Analysis of cell-cycle specific localization of the Rdi1p RhoGDI and the structural determinants required for Cdc42p membrane localization and clustering at sites of polarized grow...

ARTICLE *in* CURRENT GENETICS · JULY 2004

Impact Factor: 2.68 · DOI: 10.1007/s00294-004-0505-9 · Source: PubMed

9 AUTHORS, INCLUDING:

Kurt A Toenjes
Montana State University Billings
12 PUBLICATIONS **368** CITATIONS
SEE PROFILE

Sergio E Morales
University of Otago
28 PUBLICATIONS **382** CITATIONS
SEE PROFILE

Matthew F Whelihan
University of North Carolina at Chapel Hill
14 PUBLICATIONS **270** CITATIONS
SEE PROFILE

Douglas I Johnson
University of Vermont
43 PUBLICATIONS **3,223** CITATIONS
SEE PROFILE

Curr Genet (2004) 45: 339–349
DOI 10.1007/s00294-004-0505-9

RESEARCH ARTICLE

**Tamara J. Richman · Kurt A. Toenjes
Sergio E. Morales · Karen C. Cole · Ben T. Wasserman
Chad M. Taylor · Jacob A. Koster
Matthew F. Whelihan · Douglas I. Johnson**

Analysis of cell-cycle specific localization of the Rdi1p RhoGDI and the structural determinants required for Cdc42p membrane localization and clustering at sites of polarized growth

Received: 20 February 2004 / Revised: 23 March 2004 / Accepted: 27 March 2004 / Published online: 17 April 2004

Abstract The Cdc42p GTPase regulates multiple signal transduction pathways through its interactions with downstream effectors. Specific functional domains within Cdc42p are required for guanine-nucleotide binding, interactions with downstream effectors, and membrane localization. However, little is known about how Cdc42p is clustered at polarized growth sites or is extracted from membranes by Rho guanine-nucleotide dissociation inhibitors (RhoGDIs) at specific times in the cell cycle. To address these points, localization studies were performed in *Saccharomyces cerevisiae* using green fluorescent protein (GFP)-tagged Cdc42p and the RhoGDI Rdi1p. GFP-Rdi1p localized to polarized growth sites at specific times of the cell cycle but not to other sites of Cdc42p localization. Overexpression of Rdi1p led to loss of GFP-Cdc42p from internal and plasma membranes. This effect was mediated through the Cdc42p Rho-insert domain, which was also implicated in interactions with the Bni1p scaffold protein. These data suggested that Rdi1p functions in cell cycle-specific Cdc42p membrane detachment. Additional genetic and time-lapse microscopy analyses implicated nucleotide binding in the clustering of Cdc42p. Taken together, these results provide insight into the complicated nature of the relationships between Cdc42p localization, nucleotide binding, and protein–protein interactions.

Keywords RhoGDI · Cell polarity · Cdc42p · *Saccharomyces cerevisiae* · Green fluorescent protein

Communicated by S. Hohmann

T. J. Richman · K. A. Toenjes · S. E. Morales · K. C. Cole
B. T. Wasserman · C. M. Taylor · J. A. Koster
M. F. Whelihan · D. I. Johnson (✉)
Department of Microbiology and Molecular Genetics and the Markey Center for Molecular Genetics, University of Vermont, 95 Carrigan Dr., 202 Stafford Hall, Burlington, VT 05405, USA
E-mail: douglas.johnson@uvm.edu
Tel.: +1-802-6568203
Fax: +1-802-6568749

Present address: T. J. Richman
Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation St., FL 8, Worcester, MA 01605-2324, USA

Introduction

The Rho-type GTPase Cdc42p mediates various signal transduction pathways that regulate cell polarity, actin rearrangements, and cell cycle progression in most, if not all, eukaryotic cells (Etienne-Manneville and Hall 2002; Johnson 1999). Three inherent Cdc42p properties control these various functions: guanine-nucleotide binding and hydrolysis, interactions with regulators and downstream effectors, and intracellular targeting. Each of these properties is associated with specific structural domains within Cdc42p (Fig. 1). Guanine-nucleotide binding and hydrolysis depends primarily on three domains (Vetter and Wittinghofer 2001): (1) residues 5–20, which constitute the conserved P-loop that interacts with the β,γ-phosphates of the bound nucleotide, (2) residues 53–62, known as the switch II domain, which make contacts with the γ-phosphate and thus functions in GTPase-activating protein (GAP)-mediated GTP hydrolysis, and (3) residues 111–118, which interact with the guanine ring. Interactions with downstream effectors occur primarily through the switch I effector domain (residues 26–50), although other residues mediate interactions with some effectors and regulators. The Rho-insert domain (residues 122–135), a ca. 13-amino-acid region unique to Rho-type GTPases, is implicated in the activation of various downstream effectors (Diebold and Bokoch 2001; Joneson and Bar-Sagi 1998; McCallum et al. 1996; Walker and Brown 2002; Walker et al. 2000), in cellular transformation (Wu et al. 1998), and in providing stability to the nearby guanine-nucleotide binding

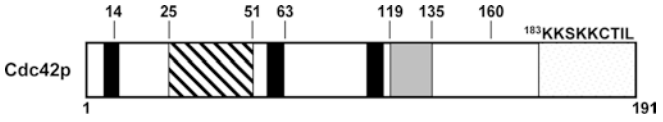


Fig. 1 Cdc42p functional domains. *Black boxes* represent residues 5–20, 53–62, and 111–118, implicated in nucleotide binding and hydrolysis. The *striped box* represents residues 26–51, defining the switch I effector domain, and the *gray box* represents residues 122–135, defining the Rho-insert domain. The *stippled box* represents the ^{183}KKSKKCTIL membrane localization domain

domain that coordinates guanine ring binding (Hoffman et al. 2000).

Localization and attachment of Cdc42p to membranes is essential for its function (Ziman et al. 1991, 1993) and depends on the carboxyl-terminal membrane localization domain (residues 183–191), encompassing the geranylgeranylated ^{188}Cys residue and the ^{183}KKSKK polylysine domain (Davis et al. 1998; Finegold et al. 1991; Ziman et al. 1993). Functional green fluorescent protein (GFP)-Cdc42p fusions in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosacharomyces pombe* have been localized to the entire plasma membrane and internal membranes, including the nuclear and vacuole membranes (Merla and Johnson 2000; Richman et al. 2002). In *Sac. cerevisiae*, GFP-Cdc42p also clustered at polarized growth sites at two distinct stages of the cell cycle: the incipient bud site in G_1/S and the mother-bud neck region at cytokinesis (Richman et al. 1999, 2002). Mutational analyses indicated that Cdc42p has essential signaling functions at both of these times in the cell cycle (Johnson 1999; Richman and Johnson 2000; Richman et al. 1999), correlating clustering to functional activity. The carboxyl-terminal localization domain was sufficient for membrane localization but not for clustering (Richman et al. 2002), indicating that residues amino-terminal to the localization domain were involved in clustering.

The involvement of protein–protein interactions or nucleotide binding in regulating Cdc42p localization has not been definitively shown. However, nucleotide binding was implicated in clustering through analysis of constitutively active, GTP-bound GFP-Cdc42^{G12V}p, which clustered at multiple sites and persisted at those sites longer than wild-type GFP-Cdc42p (Richman et al. 2002). Furthermore, the guanine-nucleotide exchange factor (GEF) Cdc24p localized to the same polarized growth sites as Cdc42p (Butty et al. 2002; Nern and Arkowitz 1999; Richman et al. 2002; Toenjes et al. 1999), suggesting a possible relationship between Cdc42p activation to a GTP-bound state and clustering. Cdc42p clustering was not affected in strains deleted for the downstream effectors Cla4p, Bni1p, Gic1p, and Gic2p, but the Bni1p-forming homologue and the filament-forming septin proteins were implicated in the timing and positioning, respectively, of mother-bud neck clustering (Richman et al. 2002).

Mammalian Rho guanine-nucleotide dissociation inhibitors (RhoGDIs) have been shown to stimulate the release of Rho-type GTPases from cellular membranes (Hori et al. 1991; Nomanbhoy and Cerione 1996; Wu et al. 1997) and to block GDP dissociation and inhibit GTP hydrolysis by antagonizing the actions of GEFs and GAPs (Hart et al. 1992). Stimulation of membrane release requires the binding of RhoGDI to the geranylgeranylated carboxyl-terminus of Cdc42p and interactions between an "acidic patch" in RhoGDI and the polylysine region (Hoffman et al. 2000; Nomanbhoy et al. 1999). The ability of RhoGDI to block GDP dissociation and inhibit GTP hydrolysis is thought to be a consequence of interactions between RhoGDI amino-terminal residues and the Cdc42p switch I and II regions (Hoffman et al. 2000). Thus, RhoGDIs provide a link between nucleotide binding and membrane localization. *Sac. cerevisiae* has a single RhoGDI, Rdi1p; and previous studies showed that deletion of *RDI1* had no effect on cellular morphogenesis, but overexpression of Rdi1p led to lethality with an increased amount of Cdc42p in cytosolic fractions (Koch et al. 1997; Masuda et al. 1994). These data suggest that Rdi1p is not important for Cdc42p membrane localization and attachment, which is essential for Cdc42p function. However, these data are consistent with Rdi1p activity being important for regulating the extraction of Cdc42p from membranes, a controlled process that can have a negative impact on Cdc42p essential functions and cell viability when exacerbated by Rdi1p overexpression.

The objectives of this study were to examine the role of the Rdi1p RhoGDI in Cdc42p membrane localization and to look more closely at the potential roles of protein–protein interactions and nucleotide binding in Cdc42p clustering at polarized growth sites. GFP-Rdi1p localized to sites of Cdc42p localization only at specific times in the cell cycle, i.e., the tips of small buds in G_1/S and the mother-bud neck region during cytokinesis. Overexpression of the Rdi1p RhoGDI led to a decrease in Cdc42p membrane localization, but did not affect clustering, suggesting that Rdi1p functions in membrane extraction but not membrane attachment. The Rdi1p-dependent release of Cdc42p from membranes required the Cdc42p Rho-insert domain, which was also implicated in Cdc42p–Bni1p interactions. Localization patterns of truncated GFP-Cdc42p and the temporal co-localization of Cdc42p and its guanine-nucleotide exchange factor Cdc24p also supported a model in which nucleotide binding regulated clustering. Collectively, these studies shed light on the unique and overlapping functions of Cdc42p domains.

Materials and methods

Reagents, media, and strains

Enzymes, polymerase chain reaction (PCR) kits, and other reagents were obtained from standard commercial

sources and used as specified by the suppliers. Oligonucleotide primers for sequencing and PCR were obtained from Qiagen Operon (Alameda, Calif.) and are available upon request. Growth media, maintenance of bacterial and yeast strains, and yeast transformations were described by Sambrook et al. (1989) and Sherman et al. (1986). Selection of transformants was on synthetic complete (SC) dropout medium lacking specified amino acid(s) and containing 2% glucose as a carbon source. For galactose induction, cells were grown in medium containing 2% raffinose with 2% galactose instead of 2% glucose. The yeast strains used are listed in Table 1.

DNA and protein analysis

Recombinant DNA manipulations (Sambrook et al. 1989) and plasmid isolation from *Escherichia coli* (Birnboim and Doly 1979) were performed as described by Davis et al. (1998). Automated DNA sequencing at the Vermont Cancer Center DNA Sequencing Facility was used to sequence all gene constructs. Site-directed mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.A). The plasmids used are listed in Table 2. Plasmid p415MET(GFP-*RDI1*) was constructed by inserting a *Bam*HI-linked ca. 600-bp PCR-generated *RDI1* fragment from pRS316(*RDI1*) into *Bam*HI-cleaved p415MET(GFP). Using the QuikChange kit, the T17N and R133G mutations were incorporated separately into p415MET(GFP-*CDC42*) and the R133G mutation was incorporated into pRS315(*CDC42*) and pRS306 (*CDC42*). The cycling parameters for the mutagenesis were 12 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 17 min. Primer sequences are available upon request.

The starting template for the creation of amino-terminal truncations was p415MET(GFP-*CDC42*), which has a *Bam*HI restriction site immediately 5′ of the *CDC42* ORF (Fig. 4). A second *Bam*HI restriction site was incorporated at the desired site in the *CDC42* ORF and the resulting plasmid was digested with *Bam*HI to remove the intervening coding region and then religated, ensuring that the integrity of the GFP-Cdc42p fusion remained intact and the designated coding region was removed. The same starting template was used for the creation of internal *cdc42* deletions, but the *Bam*HI site immediately 5′ of the *CDC42* ORF was first destroyed and new *Bam*HI restriction sites were then incorporated flanking the sequences to be removed. The resulting plasmid was digested with *Bam*HI to remove the intervening coding region and the gapped plasmid was religated with the GFP-Cdc42p fusion remaining intact.

Expression and molecular weights of all truncated proteins were verified by immunoblotting methods. Protein was isolated from wild-type strain TRY11-7D expressing the various GFP-Cdc42p constructs and immunoblot analysis was performed using anti-GFP antibody (Roche, Indianapolis, Ind.) as described by Toenjes et al. (1999). Detection of GTP-bound wild-type and mutant GFP-Cdc42p constructs was through the EZ-Detect Cdc42 activation kit (Pierce Biotechnology,

Table 1 Yeast strains used in this study. To create strain WRY1, the integrating plasmid pRS306 (*cdc42*^{R133G}), which was linearized within the *URA3* gene, was transformed into *CDC42*/Δ*cdc42*::TRP1 heterozygous diploid DJD6-11; and stable Ura$^+$ transformants had *cdc42*^{R133G}, under the control of the endogenous *CDC42* promoter, integrated at the *ura3* locus. WRY1-8A is a spore of WRY1. TRY4-2A, TRY4-7B and TRY4-3D were generated from tetrad dissection of SY3032 (Stevenson et al. 1995). TRY4-3D-H was generated by integrating a *ura3:HIS3* fragment into TRY4-3D

Strain	Genotype	Reference or source
DJTD2-16A	*MATa cdc42-1 his4 leu2 ura3 trp1*	Johnson and Pringle (1990)
Y147	*MATa cdc24-4 his3 leu2 ura3*	Bender and Pringle (1989)
DJD6-11	*MATa*/*MATα his3*Δ200/+ *his4*/+ *ade2-101*/+ *leu2*/+ *lys2-801*/*lys2-801 ura3-52*/*ura3-52 trp1-Δ1*/*trp1-Δ101 can1*/+ *cdc42*Δ::*TRP1*/+	Miller and Johnson (1997)
WRY1	*MATa*/*MATα his3*Δ200/+ *his4*/+ *ade2-101*/+ *leu2*/+ *lys2-801*/*lys2-801 trp1-Δ1*/*trp1-Δ101 can1*/+ *ura3-52:cdc42*^{R133G}*:URA3*/*ura3-52 cdc42*Δ::*TRP1*/+	This study
WRY1-8A	*MATa lys2-801 trp1-Δ1 ura3-52:cdc42*^{R133G}*:URA3 cdc42*Δ::*TRP1*	This study
TRY11-7D	*MATα his leu2 ura3 trp1*	Richman et al. (1999)
W303-1A	*MATa his3-11,5 ade2-101 leu2-3,112 trp1-Δ1 ura3-1 can1-100*	R. Rothstein
EGY48-p1840	*MATα his3 ura3 trp1 integrated lexAop-LEU2 integrated lexAop-lacZ*	Gyuris et al. (1993)
MJ398	*MATa his3 leu2 ura3 trp1 ade2 gic1*::URA3 gic2:his::URA3	Brown et al. (1997)
RAK21	*MATa ade2 his3 leu2 trp1 ura3 can1 bar1*::HIS3	Ziman et al. (1993)
RG13340	*MAThis3 leu2 lys2 ura3 bem1*::G418	Research Genetics
TRY4-2A	*MATa his3 leu2 ura3 trp1 rga1*::URA3	This study
TRY4-7B	*MATα his3 leu2 ura3 trp1 bem3*::LEU2	This study
TRY4-3D	*MATα his3 ade1 leu2 ura3 trp1 bem3*::LEU2 rga1::URA3	This study
TRY4-3D-H	*MATα his3 ade1 leu2 ura3 trp1 bem3*::LEU2 rga1::ura3::HIS3	This study
OHNY-TM1	*MATa his3 ade2 leu2 ura3 rdi1*::HIS3	Masuda et al. (1994)
ABY973	*MATa*/*MATα his3-*Δ*200*/*his3-*Δ*200 leu2-3,112*/*leu2-3,112 lys2-801*/*lys2-801 ura3-52*/*ura3-52 trp1-1(am)*/*trp1-1(am) tpm2*::HIS3/tpm2::HIS3	A. Bretscher
ABY971	*MATa*/*MATα his3-*Δ*200*/*his3-*Δ*200 leu2-3,112*/*leu2-3,112 lys2-801*/*lys2-801 ura3-52*/*ura3–52 trp1-1(am)*/*trp1-1(am) tpm1-2*^{ts}::LEU2/tpm1-2^{ts}::LEU2 tpm2::HIS3/tpm2::HIS	A. Bretscher

Table 2 Plasmids used in this study. Plasmids containing Cdc42p truncations and internal deletions (Fig. 4) are not listed but were derived from plasmid p415MET(GFP-*CDC42*); see Materials and methods

Plasmid	Reference or Source
p415MET(GFP)	Toenjes et al. (1999)
p415MET(GFP-*CDC42*)	Richman and Johnson (2000)
p415MET(GFP-*cdc42*^{T17N})	This study
p415MET(GFP-*cdc42*^{R133G})	This study
p415MET(GFP-*cdc42*^{C188S})	Richman et al. (2002)
p415MET(GFP-KKSKKCTIL)	Richman et al. (2002)
p415MET(GFP-CTIL)	Richman et al. (2002)
p415MET(YFP-*CDC24*)	Richman et al. (2002)
p416MET(GFP-*CDC42*)	Richman and Johnson (2000)
p416MET(CFP-*CDC42*)	Richman et al. (2002)
pKT10(*RDI1*)	Masuda et al. (1994)
pRS316(*RDI1*)	This study
p415MET(GFP-*RDI1*)	This study
pRS315(*CDC42*)	Ziman et al. (1991)
pRS315(*CDC42*^{R133G})	This study
pRS306(*CDC42*)	Ziman et al. (1991)
pRS306(*CDC42*^{R133G})	This study
pRS315(p *GAL-CDC42*)	Ziman et al. (1991)
pRS315(*CDC24*)	Ziman and Johnson (1994)
pEG202(*CDC42*)	Stevenson et al. (1995)
pEG202(*cdc42*^{C188S})	Stevenson et al. (1995)
pEG202(*cdc42*$^{D118A,\ C188S}$)	Stevenson et al. (1995)
pEG202(*cdc42*$^{G12V,\ C188S}$)	Stevenson et al. (1995)
pEG202(*cdc42*$^{R133G,\ C188S}$)	This study
pJG4-5(*BNI1*)	Evangelista et al. (1997)
pJG4-5(*CLA4*)	Cvrcková et al. (1995)
pRL222(*STE20*)	M. Whiteway
pJG4-5(*GIC1*)	Chen et al. (1997)
pJG4-5(*GIC2*)	Chen et al. (1997)

Rockford, Ill.), which is based on the ability of GTPγS-bound Cdc42p to preferentially interact with a p21-binding domain (PBD) peptide conjugated to glutathione-*S*-transferase (GST) and bound to glutathione-agarose beads (Benard et al. 1999). GTPγS-bound GFP-Cdc42p was observed by immunoblotting using anti-GFP antibodies (1:500 dilution).

Random mutagenesis and two-hybrid analysis

A PCR-based mutagenesis protocol (Miller and Johnson 1997) was performed with *Taq* polymerase to produce random mutations in *CDC42* that inhibited Bni1p interactions. A 2,279-bp fragment, containing the *CDC42* coding sequence plus the ADH promoter, LexA-binding domain, and ADH terminator non-*CDC42* flanking regions, was obtained by mutagenic PCR from pEG202(*CDC42*). The pool of mutagenized fragments was co-transformed with pEG202(*CDC42*), which had been cut with *Eco*RI, into the two-hybrid strain EGY48-p1840. Through gap repair, the mutagenized fragments, designated *cdc42*^{PCRMUT}, recombined with the gapped plasmid, reconstituting pEG202(*cdc42*^{PCRMUT}). The resulting transformants were mated with W303-1A containing pJG4-5(*BNI1*), selected on SC-His-Trp medium containing 2% galactose with 2% raffinose at 23°C, and screened for interactions using two-hybrid lifts as described by Davis et al. (1998). Plasmids were recovered from diploids showing reduced two-hybrid interactions and were subjected to PCR to verify that the plasmid contained a *cdc42* insert. Such plasmids were then retransformed into EGY48-p1840 and mated with W303-1A containing pJG4-5(*BNI1*) to verify the interaction phenotype. The different *cdc42*^{PCRMUT} inserts were sequenced to determine what mutations were incorporated. Using the same mating and two-hybrid procedure, the confirmed pEG202(*cdc42*^{PCRMUT}) plasmids were tested with pJG4-5(*CLA4*), pRL222(*STE20*), pJG4-5(*GIC1*), pJG4-5(*GIC2*), and pRL222(*STE20*). Strains containing pRL222(*STE20*) and the various pEG202(*CDC42*) constructs were selected on SC-His-Leu containing 2% glucose.

Photomicroscopy

Cells were grown in the appropriate liquid media to mid-log phase, collected by centrifugation, sonicated, and examined morphologically. Methods for preparing and staining cells with FM4-64 were described by Murray and Johnson (2001). Cells containing the various GFP-Cdc42p constructs were grown in SC-Ura-Met or SC-Leu-Met medium as appropriate for expression from the methionine-repressible promoter. Time-lapse photomicroscopy using phase-contrast optics and an Omega XF100 optical filter cube to visualize GFP fluorescence was performed on an E400 Nikon microscope (Omega Optical, Brattleboro, Vt.), as described by Richman et al. (2002). Digital cell images were obtained and analyzed as described by Merla and Johnson (2001). Where indicated, cells from the same culture but different fields were assembled into collages using Adobe Photoshop ver. 7.0.

Results

Localization of GFP-Rdi1p to tips of enlarging buds and mother-bud neck regions

Based on the multiple functions of RhoGDIs in regulating Cdc42p localization and activation, it is posited that Rdi1p either co-localizes with Cdc42p at all locations throughout the cell cycle or only co-localizes when it is necessary to extract Cdc42p from membranes (i.e., inactivate Cdc42p). To examine this question, a GFP-Rdi1p fusion protein was localized. In an asynchronous culture of cells, GFP-Rdi1p had a general cytoplasmic localization, which was in agreement with previously published immunofluorescence localization of a hemagglutinin (HA)-tagged Rdi1p (Koch et al. 1997). However, GFP-Rdi1p was also observed at the tips of small buds during S phase and as a band at the mother-bud neck region during cytokinesis (Fig. 2a), times in the cell cycle when Cdc42p would be predicted to be active. GFP-Rdi1p was not consistently observed at other sites

of Cdc42p localization, such as around the periphery of the cell, at internal membranes, or at sites of incipient bud formation. These data indicate that Rdi1p localizes to sites of Cdc42p localization only at specific times in the cell cycle.

Stimulation of GFP-Cdc42p release from membranes by the Rdi1p RhoGDI required the Rho-insert domain

Previous studies have shown that Rdi1p overexpression leads to lethality, with an increased amount of Cdc42p in cytosolic fractions (Koch et al. 1997; Masuda et al. 1994). The nature of the lethality was not determined but it is consistent with a model in which excess Rdi1p improperly removed Cdc42p from membranes, a potentially lethal event. To analyze the effects of Rdi1p overexpression on Cdc42p membrane localization and clustering, a plasmid containing *RDI1* under a galactose-inducible promoter was transformed into wild-type strain TRY11-7D along with a GFP-*CDC42* vector. Under non-inducing (glucose) growth conditions, GFP-Cdc42p membrane localization and clustering were comparable with wild-type cells without the *RDI* vector (Fig. 2b). After galactose induction of Rdi1p expression for up to 24 h, ca. 50% of the cells showed diffuse cytosolic fluorescence and no membrane localization or clustering (Fig. 2b). These results further supported the model in which excess Rdi1p can stimulate the removal of Cdc42p from membranes. Although overexpression of Rdi1p has been shown to be lethal, *rdi1*Δ cells did not exhibit any defects in cell viability or morphology (Masuda et al. 1994), suggesting that Cdc42p localization would not be affected by the loss of Rdi1p. Consistent with this assertion, GFP-Cdc42p localized to membranes and clustered normally at polarized growth sites in *rdi1*Δ cells (data not shown).

Cdc42p Rho-insert domain is necessary for Rdi1p function

To define the Cdc42p region required for Rdi1p action, the effects of Rdi1p overexpression on the localization of truncated Cdc42 proteins were observed. Rdi1p overexpression led to the removal of GFP-Cdc42$^{119–191}$p from membranes (see Fig. 4a for Cdc42p truncation mutants), with ca. 44% of cells showing only cytoplasmic fluorescence and no membrane fluorescence upon induction with galactose for 7.5 h (Fig. 2c). However, GFP-Cdc42$^{135–191}$p (Fig. 2c) and GFP-Cdc42$^{160–191}$p (see Figs. 4, 5; data not shown) were still observed around the periphery of cells. These results suggested that residues 119–135, which define the Rho-insert domain, were necessary for Rdi1p-dependent stimulation of Cdc42p removal from membranes. However, based on the structural characterization of the mammalian Cdc42p–RhoGDI complex (Hoffman et al. 2000), RhoGDI does not bind to the Rho-insert domain.

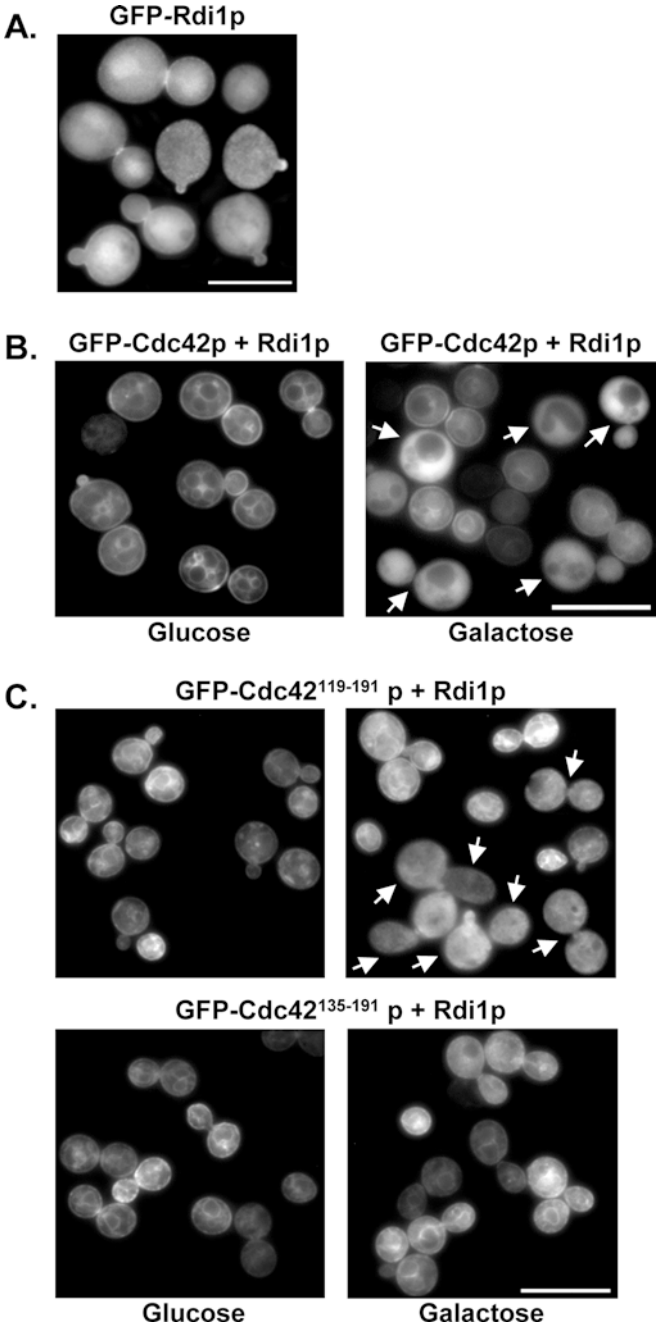


Fig. 2 a–c Overexpression of Rdi1p leads to accumulation of GFP-Cdc42p in the cytoplasm. *Bars* 10 μm. **a** Subcellular localization of GFP-Rdi1p. Plasmid p415MET(GFP-*RDI1*) was transformed into strain RAK21 and cells from an asynchronous culture were viewed by fluorescence microscopy. **b** Plasmids p415MET(GFP-*CDC42*) and pKT10(*RDI1*) were transformed into wild-type strain TRY11-7D. Cells shown were grown in Sc-Leu-Ura-Met medium containing 2% glucose (*left panel*) or Sc-Leu-Ura-Met medium containing 2% raffinose with 2% galactose (*right panel*) for 7 h. **c** Experiment was done as in **b** but with plasmids p415MET(GFP-*cdc42*$^{119–191}$) (*upper panels*) or p415MET(GFP-*cdc42*$^{135–191}$) (*lower panels*). Cells shown were observed at 7.5 h post-induction. *Arrows* in **b** and **c** point to cells that did not display membrane GFP-Cdc42p fluorescence

Therefore, the Rho-insert domain effects on RhoGDI function are most probably indirect and may require the involvement of another binding partner.

A.

Cdc42p (pEG202-BD) Mutation	Cdc42p Effector Proteins (pJG4-5-AD)				
	Bni1p	Cla4p	Ste20p	Gic1p	Gic2p
WT	+	+	+	+	+
R133G	-	+	+	+	+
D118A	-	-	-	-	-

B.

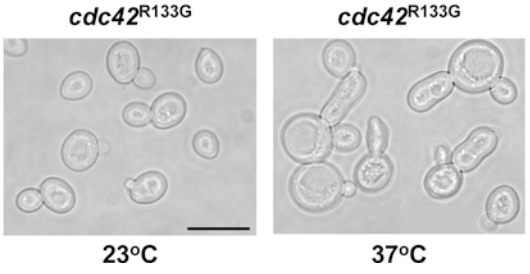
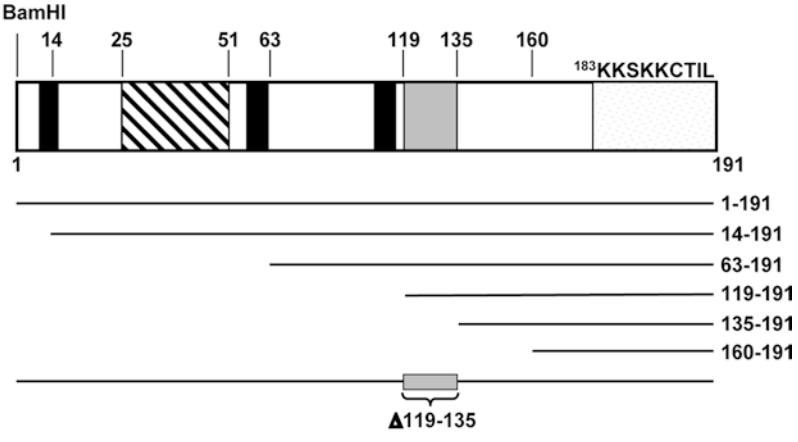


Fig. 3 **a** The *cdc42*^{R133G} mutation specifically blocked two-hybrid protein interactions with Bni1p. *Plus sign* indicates wild-type (WT) interaction, *minus sign* indicates no interaction. The D118A mutation keeps Cdc42p in a GDP-bound state and blocks interactions with all tested effectors (Richman et al. 1999). **b** Expression of Cdc42^{R133G}p affected bud morphology at 37°C. Strain WRY1-8A was grown to early log phase at 23°C and half the culture was then shifted to 37°C for 7 h and observed by microscopy. *Bar* 10 μm

Rho-insert domain mutation interfered with Bni1p formin interactions

One possible candidate for a RhoGDI binding partner that might interact with the Rho-insert domain is the Bni1p scaffold protein. To explore this possibility, we carried out a random mutagenesis/two-hybrid protein interaction study looking for mutations in Cdc42p that specifically abrogated the interactions between Cdc42p and Bni1p (see Materials and methods for details). In this study, the R133G mutation within the Rho-insert domain was isolated. The R133G mutation abolished interactions with Bni1p, but retained wild-type interactions with Cla4p, Ste20p, Gic1p, and Gic2p (Fig. 3a), other effector proteins that had previously been shown to interact with Cdc42p through the switch I effector domain. GFP-Cdc42^{R133G}p was capable of binding GTP within the cell (see Fig. 4c) and displayed normal clustering and membrane localization (data not shown), indicating that the Arg^{133} residue did not have a specific role in nucleotide binding or localization. Expression of *cdc42*^{R133G} was able to complement the *cdc42-1*^{ts} and *cdc42*Δ mutants, indicating that Cdc42^{R133G}p encoded a functional protein. Cells harboring *cdc42*^{R133G} as the sole copy of Cdc42p grew at 16, 23, 30, and 37°C and

A.



B.

GFP-Cdc42p	Observed GFP-Cdc42p Localization		
	Internal Membranes	Plasma Membrane	Clustering
WT	++	++	++
aa 14-191	+	punctate	-
aa 63-191	++	++	-
aa 119-191	++	++	-
aa 135-191	++	++	-
aa 160-191	++	++	-
Δ119-135	-	-	-
KKSKKCTIL	++	++	-
C188S	-	-	-

C.

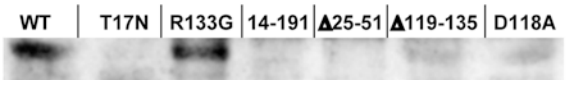


Fig. 4 **a–c** Amino-terminal truncations and internal deletions. **a** *Lines* represent the regions of Cdc42p fused to GFP and the *box* labeled Δ119–135 represents the internal deletion of the Rho-insert domain. **b** p415MET(GFP-*CDC42*) plasmids containing full-length Cdc42p or the codons for the residues listed were transformed into wild-type strain TRY11-7D and transformants were observed by fluorescence microscopy. *Double plus* indicates proper localization and strong fluorescence, *single plus* indicates proper localization but reduced fluorescence, *minus* indicates no specific targeting observed. **c** GTP-binding of GFP-Cdc42p mutant proteins. TRY11-7D cells containing the indicated GFP-Cdc42p mutant proteins were grown in SC−Leu + Met and cell lysates were prepared and then incubated with 0.1 mM GTPγS prior to incubation with GST-PBD and glutathione-agarose resin. The T17N and D118A mutant proteins were negative controls that did not bind GTP and the Δ25–51 protein was a negative control that does not bind the GST-PBD. The result is representative of three independent experiments

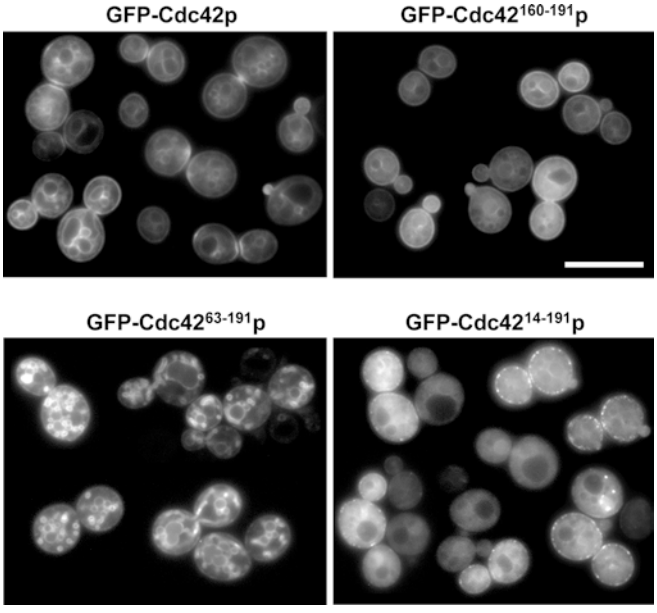


Fig. 5 Localization of truncated proteins. p415MET vectors containing the indicated wild-type or truncation GFP-*CDC42* constructs were transformed into wild-type TRY11-7D cells. *Bar* 10 μm

showed normal morphologies at all temperatures except 37°C. Cells grown at 37°C exhibited abnormal morphologies, including 28% short-elongated budded cells and 28% multi-budded cells (Fig. 3b, right panel), suggesting that Cdc42p–Bni1p interactions played an important role in normal bud growth during the cell cycle. However, overexpression of Rdi1p led to GFP-Cdc42^{R133G}p removal from membranes to the same extent as wild-type GFP-Cdc42p (data not shown), indicating that the R133G mutation did not significantly affect interactions with the Rdi1p RhoGDI and suggesting that Bni1p does not function in RhoGDI-dependent extraction of Cdc42p.

Cdc42p interactions with downstream effectors were not required for localization or clustering

Proper GFP-Cdc42p localization was previously observed in the *cla4*Δ, *gic1*Δ, *gic2*Δ, and *bni1*Δ effector mutants (Richman et al. 1999, 2002), suggesting that other single or multiple interactions with downstream effectors or regulators may be important for localization. To expand on these studies, GFP-Cdc42p localization was observed in other mutant backgrounds. GFP-Cdc42p localized and clustered normally in a *gic1*Δ *gic2*Δ double mutant and in cells deleted for the Bem1p scaffold protein (data not shown). Normal localization and clustering was previously observed in myosin *myo1*Δ, late secretion *sec1*^{ts} and *sec6*^{ts}, and septin *cdc12-6*^{ts} mutants (Richman et al. 1999, 2002); and the GFP-Cdc42p expressed in either tropomyosin *tpm2*Δ single mutants or *tpm1-2*^{ts} *tpm2*Δ double mutants showed no defects in localization or clustering (data not shown). Interestingly, *tpm1-2*^{ts} *tpm2*Δ mutant cells shifted to restrictive temperatures showed normal clustering for up to 1 h. Actin cables are disrupted within 10 min in this mutant (Pruyne et al. 1998), suggesting that actin cables are not required for clustering. Taken together, these results indicated that interactions between Cdc42p and Bni1p, Bem1p, Cla4p, Gic1p, and Gic2p are not required for proper localization and that defects in the actin cytoskeleton or polarized secretion do not affect membrane localization or clustering.

Truncated Cdc42p unable to bind guanine nucleotides does not cluster

To uncover the region(s) or specific amino acid(s) required for Cdc42p clustering, a series of Cdc42p amino-terminal truncations, internal deletions, and point mutations were examined (Figs. 4, 5). Deletions were made amino-terminal to the ^{183}KKSKKCTIL domain, which was sufficient for membrane localization but not clustering (Richman et al. 2002). Sequences were systematically deleted from residue 1 to residues 14, 63, 119, 135, or 160, with each subsequent deletion representing the removal of a structural domain (see Fig. 4). An internal deletion was also created through the removal of the Rho-insert domain (residues 119–135). The molecular weight and expression of all constructs were confirmed by immunoblot analysis; and all constructs were expressed at levels comparable with wild-type GFP-Cdc42p (data not shown). However, none of the amino-terminal truncations or the internal deletion could complement the *cdc42-1*^{ts} mutant (data not shown), indicating that removal of any domain from Cdc42p negatively affected its function.

Truncated proteins containing either residues 63–191, 119–191, 135–191, or 160–191, which lack guanine-nucleotide binding domains, showed membrane localization patterns that were indistinguishable from full-length GFP-Cdc42p (Figs. 4, 5; data not shown), suggesting that nucleotide binding was not a prerequisite for membrane binding. However, none of these truncated proteins exhibited cell-cycle specific clustering at polarized growth sites, suggesting that residues 63–191 were not sufficient for clustering. Two of these constructs (63–191, 119–191) contained the Rho-insert domain, indicating that this domain was also not sufficient for clustering. However, neither membrane localization nor clustering was observed in cells expressing GFP-Cdc42$^{\Delta 119-135}$p lacking the Rho-insert domain (Fig. 4), suggesting that the Rho-insert domain was necessary for localization. GFP-Cdc42$^{\Delta 119-135}$p could not bind GTP within the cell (Fig. 4c), reinforcing the role of the Rho-insert domain in providing stability to the nearby guanine-nucleotide binding domain that coordinates guanine ring binding (Hoffman et al. 2000).

Although cells containing GFP-Cdc42$^{63-191}$p displayed normal membrane localization, a fragmented vacuolar phenotype was observed (Fig. 5), which was

confirmed by the vacuolar stain FM4-64 (data not shown). The involvement of Cdc42p and its regulators in endocytosis and vacuolar fusion is well documented (Eitzen et al. 2001; Müller et al. 2001; Murray and Johnson 2001; White and Johnson 1997). Therefore, expression of Cdc42$^{63–191}$p seemed to have a dominant-negative effect on Cdc42p function in regulating vacuolar fusion.

GFP-Cdc42$^{14–191}$p was unable to bind GTP within the cell (Fig. 4c), most probably because it lacked part of the P-loop that was necessary for GTP binding. It did not show typical plasma membrane localization or clustering, but instead displayed a punctate staining pattern around the periphery of the cell (Figs. 4b, 5). This staining pattern may be due to proper localization of GFP-Cdc42$^{14–191}$p to the plasma membrane but an inability of GFP-Cdc42$^{14–191}$p to form normal complexes at the plasma membrane. However, we cannot rule out the possibility that improper folding of this larger truncated protein promoted the formation of aggregates, even though smaller truncated proteins (i.e., 63–191 or 119–191) were localized properly to membranes.

Yellow fluorescent protein–Cdc24p appeared at the same time that cyan fluorescent protein–Cdc42p began to cluster at the incipient bud site and mother-bud neck region

Previous data (Richman et al. 2002) and data presented herein suggested that nucleotide binding had a role in Cdc42p clustering. If activation of Cdc42p to a GTP-bound state was a prerequisite for clustering, then clustering of Cdc42p and localization of its GEF Cdc24p should temporally coincide at sites of polarized growth. To test this hypothesis, cells containing both yellow fluorescent protein (YFP)-Cdc24p and cyan fluorescent protein (CFP)-Cdc42p were examined using time-lapse microscopy. Time-lapse experiments focused on clustering at mother-bud neck regions, because the separation of Cdc24p-stained nuclei during anaphase served as an excellent signal for the imminent Cdc42p clustering and Cdc24p targeting at the mother-bud neck region (Toenjes et al. 1999). Time-lapse photomicrographs of nine mother–daughter cell pairs revealed that YFP-Cdc24p appeared at the same time that CFP-Cdc42p began to cluster at the mother-bud neck region (Fig. 6). Time-lapse experiments of clustering at incipient bud sites were limited by the lack of a signal for the forthcoming appearance of CFP-Cdc42p and YFP-Cdc24p, which led to difficulties with GFP photobleaching upon multiple exposures. However, when incipient bud site co-localization was captured by time-lapse, YFP-Cdc24p appeared and CFP-Cdc42p clustered within a 3-min interval (data not shown). Together with the mother-bud neck region data, these results positioned Cdc24p localization temporally and spatially with Cdc42p clustering.

Discussion

The accumulation of GFP-Cdc42p in the cytosol upon overexpression of Rdi1p supports a role for Rdi1p in stimulating Cdc42p release from membranes, as previously observed with mammalian RhoGDIs (Koch et al. 1997; Leonard et al. 1992; Masuda et al. 1994). The general cytoplasmic localization of GFP-Rdi1p was also observed with a HA-tagged Rdi1p (Koch et al. 1997) and is consistent with its role in extracting Cdc42p from cellular membranes into the cytosol. The novel localization of GFP-Rdi1p at polarized growth sites in small buds and at the mother-bud neck region during cytokinesis suggests that an increased localized amount of

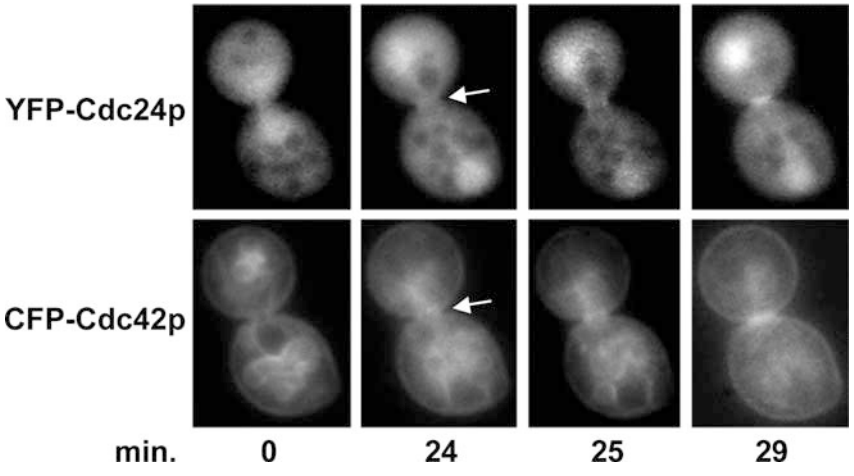


Fig. 6 Time-lapse microscopy of CFP-Cdc42p and YFP-Cdc24p co-localization. Plasmids p416MET(CFP-*CDC42*) and p415MET(YFP-*CDC24*) were transformed into wild-type strain TRY11-7D and cells were placed onto a thin-layered agar slab made with SC-Leu-Met medium. The zero time-point was set arbitrarily, representing the appearance of post-anaphase cells prior to Cdc42p clustering. YFP-Cdc24p appearance (*upper panels*, *arrow*) and CFP-Cdc42p clustering (*lower panels*, *arrow*) at the mother-bud neck region occurs at approximately the same time (24 min time-point). YFP-Cdc24p targeting to nuclei was apparent at all time-points. Photomicrographs are representative of cells followed in nine time-lapse experiments

Rdi1p is necessary for the extraction of the accumulated, clustered Cdc42p at these stages of the cell cycle (Richman et al. 2002). Interestingly, this cell-cycle specific Rdi1p localization was not observed with a HA-tagged Rdi1p (Koch et al. 1997), but this discrepancy may have been due to its intense cytoplasmic immunofluorescence signal obscuring the membrane signal. Alternatively, the HA-specific antibodies may not have been able to bind to membrane-bound Rdi1p under the experimental conditions tested, a phenomenon previously observed with immunofluorescence localization of Cdc42p (Ziman et al. 1993).

Although deletion of the Rho-insert domain abrogated the ability of Rdi1p to extract Cdc42p from membranes, it is unlikely that this was due to a direct interaction between Rdi1p and the Rho-insert domain, based on the X-ray crystal structure of the mammalian Cdc42p–RhoGDI complex (Hoffman et al. 2000). Therefore, interactions with other proteins may be important for this process. Analysis of the *cdc42*^{R133G} mutation assigned an additional function to the Rho-insert domain in mediating interactions with the potential scaffold protein Bni1p, raising the possibility that Bni1p may be involved in Rdi1p function. However, the observations that GFP-Cdc42^{R133G}p showed no defects in localization and that Rdi1p could extract Cdc42^{R133G}p from membranes suggested that interactions with Bni1p do not play a role in Rdi1p function.

Bni1p–Cdc42p interactions were not affected by various switch I effector-domain mutations (Richman and Johnson 2000; Richman et al. 1999), suggesting that a region other than the switch I effector domain was required for these interactions. The isolation of the R133G mutation from a screen designed to isolate mutations that specifically affected Cdc42p–Bni1p interactions implicated the Rho-insert domain as one of the Cdc42p regions involved in Cdc42p–Bni1p interactions. The abnormal morphological phenotype of *cdc42*^{R133G} cells grown at 37°C likewise suggested a functional role for the Rho-insert domain in regulating cell polarity and was consistent with cell polarity defects associated with *bni1* mutants (Evangelista et al. 1997; Vallen et al. 2000). Taken together with previous studies showing that the Rho-insert domain was involved in interactions with another potential scaffold protein IQGAP (McCallum et al. 1996; Wu et al. 1997), these results raise the possibility that the Rho-insert domain is important for mediating specific scaffold–Cdc42p interactions.

Multiple observations have implicated nucleotide binding as being important for Cdc42p clustering but not for membrane localization. First, the G12V mutation did not affect membrane localization, but did cause an aberrant increase in clustering (Richman et al. 2002), raising the possibility that binding of GTP could promote clustering. Also, Cdc42p and its guanine nucleotide exchange factor Cdc24p co-localized spatially (Richman et al. 2002) and temporally (Fig. 5) to sites of polarized growth, supporting a model in which Cdc24p promotes clustering through activation of Cdc42p to a GTP-bound state. Also, various truncated Cdc42 proteins predicted to be unable to bind nucleotides were able to localize properly to cellular membranes but did not cluster, supporting the importance of the nucleotide-bound state of Cdc42p for clustering. Although the regulation of nucleotide binding likely plays an important role in clustering, it is not clear whether binding of GTP is important for the initial stimulation of clustering, or the perpetuation of clustering, or both.

The results presented herein provide an important insight into the complicated nature of the relationships between Cdc42p localization, nucleotide binding, and protein–protein interactions and the domains that regulate these different functions. These data are consistent with a model in which: (1) Cdc42p is anchored at the plasma membrane around the periphery of the cell throughout the cell cycle, (2) its regional concentration at sites of actin-dependent polarized growth is significantly increased (clustering) when activated to a GTP-bound state by Cdc24p, and (3) it is extracted from the plasma membrane by Rdi1p when polarized growth is completed at the bud tip and the mother-bud neck region following cytokinesis. In this regard, Rdi1p would be acting to turn off Cdc42p-dependent signaling pathways at specific times in the cell cycle. However, the exact mechanisms by which Cdc42p is targeted to specific membranes and clustered at sites of polarized growth remain to be completely elucidated. Future studies should shed light on how multiple Cdc42p functional domains work together to control these essential Cdc42p functions.

Acknowledgements We thank Anthony Bretscher for valuable reagents, Herman Chen and Patti McClard for expert technical assistance, and members of the Johnson Laboratory for valuable discussions and critical comments on this manuscript. K.C.C. was supported in part by an undergraduate fellowship from the UVM-Howard Hughes HELiX Program. This research was supported by National Science Foundation grant MCB-0110138.

References

Benard V, Bohl BP, Bokoch GM (1999) Characterization of Rac and Cdc42 activation in chemoattraction-stimulated human neutrophils using a novel assay for active GTPases. J Biol Chem 274:13198–13204

Bender A, Pringle JR (1989) Multicopy suppression of the *cdc24* budding defect in yeast by *CDC42* and three newly identified genes including the *ras*-related gene *RSR1*. Proc Natl Acad Sci USA 86:9976–9980

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Brown JL, Jaquenoud M, Gulli M-P, Chant J, Peter M (1997) Novel Cdc42-binding proteins Gic1 and Gic2 control cell polarity in yeast. Genes Dev 11:2972–2982

Butty AC, Perrinjaquet N, Petit A, Jaquenoud M, Segall JE, Hofmann K, Zwahlen C, Peter M (2002) A positive feedback loop stabilizes the guanine-nucleotide exchange factor Cdc24 at sites of polarization. EMBO J 21:1565–1576

Chen G-C, Kim Y-J, Chan CSM (1997) The Cdc42 GTPase-associated proteins Gic1 and Gic2 are required for polarized cell growth in *Saccharomyces cerevisiae*. Genes Dev 11:2958–2971

Cvrcková F, De Virgilio C, Manser E, Pringle JR, Nasmyth K (1995) Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. Genes Dev 9:1817–1830

Davis CR, Richman TJ, Deliduka SB, Blaisdell JO, Collins CC, Johnson DI (1998) Analysis of the mechanisms of action of the *Saccharomyces cerevisiae* dominant lethal *cdc42*^{G12V} and dominant negative *cdc42*^{D118A} mutations. J Biol Chem 273:849–858

Diebold BA, Bokoch GM (2001) Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. Nat Immunol 2:211–215

Eitzen G, Thorngren N, Wickner W (2001) Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. EMBO J 20:5650–5656

Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. Nature 420:629–635

Evangelista M, Blundell K, Longtine MS, Chow CJ, Adames N, Pringle JR, Peter M, Boone C (1997) Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. Science 276:118–122

Finegold AA, Johnson DI, Farnsworth CC, Gelb MH, Judd SR, Glomset JA, Tamanoi F (1991) Protein geranylgeranyltransferase of *Saccharomyces cerevisiae* is specific for Cys-Xaa-Xaa-Leu motif proteins and requires the *CDC43* gene product, but not the *DPR1* gene product. Proc Natl Acad Sci USA 88:4448–4452

Gyuris J, Golemis E, Chertkov H, Brent R (1993) Cdi, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell 75:791–803

Hart MJ, Maru Y, Leonard D, Witte ON, Evans T, Cerione RA (1992) A GDP dissociation inhibitor that serves as a GTPase inhibitor for the Ras-like protein CDC42Hs. Science 258:812–815

Hoffman GR, Nassar N, Cerione RA (2000) Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. Cell 100:345–356

Hori Y, Kikuchi A, Isomura M, Katayama M, Miura Y, Fujioka H, Kaibuchi K, Takai Y (1991) Post-translational modifications of the C-terminal region of *rho* are important for its interaction with membranes and the stimulatory and inhibitory GDP/GTP exchange proteins. Oncogene 6:515–522

Johnson DI (1999) Cdc42: an essential Rho-type GTPase controlling eukaryotic cell polarity. Microbiol Mol Biol Rev 63:54–105

Johnson DI, Pringle JR (1990) Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J Cell Biol 111:143–152

Joneson T, Bar-Sagi D (1998) A Rac1 effector site controlling mitogenesis through superoxide production. J Biol Chem 273:17991–17994

Koch G, Tanaka K, Masuda T, Yamochi W, Nonaka H, Takai Y (1997) Association of the Rho family small GTP-binding proteins with Rho GDP dissociation inhibitor (Rho GDI) in *Saccharomyces cerevisiae*. Oncogene 15:417–422

Leonard D, Hart MJ, Platko JV, Eva A, Henzel W, Evans T, Cerione RA (1992) The identification and characterization of a GDP-dissociation inhibitor (GDI) for the CDC42Hs protein. J Biol Chem 267:22860–22868

Masuda T, Tanaka K, Nonaka H, Yamochi W, Maeda A, Takai Y (1994) Molecular cloning and characterization of yeast rho GDP dissociation inhibitor. J Biol Chem 269:19713–19718

McCallum SJ, Wu WJ, Cerione RA (1996) Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. J Biol Chem 271:21732–21737

Merla A, Johnson DI (2000) The Cdc42p GTPase is targeted to the site of cell division in the fission yeast *Schizosaccharomyces pombe*. Eur J Cell Biol 79:469–477

Merla A, Johnson DI (2001) The *Schizosaccharomyces pombe* Cdc42p GTPase signals through Pak2p and the Mkh1p–Pek1p–Spm1p MAP kinase pathway. Curr Genet 39:205–209

Miller PJ, Johnson DI (1997) Characterization of the *S. cerevisiae* *cdc42-1*^{ts} allele and new temperature-conditional-lethal *cdc42* alleles. Yeast 13:561–572

Murray JM, Johnson DI (2001) The Cdc42p GTPase and its regulators Nrf1p and Scd1p are involved in endocytic trafficking in the fission yeast *Schizosaccharomyces pombe*. J Biol Chem 276:3004–3009

Müller O, Johnson DI, Mayer A (2001) Cdc42p functions at the docking stage of yeast vacuole membrane fusion. EMBO J 20:5657–5665

Nern A, Arkowitz RA (1999) A Cdc24p–Far1p–Gbg protein complex required for yeast orientation during mating. J Cell Biol 144:1187–1202

Nomanbhoy TK, Cerione RA (1996) Characterization of the interaction between RhoGDI and Cdc42Hs using fluorescence spectroscopy. J Biol Chem 271:10004–10009

Nomanbhoy TK, Erikson JW, Cerione RA (1999) Kinetics of Cdc42 membrane extraction by Rho-GDI monitored by real-time fluorescence resonance energy transfer. Biochemistry 38:1744–1750

Pruyne DW, Schott DH, Bretscher A (1998) Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. J Cell Biol 143:1931–1945

Richman TJ, Johnson DI (2000) *Saccharomyces cerevisiae* Cdc42p GTPase is involved in preventing the recurrence of bud emergence during the cell cycle. Mol Cell Biol 20:8548–8559

Richman TJ, Sawyer MM, Johnson DI (1999) The Cdc42p GTPase is involved in a G_2/M morphogenetic checkpoint regulating the apical-isotropic switch and nuclear division in yeast. J Biol Chem 274:16861–16870

Richman TJ, Sawyer MM, Johnson DI (2002) *Saccharomyces cerevisiae* Cdc42p localizes to cellular membranes and clusters at sites of polarized growth. Eukaryot Cell 1:458–468

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sherman F, Fink GR, Hicks JB (1986) Methods in yeast genetics: laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Stevenson BJ, Ferguson B, De Virgilio C, Bi E, Pringle JR, Ammerer G, Sprague GF (1995) Mutation of *RGA1*, which encodes a putative GTPase-activating protein for the polarity-establishment protein Cdc42p, activates the pheromone-response pathway in the yeast *Saccharomyces cerevisiae*. Genes Dev 9:2949–2963

Toenjes KA, Sawyer MM, Johnson DI (1999) The guanine-nucleotide-exchange factor Cdc24p is targeted to the nucleus and polarized growth sites. Curr Biol 9:1183–1186

Vallen EA, Caviston J, Bi E (2000) Roles of Hof1p, Bni1p, Bnr1p, and Myo1p in cytokinesis in *Saccharomyces cerevisiae*. Mol Biol Cell 11:593–611

Vetter IR, Wittinghofer A (2001) Signal transduction—the guanine nucleotide-binding switch in three dimensions. Science 294:1299–1304

Walker SJ, Brown HA (2002) Specificity of Rho insert-mediated activation of phospholipase D1. J Biol Chem 277:26260–26267

Walker SJ, Wu W-J, Cerione RA, Brown HA (2000) Activation of phospholipase D1 by Cdc42 requires the Rho insert region. J Biol Chem 275:15665–15668

White WH, Johnson DI (1997) Characterization of synthetic-lethal mutants reveals a role for the *Saccharomyces cerevisiae* guanine-nucleotide exchange factor Cdc24p in vacuole function and Na^+ tolerance. Genetics 147:43–55

Wu WJ, Leonard DA, Cerione RA, Manor D (1997) Interaction between Cdc42Hs and RhoGDI is mediated through the Rho insert region. J Biol Chem 272:26153–26158

Wu WJ, Lin R, Cerione RA, Manor D (1998) Transformation activity of Cdc42 requires a region unique to Rho-related proteins. J Biol Chem 273:16655–16658

Ziman M, Johnson DI (1994) Genetic evidence for a functional interaction between *S. cerevisiae CDC24* and *CDC42*. Yeast 10:463–474

Ziman M, O'Brien JM, Ouellette LA, Church WR, Johnson DI (1991) Mutational analysis of *CDC42Sc*, a *Saccharomyces cerevisiae* gene that encodes a putative GTP-binding protein involved in the control of cell polarity. Mol Cell Biol 11: 3537–3544

Ziman M, Preuss D, Mulholland J, O'Brien JM, Botstein D, Johnson DI (1993) Subcellular localization of Cdc42p, a *Saccharomyces cerevisiae* GTP-binding protein involved in the control of cell polarity. Mol Biol Cell 4:1307–1316