Biochemical and Biophysical Research Communications 405 (2011) 74–78

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Benoît Odaert [a,1], Valérie Prouzet-Mauleon [b,1], Jean-William Dupuy [c], Marc Crouzet [b], Marc Bonneu [c], Xavier Santarelli [d], Aurélie Vieillemard [b], Didier Thoraval [b], François Doignon [b], Michel Hugues [b,*]

[a] Université de Bordeaux, Chimie et Biologie des Membranes et des Nanoobjets, CNRS UMR 5248, Pessac 33607, France
[b] Université de Bordeaux, Institut de Biochimie et de Génétique Cellulaires, CNRS UMR 5095, Bordeaux 33000, France
[c] Université de Bordeaux, Centre de Génomique Fonctionnelle Bordeaux, Pôle protéomique, Bordeaux 33000, France
[d] Université de Bordeaux, EA 4135, ENSTBB, 146 rue Léo Saignat, Bordeaux 33000, France

ARTICLE INFO

Article history:
Received 28 December 2010
Available online 5 January 2011

Keywords:
Saccharomyces cerevisiae
RhoGAP
Rgd1
NMR
Phosphoinositides
Circular dichroïsm

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.

* Corresponding author. Address: RDPR, Institut de Biochimie et de Génétique Cellulaires, Université Bordeaux 2, case 64, 146 rue Léo Saignat, 33076 Bordeaux cedex, France. Fax: +33 5 57 57 47 19.
E-mail address: michel.hugues@u-bordeaux2.fr (M. Hugues).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.12.130

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