A conserved G protein (Drg1p) plays a role in regulation of invasive filamentation in *Candida albicans*

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During infection, the opportunistic fungal pathogen *Candida albicans* grows invasively into the tissues of its host, forming filaments that penetrate the host tissue. To search for genes that are important for invasive filamentation, a screen for mutants that were defective in invasion of agar medium was conducted. A mutant carrying an insertion mutation in the locus of a gene, termed here *DRG1*, was identified. *DRG1* encodes a highly conserved cytoplasmic G protein, with orthologues in the genomes of organisms from humans to yeast and archaea. *C. albicans* strains lacking Drg1p were defective in producing filaments that penetrated agar media, but produced filaments normally under other conditions, such as during liquid growth. When inoculated intravenously into mice, the *drg1* null mutant caused delayed lethality accompanied by delayed invasive growth in the kidneys of the host, in comparison with those of the wild-type strain. These results implicate Drg1p in the control of invasive filamentation in the laboratory, and in the progression of invasive disease in the host.

INTRODUCTION

Candida albicans is one of the most important opportunistic fungal pathogens in humans. *C. albicans* causes diseases ranging from mild superficial infections to more severe bloodstream infections that may lead to systemic disease. Examples of superficial infections include oral thrush found in young children, and vaginal yeast infections in women. Systemic infections usually occur in immunocompromised hosts whose immune systems may be suppressed by chemotherapy, weakened by other diseases, or not fully developed.

During infection, *C. albicans* grows invasively into the tissues of its host. *C. albicans* is a dimorphic fungus and, during invasive growth, typically assumes a filamentous morphology. Under other conditions, *C. albicans* is found in a yeast form. The ability to convert from yeast-form cells to filamentous hyphae (elongated cells lacking constrictions at the septa) and pseudohyphae (elongated cells with constrictions at the septa) is believed to be important for invasiveness and virulence (reviewed by Kumamoto & Vines, 2005b).

To probe the mechanisms that allow *C. albicans* to invade host tissue, laboratory studies have made use of *C. albicans*

invasion into agar matrices. Several proteins, including signal transduction proteins and transcription factors, which regulate invasive growth into agar have been previously described (reviewed by Kumamoto & Vines, 2005a). The bHLH transcription factor Efg1p is a negative regulator of filamentation when cells are embedded in an agar matrix (Giusani *et al.*, 2002), even though Efg1p has a critical positive role in filamentation under most other conditions (Stoldt *et al.*, 1997).

In this study, a search for *C. albicans* genes that are important for invasive filamentation in agar was undertaken. Mutations affecting the gene, orf19.5083, were found to result in reduced invasiveness. This gene is highly homologous to the *RBG1* (ribosome-interacting GTPase)/*FUN11* (function unknown now) gene of *Saccharomyces cerevisiae*, and to the gene encoding DRG1 (developmentally regulated GTP-binding protein) of *Xenopus laevis* (Kumar *et al.*, 1993) and other species. The *C. albicans* gene will be referred to as *DRG1* in this paper.

Previous studies have suggested that the function of DRG1 may be related to growth control. For example, ectopic expression of mouse DRG1 enhances oncogenic transformation by c-myc and ras (Mahajan *et al.*, 1996). Mouse DRG1 interacts with an oncogene for stem cell acute lymphoblastic leukaemia, TAL1/SCL1 (Mahajan *et al.*, 1996). TAL1/SCL1 encodes a bHLH transcription factor whose HLH domain interacts with the C-terminal region of DRG1. Thus, *DRG1* may influence growth control by

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; REMI, restriction-enzyme-mediated integration.

binding and regulating transcription factors. Expression of DRG1 also correlates with growth; *DRG1* is highly expressed during embryonic development in *Xenopus* (Kumar *et al.*, 1993) and mice (Sazuka *et al.*, 1992), and is expressed at higher levels in growing versus non-growing plant cells (Devitt *et al.*, 1999). Despite these clues, the function of DRG1 is not well understood in any species.

In this study, we showed that a *C. albicans* *drg1* mutant was attenuated in invasiveness in an agar matrix, but was capable of hyphal growth under other conditions. In addition, the *drg1* null mutant exhibited delayed invasiveness in host tissue, and delayed lethality during infection. As invasive growth is a contact-dependent response, these findings suggest that Drg1p functions in contact-dependent signaling or responses.

METHODS

Strains. *C. albicans* strain CAI-4 (Δ *ura3::imm434/ura3::imm434*) (Fonzi & Irwin, 1993) was used to construct all *C. albicans* strains (listed in Table 1).

To construct *drg1* null mutants by the Ura-blaster method (Fonzi & Irwin, 1993), plasmids pXC110 and pXC111, encoding 5' and 3' fragments of the *DRG1* locus flanking *hisG-URA3-hisG*, were digested with *KpnI* and *HindIII*. Strains were characterized by Southern blotting

to verify that all alleles of *DRG1* were deleted. For complementation analysis, *Ura*[−] derivatives of *drg1* null mutants were integratively transformed with *BsaBI*-digested pXC155.

For cloning, *Escherichia coli* strains XL-1 Blue or JM109 were used.

Media and growth conditions. For culturing *C. albicans*, standard media included YP (1% yeast extract and 2% bacto peptone) with either 2% glucose (YPD) or 2% sucrose (YPS), or CM-Ura (complete minimal medium lacking uridine and uracil) (Ausubel *et al.*, 1989). For solid media, 2% agar was added. For selection of *Ura*[−] strains, CM-Ura medium was supplemented with 60 μ g uridine ml^{−1} and 1 mg 5-fluoroorotic acid ml^{−1} (Kelly *et al.*, 1987). For the colony morphology assay, YPS medium containing 1% agar (Difco) was used (Brown *et al.*, 1999). Synthetic low ammonium, Lee's, proline and GlcNAc media were prepared as described by Brown *et al.* (1999).

E. coli strains were cultured in L broth or on L plates (Miller, 1972), with ampicillin added to a concentration of 100 μ g ml^{−1}.

Construction of plasmids. Synthetic oligonucleotides are described in Table 2. pXC110 and pXC111 were constructed from p510 (<http://www.sacs.ucsf.edu/home/JohnsonLab/burk/Candisrupt.html>) (kind gift of B. Braun and A. Johnson, University of California, San Francisco). For pXC110, the 5' *DRG1* fragment was amplified from chromosomal DNA with primers XC-01 and XC-02. The DRG 3' fragment was amplified with primers XC-03 and XC-04. pXC111, used to delete the last allele of *DRG1*, contained *DRG1* sequences that were within the sequences deleted by pXC110. For

Table 1. Fungal strains used in this study

Strain	Genotype	Source or reference
<i>C. albicans</i>		
CAI-4	SC5314 <i>ura3/ura3</i>	Fonzi & Irwin (1993)
CKY101	CAI-4 [<i>ade2::pDBI52 (URA3)</i>]	Brown <i>et al.</i> (1999)
CKY136	CAI-4 <i>efg1::his/efg1::his</i> [<i>ade2::pDBI52 (URA3)</i>]	Giusani <i>et al.</i> (2002)
HLC74	CAI-4 <i>efg1::his/efg1::his</i> [<i>leu2::HLB134 (EFG1)</i>]	Lo <i>et al.</i> (1997)
XC101	CAI-4, [<i>leu2::pRC3915 (URA3)</i>] (two copies of chromosome 1)	Chen <i>et al.</i> (2004)
XC121	CAI-4, [<i>ade2::pXC154 (URA3)</i>] (two copies of chromosome 1)	This study
XC210	Insertion of pXC132 into the chromosome of CAI-4	This study
XC211	Insertion of pXC132 into the chromosome of CAI-4	This study
XC301	CAI-4, <i>drg1Δ::his-URA3-his/DRG1/DRG1</i>	This study
XC302	CAI-4, <i>drg1Δ::his/DRG1/DRG1</i>	This study
XC303	CAI-4, <i>drg1Δ::his/drg1Δ::his-URA3-his/DRG1</i>	This study
XC304	CAI-4, <i>drg1Δ::his/drg1Δ::his/DRG1</i>	This study
XC305	CAI-4, <i>drg1Δ::his/drg1Δ::his/drg1Δ::his-URA3-his</i>	This study
XC306	CAI-4, <i>drg1Δ::his/drg1Δ::his/drg1Δ::his</i>	This study
XC308	CAI-4, <i>drg1Δ::his/drg1Δ::his</i> (two copies of chromosome 1)	This study
XC413	XC308 [<i>leu2::pRC3915 (URA3⁺)</i>]	This study
XC414	XC308 [<i>drg1::pXC155 (URA3⁺, DRG1⁺)</i>]	This study
XC415	XC308 [<i>ade2::pXC154 (URA3⁺)</i>]	This study
XC416	XC308 [<i>drg1::pXC156 (URA3⁺, myc-DRG1)</i>]	This study
XC310	CAI-4, <i>drg1Δ::his/drg1Δ::his/efg1Δ::his/efg1Δ::his</i> [<i>leu2::pRC3915</i>]	This study
XC311	CAI-4, <i>drg1Δ::his/drg1Δ::his/efg1Δ::his/efg1Δ::his</i> [<i>leu2::HLB134 (EFG1)</i>]	This study
<i>S. cerevisiae</i>		
EGY40	<i>MATα ura3-1 his3-11 trp1-1 leu2-3,112</i>	Golemis <i>et al.</i> (1996)

Table 2. Synthetic oligonucleotides used in this study

Name	Sequence (5'–3')*
XC-01	cccAAGCTTtcttagtagtggtgaagt (<i>Hind</i> III)
XC-02	aaCTGCAGtgctagcccttgaagtaac (<i>Pst</i> I)
XC-03	gcGAGCTCaatgtcaagtagatccaaatag (<i>Sac</i> I)
XC-04	gtgtatGGTACCTcggttaagca (<i>Kpn</i> I)
XC-05	cccAAGCTTactaatcatgactaccgtgg (<i>Hind</i> III)
XC-06	aaCTGCAGcagcttctgaatgggtcct (<i>Pst</i> I)
XC-07	gcGAGCTCccaccaagacttgtaag (<i>Sac</i> I)
XC-08	cggGGTACCTcgttctctattgaagagtga (<i>Kpn</i> I)
XC-09	cGAGCTCggtccagttctggtcgagtga (<i>Sac</i> I)
XC-10	ccTTAATTAAggttcagcttggtgatcttg (<i>Pac</i> I)
XC-11	gattactcaaaaggGCTAGCa (<i>Nhe</i> I)
XC-12	ttaattaacccgggagccgcatgattagtgggtctttt
XC-13	cggatccccgggttaattaa
XC-14	tttctccaggtagtcattgattgattgat
XC-15	ggtGGATCCtttctcaaaatcgtaatacat (<i>Bam</i> HI)
XC-16	ggtGGATCCtcttcaaaatcgtaatacatct (<i>Bam</i> HI)
XC-17	ggtGAATTCaagaaccactaaatcatgact (<i>Eco</i> RI)
XC-18	cgGAATTCtaacgtattctataccattac (<i>Eco</i> RI)
XC-19	cgGGATCCgatgtacacgaatgatattattac (<i>Bam</i> HI)
XC-20	ttgacaggagccttagatgga
XC-21	agaagaagaacagcgtaggtt
XC-22	caacacatagattgcaggaatg
XC-23	tcactacattgacaacagtc
XC-24	cgGAATTCgatgctactactacgtaaatga (<i>Eco</i> RI)
XC-25	aaCTGCAGgatatcgtcgacatgcatgga (<i>Pst</i> I)

*Restriction sites (listed in parentheses) introduced into primer sequences for cloning purposes are shown in upper-case type.

construction of pXC111, a *DRG1* 5' fragment was amplified using primers XC-05 and XC-06, and a 3' fragment was amplified using primers XC-07 and XC-08.

pXC155 (*DRG1*⁺) and pXC154 (empty vector) were used for complementation analysis. To construct pXC155, *DRG1* was amplified by PCR using primers XC-09 and XC-10. After digestion with *Sac*I and *Pac*I, the fragment was ligated into *Sac*I/*Pac*I-digested pNUB1 (pNEB193 carrying the *C. albicans* *Xba*I to *Sac*I *URA3* fragment cloned into the *Nde*I site). pXC154 (vector-only control) was generated by deleting the maltase promoter from pDBI52, and carried the *C. albicans* *URA3* gene and a fragment of *C. albicans* *ADE2*. For construction of pXC156, encoding a myc-tagged *DRG1* allele, PCR fragments were generated using primers XC-11 and XC-12 with pXC155 (*DRG1*⁺) as a template, and using primers XC-13 and XC-14 with pFA6a-13Myc-kanMX6 (Longtine *et al.*, 1998) as a template. These two PCR fragments were mixed and amplified with primers XC-11 and XC-14, generating a fragment that contained part of the *DRG1* promoter, 13Myc, and part of the *DRG1* ORF. This fragment was digested with *Nhe*I and *Btg*I, and ligated into *Nhe*I/*Btg*I-digested pXC155 (*DRG1*⁺), generating pXC156. For transformation, pXC156 was digested with *Bsa*BI.

lexA-DRG1 was constructed by PCR amplification of the *DRG1* gene with primers XC-15 and XC-16, and cloning of the PCR product into vector pEG202 (Golemis *et al.*, 1996). *DRG1-lexA* was constructed by PCR amplification of the *DRG1* gene with primers XC-17 and XC-16, and cloning of the PCR product into vector pNlexA (Golemis *et al.*,

1996). *GAL4AD-EFG1* was constructed by PCR amplification of *EFG1* with primers XC-18 and XC-19, and cloning of the PCR product into vector pGAD-C2 (Giusani *et al.*, 2002; James *et al.*, 1996). plexA-Fos and pC1.1.2, encoding *GAL4AD-SLK19* (control fusion proteins that are not expected to interact with either Efg1p or Drg1p), were generously provided by R. Brent (Massachusetts General Hospital, Boston) and R. Kamieniecki (Tufts University, Boston).

pXC132, containing *Bam*HI, *Nsi*I, *Sal*I and *Eco*RV sites upstream of a promoterless *yEGFP* gene that was fused to the 3' UTR of *ACT1* in a pNUB1 backbone, was used for insertional mutagenesis.

DNA isolation and analysis. PCR, restriction digestion and gel electrophoresis were performed by standard methods, as described elsewhere (Sambrook *et al.*, 1989). Automated DNA sequencing was performed by Michael Berne and coworkers at the Tufts University Core Facility. Chromosomal DNA isolation and Southern blot hybridization were performed by standard methods, as described previously (Burke *et al.*, 2000; Sambrook *et al.*, 1989). DNA probes were labelled with [α -³²P]dATP by random priming.

To demonstrate that the *DRG1* gene had been deleted, a probe corresponding to a sequence upstream of the *DRG1* ORF was generated by PCR using primers XC-20 and XC-21, and was used to probe a Southern blot of *Msi*I-digested chromosomal DNA from candidate strains. To verify that all copies of *DRG1* had been deleted, a second probe, corresponding to sequences within the *DRG1* ORF, was generated by PCR using primers XC-22 and XC-23, and used to probe the same blot.

For inverse PCR, genomic DNA from strain XC210 was digested with *Rsa*I, *Hae*I or *Alu*I separately. Following ligation, the circular DNA products were used as templates for PCRs using primers XC-24 and XC-25 (homologous to sequences within pXC132). The PCR products were purified from a 1% agarose gel, and the DNA was sequenced using the primers described above.

Electroporation and restriction-enzyme-mediated integration (REMI) of *C. albicans*. The method used was that described by Brown *et al.* (1996), with minor modifications. Cells were grown to OD₆₀₀ ~0.5–0.6, harvested, and treated with 0.1 M lithium acetate, 0.01 M Tris/HCl, pH 7.5, and 1 mM EDTA for 45 min at 30 °C. DTT was added to a concentration of 2 mM, and the cells were incubated for an additional 15 min at 30 °C. After washing with cold water, and with cold 10% (v/v) glycerol, cells were resuspended in a small volume of ice-cold 10% glycerol. For REMI, 20 μ g plasmid DNA was digested with *Bam*HI and then precipitated using ethanol. One hundred units of *Bam*HI mixed with the digested DNA were added to 40 μ l concentrated strain CAI-4 cells kept on ice. The mixture of cells and DNA was electroporated at 1.5 kV, 200 Ω and 25 μ F, and plated on CM-Ura. For homologous recombination, 10 μ g digested plasmid DNA was added to 40 μ l concentrated cells, and electroporated as above.

Analysis of filamentation. For analysis of filamentation under embedded conditions, actively growing cells in YPD medium (~400 c.f.u.) were mixed with molten YPS agar, and plated. Plates were incubated for several days. At various times, 100–200 embedded colonies were examined, and the percentage of filamentous colonies was determined (a colony that contained at least 20 filaments protruding from the periphery of the colony was arbitrarily defined as a filamentous colony). Colonies were photographed with a Nikon E400 microscope at $\times 4$ magnification.

To study filamentous growth in 10% serum and Lee's, proline and GlcNAc media, strains were grown in CM-Ura liquid medium at room

temperature, washed, starved in water, and added to each medium. After incubation at 37 °C for 6 h, cells were observed microscopically, and the percentage of filamentous cells was determined.

Protein localization. Immunofluorescence methods were based on standard methods used for *S. cerevisiae* (Pringle *et al.*, 1991), with modifications. Briefly, *C. albicans* cells expressing N-terminally myc-tagged Drg1p were grown in liquid medium, or were embedded in agarose. Cells were fixed in formaldehyde (4.4%) for 2 h. After fixation, embedded cells were released from the agarose by crushing. After zymolyase treatment, cells were added to wells of polylysine-coated slides, and processed for immunofluorescence with anti-myc antibody clone 4A6 (Upstate), using standard methods. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI), observed by fluorescence microscopy, and photographed using the $\times 60$ objective.

Intravenous inoculation of mice. *C. albicans* cells were grown for 24 h, and harvested by centrifugation. The cells were washed three times with PBS, counted, and adjusted to a density of 5×10^6 cells ml⁻¹; and 0.2 ml of the cell suspension was injected into the lateral tail vein of female CF1 mice (18–20 g body weight; Charles River Laboratories). In each experiment, four mice were inoculated per strain, and this experiment was repeated three times. Mice were observed twice daily after infection with *C. albicans*. In two experiments, some animals were sacrificed, and their kidneys were dissected, fixed in buffered formalin, sectioned, and stained with haematoxylin and eosin or Gomori methenamine silver.

Two-hybrid analysis. *S. cerevisiae* strain EGY40 carrying the *lacZ* reporter plasmid pSH18-34 (Golemis *et al.*, 1996) was transformed with the *lexA* plasmids pEG202, *plexA-DRG1*, pNLexA, p*DRG1-lexA* and *plexA-Fos*, and *GAL4AD* plasmids pGAD-C2, pKLEF4 and pC1.1.2, using standard methods (Ausubel *et al.*, 1989). For assays of β -galactosidase activity, cells grown to exponential phase in synthetic complete medium lacking uracil, histidine and leucine were harvested, and β -galactosidase activity was determined using ONPG as a substrate, as described elsewhere (Clontech, 2001). Analyses were performed in triplicate.

RESULTS

An insertion mutation results in defective filamentation under embedded conditions

In this study, random heterozygous insertion mutations were generated using REMI, as described in Methods. From the first 579 REMI transformants, two interesting strains, termed XC210 and XC211, that exhibited defects in invasive filamentation were identified. These two mutants came from the same REMI pool, which was composed of 44 transformants. Southern blot analysis of the location of the construct indicated that the two strains were probably siblings (data not shown).

Following embedding in YPS agar, and growth at 25 °C, the mutant strains XC210 and XC211 were strongly defective in filamentous growth, in comparison to wild-type strain CKY101 (CAI-4, Ura⁺) (data not shown). Despite the strong defect in filamentous growth under matrix-embedded conditions, no obvious defect in growth rate was observed when strains XC210, XC211 and CKY101 were grown in YPD liquid medium, and the defect in filamentous growth was not observed when strains XC210 and XC211

were grown in 10% serum, or in Lee's, proline or GlcNAc medium at 37 °C (data not shown). Together, these results suggest that the gene disrupted by insertion of pXC132 in strains XC210 and XC211 plays a role in filamentous growth specifically under matrix-embedded conditions.

Strain XC210 contains an insertion in the *DRG1* locus

To find the location of the insertion site in strain XC210, inverse PCR and DNA sequencing were performed, as described in Methods. The inserted DNA construct was found to be located 80 bp upstream of the ORF orf19.5083. This ORF is very similar to those termed *DRG1* in other eukaryotic species; therefore, we termed the *C. albicans* gene *DRG1*.

In *C. albicans*, *DRG1* encoded a protein of 368 aa with characteristic G1–G5 motifs of GTP-binding proteins (Fig. 1). Outside of these motifs, Drg1p is not very similar to the existing subfamilies of GTP-binding proteins, such as heterotrimeric G proteins or ras-like G proteins. As suggested by Sazuka *et al.* (1992), Drg1p represents a novel class of GTP-binding proteins.

drg1 null mutants are defective in filamentation under embedded conditions

To study the function of *DRG1*, *drg1* null mutants were constructed (Fig. 2). The starting strain, CAI-4, was trisomic for chromosome 1, and *DRG1* mapped to chromosome 1. Therefore, the initial heterozygous mutants contained one deleted copy of *DRG1* and two intact copies (e.g. XC301 and XC302; Fig. 2). The second-round heterozygous mutants, e.g. XC303 and XC304, contained two deleted copies of *DRG1* and one intact copy (Fig. 2). Both types of heterozygous mutants had no obvious defect in filamentous growth when they were grown embedded in YPS agar at 25 °C (Fig. 3). Since the original insertion mutants were heterozygous for the insertion, these strains probably carried additional mutations.

Five null mutants constructed from two heterozygotes were characterized; some were trisomic (XC306; Fig. 2), and others had lost one copy of *DRG1* (XC308; Fig. 2). To demonstrate that the phenotypes of these mutants reflected the absence of *DRG1*, an intact *DRG1* gene under the control of its native promoter was transformed into each of the null mutants (XC414; Fig. 2). The phenotype of XC415, a *drg1* null mutant carrying two copies of the *drg1* locus, is shown in Fig. 3. The *drg1* null mutant, carrying *URA3* either at its native locus, at *ADE2*, or at *LEU2*, was defective in filamentation under embedded conditions at 30 °C (Fig. 3) and at 25 °C (data not shown). The filamentous growth defect of the *drg1* null mutant could be partially complemented by reintroduction of the *DRG1*⁺ gene at its native locus, under the control of its native promoter (Fig. 3). A representative colony of each strain grown embedded in YPS agar is shown in Fig. 3(B–D). The *drg1*/

Drg1p (<i>C. albicans</i>)	M.TTVEKIKAIEDEMAKTQKNKATSFHLGQLKAKLAKLRRELLSE
Drg1p (<i>M. musculus</i>)	-SG-LA--AE--A---R-----AH---L---R-----ITP
Drg1p (<i>C. albicans</i>)	GS GSGGGGAGVGFDFVARTGVATVGVGFVSGKSTLLSKLTGTHS
Drg1p (<i>M. musculus</i>)	.K-G----P-E-----K--D-RI-----N-A-VY-
Drg1p (<i>C. albicans</i>)	EAAAYEFTTLTTPGTIKYKAKIQMLDLPGIIEGAKDGKGRGRQ
Drg1p (<i>M. musculus</i>)	-V-----V-R-----L-----
Drg1p (<i>C. albicans</i>)	VIAVARSVNLIFLVLDVNKPLQHKKIIEHELEGMGIRINKEPPNI
Drg1p (<i>M. musculus</i>)	-----TC---LI---L---G-----F---L-SK----
Drg1p (<i>C. albicans</i>)	VITKKDKGGINITNTVPLTHLDNDEIRAVMSEYKINSANIAFRCD
Drg1p (<i>M. musculus</i>)	GFK-----L-A-C-QSE--AETVKSILA---HN-DVTL-S-
Drg1p (<i>C. albicans</i>)	ATVDDLIDAIEAKARKYIPAIYVLNKIDSFSIEELNLLAKIPDAI
Drg1p (<i>M. musculus</i>)	--A-----VV-GN.-V---C-----QI-----DIIY-V-HCV
Drg1p (<i>C. albicans</i>)	PISSANGWNLDLLQMMWDKLLVRVYTKPKGKLPDFNEPVLRS
Drg1p (<i>M. musculus</i>)	---AHHR--F---EKI--Y-----I-----Q---YTS---PY
Drg1p (<i>C. albicans</i>)	DRCTVEDFCNSIHKSLVEDFRNATVYGTSVKHQPQIVGLNHQLED
Drg1p (<i>M. musculus</i>)	S-T-----MK---N-IKE-KY---W-L---N--K--KD-T---
Drg1p (<i>C. albicans</i>)	EDVITILKK
Drg1p (<i>M. musculus</i>)	-----V--

Fig. 1. *C. albicans* Drg1p. Alignment of *C. albicans* Drg1p and mouse (*Mus musculus*) DRG1 is shown. Dashes indicate identical amino acids and dots indicate spaces. Solid lines above sequences indicate the characteristic G1 to G5 motifs of G proteins (Schenker *et al.*, 1994). Boxed residues are important for binding murine DRG1 to the bHLH transcription factor TAL1.

drg1/drg1 homozygous null mutants also exhibited a delay in filamentous colony formation under matrix-embedded conditions, and these defects were partially complemented by addition of wild-type *DRG1*⁺ (data not shown). When

the growth rates of the *drg1* null mutants were compared with those of the wild-type strains during growth in YPS + uridine liquid medium, there were no growth defects for any of the *drg1* mutants (data not shown). These results

Fig. 2. Southern blot analysis of *drg1* null mutants. (A) Restriction map of the *DRG1* locus (not drawn to scale). Horizontal lines depict chromosomal DNA, and black boxes indicate the *DRG1* ORF. Dashed lines delimit the sequences that were deleted and replaced by the *hisG-URA3-hisG* URA-blaster construct. The dotted line indicates the position of the upstream probe used for Southern blotting. (B) Southern blot of *MspI*-digested chromosomal DNA probed with the upstream probe. Positions of the wild-type *DRG1* allele (5.0 kb) and the two *drg1Δ::hisG-URA3-hisG* alleles (3.6 and 3.9 kb) are indicated on the right. XC306 is trisomic, and XC308 has lost the allele that gives the 3.9 kb band. The two bands in the XC414 sample exhibited equal intensity, consistent with two copies of the *DRG1* locus. Lanes: a, CAI-4; b, XC301; c, XC302; d, XC303; e, XC304; f, XC306; g, XC308; h, XC414. (C) Southern blot of *MspI*-digested chromosomal DNA probed with the ORF probe. In lane c, the null mutant completely lacked the *DRG1* ORF and failed to yield a band. Lanes: a, CAI-4; b, XC304; c, XC305.

Fig. 3. Defective invasive filamentation in agar medium. Cells were grown embedded in agar medium as described in Methods. (A) After 1 day of growth at 30 °C, 100–200 colonies were examined, and the percentage of filamentous colonies was determined as described in Methods. (B–D) Representative colonies: (B) XC121 (WT), (C) XC415 (*drg1* null) and (D) XC414 (*DRG1* complemented strain).

support the notion that *DRG1* is important for filamentous growth when *C. albicans* is embedded in a matrix.

Filamentous growth of *drg1* null mutants containing two or three copies of chromosome 1 was further tested under different conditions known to induce *C. albicans* filamentation. The *drg1* null mutant was defective in filamentous growth when embedded in different media (e.g. YPS, Spider or low ammonium minimal), and grown at either 25 or 30 °C. At 37 °C, *drg1* null mutants exhibited defects in filamentation when embedded in low ammonium minimal medium, but not in other media. However, defects in filamentous growth were not observed at 37 °C when cells were grown on the surface of agar media (YPS, Spider, low ammonium minimal), or grown in liquid media (serum, Lee's, proline or GlcNAc). Therefore, these results indicate that *DRG1* is not needed to promote filamentous growth under all conditions.

myc-tagged Drg1p was primarily localized in the cytoplasm

To study the localization of Drg1p in *C. albicans*, pXC156, encoding an N-terminally myc-tagged derivative of *DRG1*, was used. The derivatized gene was recombined into the genome of strain XC308 (*drg1* null mutant) at its native locus under the control of its native promoter, as described in Methods. The derivatized mutant gene was biologically

Fig. 4. Immunolocalization of myc-tagged DRG1p. XC416 encoding *myc-DRG1* was grown in either liquid medium (A, C) or embedded in agarose (B, D). Cells were fixed and processed for immunofluorescence as described in Methods. (A, B) Localization of myc-tagged Drg1p visualized with anti-myc clone 4A6 and anti-mouse IgG conjugated to Texas red. (C, D) Overlay of DAPI-stained nuclei and Texas red-stained myc-Drg1p. Arrows indicate positions of DAPI-stained nuclei, which exhibit reduced staining for myc-Drg1p.

active and complemented the *drg1* null mutation (Fig. 3). In contrast, a C-terminally green fluorescent protein (GFP)-tagged *DRG1* derivative failed to complement the *drg1/drg1* mutation (data not shown); suggesting that modification of the C terminus is not compatible with this function.

Cells expressing myc-*DRG1* or untagged *DRG1* were grown in liquid medium, or were embedded in agarose. These cells were fixed and processed for indirect immunofluorescence with anti-myc clone 4A6 (Upstate) and Texas red-conjugated anti-mouse IgG secondary antibody. As shown in Fig. 4, most of the cells exhibited perinuclear and cytoplasmic staining. In the majority of cells, the DAPI-stained nuclei were deficient in myc-Drg1p staining. When cells expressing untagged *DRG1* were stained, no fluorescent signal was observed (data not shown). Therefore, we conclude that Drg1p exhibits a predominantly cytoplasmic localization, consistent with the results of studies of murine and human DRG1 (Sazuka *et al.*, 1992; Sommer *et al.*, 1994).

Analysis of cells carrying a transcriptional fusion between the *DRG1* promoter region and the gene encoding yEGFP showed that expression of the GFP reporter was similar when cells were grown as yeast cells in liquid medium, or when cells formed embedded filaments (data not shown). Therefore, expression from the *DRG1* promoter did not differ strongly between yeast cells and embedded hyphae.

The *drg1* null mutant caused delayed lethality and exhibited reduced invasiveness following intravenous inoculation of mice

The *drg1* null mutant strain XC413, containing two copies of chromosome 1, was used to test the effect of a *drg1* deletion on the virulence of *C. albicans*. Strains XC101, XC413 (*drg1* null) and XC414 (*DRG1* complemented mutant) were intravenously inoculated into mice, as described in Methods. On day 7, nine of 10 mice inoculated with strain XC413 (*drg1* null mutant) survived, as opposed to four of 12 mice inoculated with strain XC101 (WT), or seven of 11 mice inoculated with XC414 (*DRG1*⁺ complemented). Fisher's exact test showed that the mutant was significantly different from the wild-type strain ($P=0.01$). At later time points, mice inoculated with the *drg1* null mutant succumbed to infection (Fig. 5A); therefore, the mutant strain exhibited delayed lethality. The location of the *URA3* gene in XC414 (*DRG1*⁺ complemented strain) differed from its location in XC101 (WT), and may have influenced the virulence of the complemented strain, as observed previously (Brand *et al.*, 2004; Lay *et al.*, 1998).

To determine whether the *drg1* null mutant exhibited reduced invasiveness, surviving intravenously inoculated mice were sacrificed at 7 days post-inoculation, and their kidneys were examined histologically. As shown in Fig. 5(B), in mice that survived to day 7, strain XC101 (*DRG1*⁺/*DRG1*⁺) exhibited extensive invasive growth in the renal papilla. Also, the kidney cortex showed evidence of interstitial inflammation with some damage to tubules, but organisms were not observed (data not shown). In contrast, when kidneys of mice inoculated with the *drg1* null mutant were examined, organisms were not observed (Fig. 5C). Kidneys of mice inoculated with the complemented strain resembled those of mice inoculated with wild-type organisms (Fig. 5D). These results show that at an early time point following intravenous inoculation of a host, the *drg1* null mutant exhibited reduced invasiveness.

In the absence of Efg1p, a repressor of embedded filamentation, the *drg1* null mutant phenotype was not observed

Human and mouse DRG1 proteins have been shown to bind bHLH transcription factors via their C-terminal regions. The C-terminal region of *C. albicans* Drg1p contains the residues that are required for binding of murine DRG1 to TAL1/SCL1 (Fig. 1). Therefore, because the bHLH transcription factor Efg1p is an important negative regulator of filamentation during growth under embedded conditions (Giusani *et al.*, 2002), interaction between *C. albicans* Drg1p and Efg1p was analysed using the two-hybrid system. A *lexA*–*DRG1* fusion was tested for interaction with a *GAL4AD*–*EFG1* fusion. As shown in Table 3, while control strains exhibited low levels of the reporter β -galactosidase, strains containing both *DRG1* and *EFG1* fusions exhibited approximately two- to threefold higher activity. In contrast, C-terminally tagged *DRG1*–*lexA* failed to exhibit

Fig. 5. Delayed lethality caused by the *drg1* null mutant. (A) Cells of strains XC101 (*DRG1*/*DRG1*) (●, WT), XC413 (*drg1*Δ::his/*drg1*Δ::his) (▲, *drg1* null) and XC414 (*drg1*Δ::his/*drg1*Δ::his/*DRG1* complemented) (■, Comp, dotted line) were inoculated intravenously into 10–12 mice, and survival as a function of time post-inoculation was monitored. Some animals were sacrificed for histological analysis of kidneys. All mice from three experiments are shown in the graph. (B–D) Mice surviving at day 7 were sacrificed, and kidneys were fixed, sectioned and stained with Gomori methenamine silver staining. Sections were photographed with the ×40 objective. (B) Extensive growth of strain XC101 (WT) in the renal papilla is shown. (C) Organisms were not detected in the renal papilla of mice inoculated with *drg1* null strain XC413. (D) Complemented mutant XC414 exhibiting growth in the renal papilla.

a two-hybrid interaction with *GAL4AD*–*EFG1* (Table 3), consistent with the notion that modification of the C terminus of Drg1p is incompatible with its function. Therefore, Drg1p and Efg1p may interact, and the effects

Table 3. Two-hybrid interaction between *DRG1* and *EFG1*

NA, Not applicable.

Sample	lexA fusion	GAL4AD fusion	Activity*	P value†
1	lexA- <i>DRG1</i>	<i>GAL4AD-EFG1</i>	8.9 ± 1.0	NA
2	Unfused‡	<i>GAL4AD-EFG1</i>	3.8 ± 1.4	0.001
3	lexA- <i>DRG1</i>	Unfused‡	3.5 ± 0.7	0.006
4	lexA- <i>DRG1</i>	<i>GAL4AD-SLK19</i> §	3.7 ± 1.1	0.004
5	lexA- <i>fos</i> §	<i>GAL4AD-EFG1</i>	3.0 ± 1.0	0.002
6	PNlexA (unfused)‡	<i>GAL4AD-EFG1</i>	3.2 ± 1.0	NA
7	<i>DRG1-lexA</i>	<i>GAL4AD-EFG1</i>	3.9 ± 1.1	NA

*Units of β-galactosidase activity ± SD.

†P value versus sample 1 determined by two-tailed *t* test.

‡Strain carrying the empty vector plasmid.

§Fusions that are not expected to interact with either Drg1p or Efg1p.

of Drg1p on invasive filamentation may be mediated through binding of Efg1p.

If Drg1p binds and regulates Efg1p, then the defect in invasive filamentation caused by deletion of *DRG1* would not be observed in the absence of Efg1p. To test this hypothesis, a *drg1 efg1* double null mutant was constructed. In contrast to a *drg1* null mutant, which was delayed in filamentation under embedded conditions (Fig. 3), an *efg1* null mutant was hyperfilamentous (Fig. 6; Giusani *et al.*, 2002). For these experiments, the samples were incubated at room temperature, rather than at 30 °C. The lower temperature resulted in slower development of filamentous colonies, allowing detection of the hyperfilamentous phenotype. The *efg1 drg1* double null mutant was also hyperfilamentous, and was indistinguishable from the *efg1* single null mutant (Fig. 6). Reintroduction of the *EFG1*⁺ gene into either the *efg1 drg1* double null mutant or the *efg1* single null mutant resulted in partial complementation of the *efg1* mutation, and reduced filamentation (Fig. 6;

Giusani *et al.*, 2002). Therefore, invasive filamentation can occur in the absence of Drg1p, and the effects of the *drg1* mutation are not observed in the absence of Efg1p. These results suggest that Drg1p plays a role in regulating the function of Efg1p.

DISCUSSION

In this communication, we demonstrate that cells lacking the highly conserved G protein Drg1p exhibit reduced invasiveness, both during growth in the laboratory and within a host. The findings that mice infected with the *drg1* null mutant survived longer, and that growth of mutant *C. albicans* cells in the mouse kidney was reduced at an early time point, indicate that the ability of *C. albicans* to grow invasively in tissue contributes to the progression of lethal infection. Previous studies have similarly linked tissue invasiveness and virulence (e.g. Felk *et al.*, 2002).

DRG1 is a highly conserved gene with orthologues in the genomes of species from archaea to humans. The *S. cerevisiae* orthologue Rbg1p/Fun11p has been shown to bind to a polysome-associated protein (Fleischer *et al.*, 2006). *C. albicans* Drg1p may similarly interact with polysome-associated proteins, as well as with other proteins.

Mutants lacking Drg1p are capable of forming filaments under a wide range of conditions, demonstrating that Drg1p is not strictly needed for formation of filamentous cells. However, the mutant strain was defective in responding to the cues that normally promote filamentation when the organism is growing embedded within agar media. These results suggest that Drg1p is involved in the mechanism for sensing or responding to signals arising from growth within agar media. One of the important cues that fungal cells experience during growth within agar is contact (Kumamoto, 2005). Therefore, since G proteins typically function as molecular switches, with active and inactive forms, the activation state of Drg1p may change when cells sense contact with agar media or with host tissue, and Drg1p

Fig. 6. Bypass of the *drg1* mutant defect by deletion of *EFG1*. Cells were embedded in agar medium, and incubated at room temperature. After 2 days, 100–200 colonies were observed and scored for filamentation. Graph shows percentage filamentous colonies for the following strains: WT, XC121; *efg1* null, CKY136; *efg1/EFG1* complemented, HLC74; *drg1 efg1* double null, XC310 (two isolates analysed); and *drg1 efg1/EFG1*, XC311 (two isolates analysed). Two different isolates of each strain were analyzed; the result obtained with one isolate is shown in grey and the other in black.

may participate in contact-dependent signalling or responses.

In mammalian cells, *Drg1p* has been previously implicated in processes related to growth control and oncogenic transformation. For example, *DRG1* expression is associated with proliferating cells. In the mouse, *DRG1* is relatively highly expressed in embryonic cells, and has lower expression in cells after birth (Sazuka *et al.*, 1992). In plants, *DRG* is more highly expressed in growing cells as opposed to non-growing cells (Devitt *et al.*, 1999). These findings suggest that *DRG1* plays a role in a process that is necessary in proliferating cells.

In addition, ectopic expression of mouse *DRG1* enhances transformation of rat embryonic fibroblasts by c-myc and ras (Mahajan *et al.*, 1996). Since insensitivity to contact-dependent regulation of cellular proliferation is a characteristic of cancer cells, ectopic expression of *DRG1* may contribute to the dysregulation of this normal control mechanism, enhancing transformation. Therefore, by analogy with the function of *Drg1p* in *C. albicans*, *DRG1* in mammalian cells may play a role in contact-dependent signalling, or in the response of mammalian cells to contact-dependent cues.

Previous studies have demonstrated binding of mouse or human *DRG* proteins to the transcription factor oncogene *TAL1/SCL1*. This binding is mediated through the HLH region of *TAL1* and the C-terminal region of *DRG* (Mahajan *et al.*, 1996). *C. albicans* *Drg1p* exhibited a two-hybrid interaction with the bHLH transcription factor *Efg1p*, an important negative regulator of filamentation under embedded conditions. Since deletion of *DRG1* resulted in delayed filamentation under embedded conditions, binding of *Efg1p* by *Drg1p* may be a mechanism that contributes to relief of negative regulation by *Efg1p*. Consistent with this possibility, deletion of *EFG1* bypassed the defect in filamentation caused by deletion of *DRG1*.

Efg1p function appears to be tightly regulated through numerous mechanisms. At the transcriptional level, expression of the *EFG1* gene is negatively autoregulated through binding of *Efg1p* to the TATA box region of its promoter (Tebarth *et al.*, 2003). At the protein level, *Efg1p* is activated by phosphorylation by protein kinase A (Bockmuhl & Ernst, 2001). In addition, although *Efg1p* is required for hyphal development in liquid medium, once hypha formation is initiated, down-regulation of *EFG1* expression is observed, and forced expression of *EFG1* during hyphal growth inhibits hyphal development (Tebarth *et al.*, 2003). The findings described in this communication reveal another possible mechanism for regulation of *Efg1p*: binding by *Drg1p*. *Drg1p* is cytoplasmically localized and, therefore, may regulate *Efg1p* by affecting its localization. Many transcription factors exhibit cytoplasmic localization under certain conditions, and *Efg1p* may undergo *Drg1p*-dependent cytoplasmic localization when its activity needs to be inhibited, such as during embedded filamentation.

Taken together, these results suggest that tight control of *Efg1p* function at both the transcriptional and post-transcriptional levels is important for filamentation.

In addition to filamentation, *Efg1p* regulates expression of classical virulence factors (e.g. proteases and adhesins), as well as numerous genes involved in metabolism (Doedt *et al.*, 2004; Nantel *et al.*, 2002). Thus, the role of *Efg1p* in cellular physiology goes well beyond its role in controlling cellular morphology. In this context, the tight regulation of *Efg1p* function through multiple regulatory mechanisms allows careful control of a wide range of cellular processes that are impacted by this important transcription factor.

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