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The *Candida albicans* Rgd1 is a RhoGAP protein involved in the control of filamentous growth

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ABSTRACT

Rho proteins are essential regulators of polarized growth in eukaryotic cells. These proteins are down-regulated *in vivo* by specific Rho GTPase Activating Proteins (RhoGAP). We investigated the role of Rgd1 RhoGAP, encoded by the *Candida albicans RGD1* gene. We demonstrated that CaCdc42, CaRho3 and CaRho4 proteins had an intrinsic GTPase activity and that CaRgd1 stimulates *in vitro* GTP hydrolysis of these GTPases. Deletion of *RGD1* in *C. albicans* results in sensitivity to low pH as already described for *rgd1*Δ in *Saccharomyces cerevisiae*. The role of Rgd1 in survival at low pH is conserved in the two yeast species as the Ca*RGD1* gene complements the Sc*rgd1*Δ sensitivity. By tagging the RhoGAP with GFP, we found that CaRgd1 is localized at the tip and cortex of growing cells and during cytokinesis at the septation sites in yeast and filamentous forms. We investigated the effect of CaRgd1 on the control of the polarized growth. Removing Ca*RGD1* alleles increased filamentous growth and cells lacking CaRgd1 presented longer germ tubes. Conversely, *RGD1* overexpression restricted hyphae growth. Our results demonstrate that Rgd1 is critical for filamentous formation in *C. albicans* especially for filamentous elongation.

1. Introduction

Candida albicans is an opportunistic human fungal pathogen responsible for infections ranging from superficial mycoses to severe life-threatening bloodstream infections in vulnerable patients (Beck-Sague and Jarvis, 1993). This yeast is the fourth most common life-threatening agent for immunodeficient patients, with a mortality of around 50% (Edmond et al., 1999; Pfaller and Diekema, 2007; Safdar and Armstrong, 2002). Virulence of the yeast *C. albicans* is related to its ability to switch between the yeast form and the filamentous forms of either pseudohyphae or true hyphae (Gow et al., 2002; Sudbery et al., 2004). *C. albicans* and *Candida dubliniensis* are the only *Candida* species able to form true hyphae (Gilfillan et al., 1998; Whiteway and Bachewich, 2007). These true hyphae are unconstricted germ tubes and are induced in human infections and in media containing blood serum (Berman, 2006). Pseudohyphae consist of branching chains and elongated yeast cells and this morphological shape is shared by *C. albicans* and *Saccharomyces cerevisiae* species (Berman, 2006; Gancedo, 2001; Lo and Dranginis, 1998).

The various cellular morphologies of *C. albicans* are related to polarized cell growth and, the switches between the different shapes require the selection of a specific growth site and rearrangement of the actin cytoskeleton (Whiteway and Bachewich, 2007). Filamentous growth depends on extrinsic signals and many modulators regulate the cell polarity (Davis, 2003; Hornby et al., 2004; Kumamoto, 2005; Martin et al., 2005). The Rho proteins actively participate in differentiation processes and play a key role in hyphal growth (Bassilana and Arkowitz, 2006; Bassilana et al., 2005; Court and Sudbery, 2007; Smith et al., 2002; Ushinsky et al., 2002). As in budding yeast, Cdc42 is a major regulator of polarity control in the pathogenic yeast *C. albicans*. It acts as a molecular switch toggling between an active GTP-bound form and an inactive GDP-bound form (Bassilana et al., 2005; Johnson, 1999). The regulation of *C. albicans* Cdc42 GTPase was studied by investigating the module regulators: the GEF Cdc24 (Bassilana et al., 2005) and the RhoGAPs Bem3 and Rga2 (Court and Sudbery, 2007). In addition to Cdc42, Cdc24 also has a major role in viability, bud to hypha transition, maintenance of hyphal growth and pathogenicity in a murine model (Bassilana et al., 2003, 2005). Court and Sudbery (2007) found that cells lacking the RhoGAPs Rga2 and Bem3 display characteristics of hyphal growth in conditions that normally promote pseudohyphal growth. These mutant strains show hyperpolarized growth with a hyphal pattern of sep-

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tin organization and the presence of Spitzenkörpers. It seems that the role of Rga2 and Bem3 requires their GAP activity (Court and Sudbery, 2007).

Dunkler and Wendland (2007) recently showed that the Rho3 and Rho4 proteins in *C. albicans* are key elements in polarized growth. Specific functions for Rho3 at the hyphal tip and for Rho4 at septal sites were discovered. We have worked on the action and regulation of Rho3 and Rho4 homologs in *S. cerevisiae* for a long time (de Bettignies et al., 2001; Roumanie et al., 2000, 2002) and demonstrated the role of Rgd1 protein as the RhoGAP regulator for these GTPases (Doignon et al., 1999; Roumanie et al., 2000). Given the role of these Rho proteins in the morphogenesis of *C. albicans*, we searched for the homolog of the RhoGAP Rgd1 in this organism and aimed to demonstrate its role in this fungus. A protein similar to ScRgd1 with a similar domain organization was identified by *in silico* analysis. This protein, named CaRgd1, displayed GAP activity *in vitro* on the CaRho3 and CaRho4 GTPases but also on the CaCdc42 GTPase. The *RGD1* gene of *C. albicans* was shown to complement the sensitivity to low pH of *rgd1*Δ in *S. cerevisiae*. Given the GAP activity of CaRgd1, the influence of the *RGD1* gene on *C. albicans* polarized growth was explored and we revealed a role of CaRgd1 in filamentation. During this work we also showed that inactivation of both *RGD1* alleles in the diploid *S. cerevisiae* strain enhanced pseudohyphal growth. Thus the regulatory role of *RGD1* on filamentous growth was conserved in *S. cerevisiae* as well as in *C. albicans*.

2. Materials and methods

2.1. Media and growth conditions

Yeast extract-peptone-dextrose (YEPD), synthetic Dextrose (SD) and synthetic complete (SC) media were already described (Sherman, 1991). YEPD and SD media were supplemented with 80 mg of uridine/liter; when necessary 50 mg/l of histidine and arginine were included in the SD medium. *C. albicans* was routinely grown at 30 °C unless otherwise indicated. Solid YEPD medium was acidified to pH 2.5 by the addition of hydrochloric acid. Pseudohyphal filamentation was promoted by growing cells on YEPD pH 6 at 36 °C; for hyphal induction, 20% fetal calf serum (FCS) was added to YEPD medium and cells were incubated at 37 °C (Sudbery, 2001). Morphological assays of the colonies were performed on YEPD +20% serum, YEPS and SPHD media containing 1% agar. YEPS contained 2% sucrose instead of the glucose in YEPD (Brown et al., 1999). The SPHD medium contained 6.7 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% glucose, 50 mg/l histidine and 1.0 g/l of L-proline as sole nitrogen source (Gimeno et al., 1992). The effect of physical environment on filamentous growth (Brown et al., 1999) was studied by plating cells in three ways: (i) on the surface of YEPS medium, (ii) on the surface of YEPS medium with a second slab of YEPS agar placed over the cells (sandwiched) or (iii) mixed into the medium during agar solidification (embedded). Induction of pseudohyphae in *S. cerevisiae* was promoted by cultivating diploid cells on solid SLAHD medium supplemented with 4 mg/l histidine (Gimeno et al., 1992). Leucine and uracil were added to a final concentration of 4 mg/l when required.

2.2. Strains and plasmids

Strains are listed in Table 1. All *C. albicans* strains were derived from the BWP17 strain provided by Aaron P. Mitchell. Heterozygous and homozygous *rgd1*Δ mutant strains were constructed using the strategy developed by Enloe and coauthors (2000). The two *RGD1* alleles were sequentially deleted in the parental BWP17 strain using the *UAU1* (*ura3*Δ*3′-ARG4-ura3*Δ*5′*) cassette carried in pBME101 (Enloe et al., 2000). To construct the *rgd1::UAU1* gene disruption cassette, the *UAU1* cassette was amplified by PCR from pBME101 with primers RGD1-5DR and RGD1-3bDR to yield an *rgd1::UAU1* PCR product with 50 bp of *RGD1* homology on each end. The *arg4 ura3 his1* strain BWP17 was transformed with approximately 1 μg of the 4.15-kbp PCR product. Transformed cells were first plated onto SC-Arg medium, which selects for the *ARG4* marker. Single allele deletion of *RGD1* (*RGD1*/*rgd1::UAU1*) was expected. To screen for Arg+ Ura+ strains, three independent Arg+ transformants were grown on YEPD medium for 2 days and then plated on SC-Ura-Arg plates. The Arg+ Ura+ strains may have had a disruption of the two copies of *RGD1* (*rgd1::U*/*rgd1::UAU1*) as described by Enloe et al. (2000). Single allele (*RGD1*/*rgd1::UAU1*) and both allele (*rgd1::U*/*rgd1::UAU1*) deletions of *RGD1* were confirmed by PCR. Arg+ and Arg+ Ura+ transformants were screened for replacement of the targeted gene using a 5′ primer that annealed within the selectable marker of the UAU1 cassette (ARG4det, URAdet3′) and a 3′ primer that annealed downstream of the deleted gene (det4). Positive clones were further screened by PCR using primers that annealed upstream (det2, det2b) and downstream (det4) of the deleted gene. Finally, the complete absence of the *RGD1* gene was confirmed using PCR primers that annealed on both sides of the deleted region (det2b + det1). All the oligonucleotides used are listed in Table 2. This disruption removed essentially two thirds of the *RGD1* coding sequence with 486 base pairs 3′ of the START codon and 72 base pairs 5′ of the STOP codon remaining. Phenotype analysis was performed in each case on three independent transformants.

One copy of *RGD1* was reintroduced into the *C. albicans* homozygous *rgd1*Δ mutant strain. For this, the open reading frame of *RGD1* flanked with its own promoter and terminator regions was amplified by PCR from genomic DNA using F-promCaRGD1 and R-terCaRGD1 primers and the PCR product was cloned between *Not*I and *Sma*I of pDDB78 vector (Spreghini et al., 2003). The

Table 1
Strains used in this study.

Strain	Genotype	Source
C. albicans		
BWP17	ura3Δ::λimm434/ura3Δ::λimm43, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG	Wilson et al. (1999)
DAY286	BWP17 arg4::hisG/ hisG::arg4::ARG4-URA3	Davis et al. (2002)
CaRGD1/rgd1Δ	BWP17 RGD1/rgd1::ura3Δ-ARG4-ura3Δ	This study
Cargd1Δrgd1Δ	BWP17 rgd1::URA3/rgd1::ura3Δ-ARG4-ura3Δ	This study
Cargd1Δrgd1Δ + RGD1	ura3Δ::λimm434/ura3Δ::λimm434, his1::hisG/hisG::his1::HIS1-RGD1, arg4::hisG/arg4::hisG, rgd1::URA3/rgd1:: ura3Δ-ARG4-ura3Δ	This study
BWP17 + CaGFP-RGD1	BWP17 RGD1/RGD1 RP10/rp10::PADH-GFP-RGD1-ADHT	This study
Cargd1Δrgd1Δ + CaGFP-RGD1	BWP17 rgd1::URA3/rgd1::ura3Δ-ARG4-ura3Δ RP10/rp10:: PADH-GFP-RGD1-ADHT	This study
S. cerevisiae		
Scrgd1Δ	MATa, his3–11,15, rgd1::HIS3, trp1Δ	Our lab
FY1679	MATa/MATα; ura3–52/ura3–52, trp1Δ63/TRP1, leu2Δ1/LEU2, his3Δ200/HIS3, GAL2/GAL2	Winston et al. (1995)
Scrgd1Δ/rgd1Δ	FY1679 rgd1::KANMX4/rgd1::KANMX4	Our lab