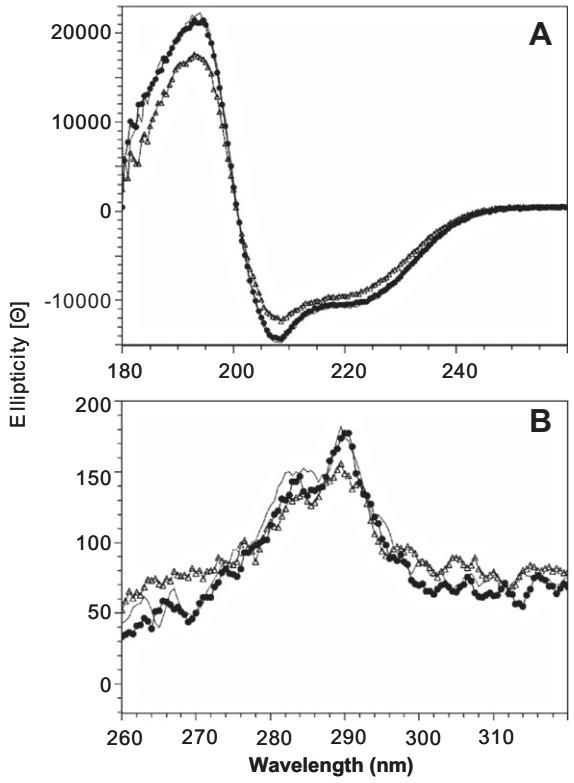

Fig. 3. Circular dichroïsm analysis. CD spectra of the RhoGAP domain (45 μM) without (●) or with 125 μM PS (dashed line) or 125 μM PI(4, 5)P_2 (Δ) at 40 °C in 20 mM Tris–HCl pH 7.3 A: Far-UV region (180–270 nm) and B: Near-UV region (260–330 nm). Ellipticity [Θ] was expressed in deg cm^2 $dmol^{-1}$.

4. Discussion

Previous work demonstrated that phospholipids were able to modulate the RhoGAP activity of the native Rgd1 protein [11], which contains both F-BAR (member of BAR family) and RhoGAP domains. In this work, we show for the first time a direct interaction between PI(4, 5)P_2 and the RhoGAP domain of Rgd1p without the contribution of the F-BAR domain, with an half maximal effective concentration of PI(4, 5)P_2 (EC_{50}) estimated to 30 μM. Circular dichroïsm experiments reveal that this interaction is strong enough to affect the dynamics of the protein at both secondary and tertiary structures. In agreement with gel filtration experiments, PS did not affect CD spectra of the protein demonstrating the specificity of the interaction between the RhoGAP

of Rgd1p domain and PI(4, 5)P_2. Although they are present in low proportion in membranes phosphoinositides are involved in the regulation of many cellular functions via their interactions with specific protein domains. BAR domain proteins are able to directly deform phosphoinositides-rich membranes to induce protrusions or invaginations. The BAR domains typically interact with cellular membranes through electrostatic interactions between the positively charged regions located close to the poles of the BAR structure and the negatively charged phospholipids headgroups [14,15]. Furthermore, a previous report pointed out a specific interaction between DLC1, a RhoGAP protein (devoid of BAR domain whose gene is mutated in some cancers), and PI(4, 5)P_2. This interaction was linked to a modulation of the GAP activity of DLC1 [16]. Moreover, PI(4, 5)P_2 is one of the phospholipids that may be involved in epithelial to mesenchymal transition of cancer cells [17]. Interestingly a FGVPL motif located near the RhoGAP domain and beside basic residues was observed in Rgd1p and conserved in several RhoGAP proteins among different eukaryotic organisms. NMR investigations addressing interaction with phosphoinositides will be useful to precise the impact of this interaction on the functionality of Rgd1p and will shed a new light on the Rgd1 RhoGAP domain organization and modulation by specific phosphoinositides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.130.

References

[1] P. Perez, S.A. Rincon, Rho GTPases: regulation of cell polarity and growth in yeasts, Biochem. J. 426 (2010) 243–253.
[2] A.L. Bishop, A. Hall, Rho GTPases and their effector proteins, Biochem. J. 348 (Pt 2) (2000) 241–255.
[3] A. Hall, Rho GTPases and the actin cytoskeleton, Science 279 (1998) 509–514.
[4] L. Van Aelst, C. D'Souza-Schorey, Rho GTPases and signaling networks, Genes Dev. 11 (1997) 2295–2322.
[5] J. Tcherkezian, N. Lamarche-Vane, Current knowledge of the large RhoGAP family of proteins, Biol. Cell 99 (2007) 67–86.
[6] F. Doignon, C. Weinachter, O. Roumanie, M. Crouzet, The yeast Rgd1p is a GTPase activating protein of the Rho3 and rho4 proteins, FEBS Lett. 459 (1999) 458–462.
[7] J.E. Adamo, G. Rossi, P. Brennwald, The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity, Mol. Biol. Cell 10 (1999) 4121–4133.
[8] H. Fernandes, O. Roumanie, S. Claret, X. Gatti, D. Thoraval, F. Doignon, M. Crouzet, The Rho3 and Rho4 small GTPases interact functionally with Wsc1p, a cell surface sensor of the protein kinase C cell-integrity pathway in *Saccharomyces cerevisiae*, Microbiology 152 (2006) 695–708.
[9] Y. Matsui, E.A. Toh, Yeast RHO3 and RHO4 ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1, Mol. Cell Biol. 12 (1992) 5690–5699.
[10] Y. Matsui, A. Toh-e, Isolation and characterization of two novel ras superfamily genes in *Saccharomyces cerevisiae*, Gene 114 (1992) 43–49.
[11] V. Prouzet-Mauleon, F. Lefebvre, D. Thoraval, M. Crouzet, F. Doignon, Phosphoinositides affect both the cellular distribution and activity of the F-BAR-containing RhoGAP Rgd1p in yeast, J. Biol. Chem. 283 (2008) 33249–33257.
[12] G. Bodenhausen, D. Ruben, Natural abundance Nitrogen-15 NMR by enhanced heteronuclear spectroscopy, Chem. Phys. Lett. 60 (1980) 185–189.
[13] Q.H.J.M. Scholtz, E.J. York, J.M. Stewart, R.L. Baldwin, Parameters of helix-coil transition theory for alanine-based peptides for varying chain lengths in water, Biochemistry 31 (1999) 1463–1470.
[14] J. Saarikangas, H. Zhao, P. Lappalainen, Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides, Physiol. Rev. 90 (2010) 259–289.
[15] M.A. Lemmon, Membrane recognition by phospholipid-binding domains, Nat. Rev. Mol. Cell Biol. 9 (2008) 99–111.
[16] P. Erlmann, S. Schmid, F.A. Horenkamp, M. Geyer, T.G. Pomorski, M.A. Olayioye, DLC1 activation requires lipid interaction through a polybasic region preceding the RhoGAP domain, Mol. Biol. Cell 20 (2009) 4400–4411.
[17] M.W. Klymkowsky, P. Savagner, Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe, Am. J. Pathol. 174 (2009) 1588–1593.