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Interaction between a Ras and a Rho GTPase Couples Selection of a Growth Site to the Development of Cell Polarity in Yeast

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Polarized cell growth requires the coupling of a defined spatial site on the cell cortex to the apparatus that directs the establishment of cell polarity. In the budding yeast *Saccharomyces cerevisiae*, the Ras-family GTPase Rsr1p/Bud1p and its regulators select the proper site for bud emergence on the cell cortex. The Rho-family GTPase Cdc42p and its associated proteins then establish an axis of polarized growth by triggering an asymmetric organization of the actin cytoskeleton and secretory apparatus at the selected bud site. We explored whether a direct linkage exists between the Rsr1p/Bud1p and Cdc42p GTPases. Here we show specific genetic interactions between *RSR1/BUD1* and particular *cdc42* mutants defective in polarity establishment. We also show that Cdc42p coimmunoprecipitated with Rsr1p/Bud1p from yeast extracts. In vitro studies indicated a direct interaction between Rsr1p/Bud1p and Cdc42p, which was enhanced by Cdc24p, a guanine nucleotide exchange factor for Cdc42p. Our findings suggest that Cdc42p interacts directly with Rsr1p/Bud1p in vivo, providing a novel mechanism by which direct contact between a Ras-family GTPase and a Rho-family GTPase links the selection of a growth site to polarity establishment.

INTRODUCTION

Within eukaryotic cells, an asymmetric reorganization of the cytoskeleton and secretory apparatus precedes and supports polarized cell growth at selected sites on the cell cortex (Drubin and Nelson, 1996). Many studies continue to identify the intra- and extracellular signals that bias growth at specific cortical locations. These cortical cues serve to position the axis of polarized growth but are usually not essential for the asymmetric organization of the specific proteins and organelles needed to reinforce the axis of polarity (reviewed in Drubin, 2000). Although central to processes in which function is dependent on polarized morphogenesis (e.g., neuronal growth, nutrient transport, cell migration, and asymmetric cell division), the linkage of a spatial cue to the molecules that regulate the establishment of cell polarity is not fully defined in any cell type.

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Cells of the budding yeast *Saccharomyces cerevisiae* provide a unique opportunity to decipher the molecular mechanism of polarized morphogenesis. During the mitotic cell cycle of *S. cerevisiae*, cell surface growth mainly takes place in the bud. This polarized growth is supported by the orientation of the secretory apparatus and cytoskeleton toward the bud. The site chosen for polarization is dependent on cell type: haploid **a** and α cells exhibit an axial budding pattern, in which mother and daughter cells bud immediately adjacent to the site selected for budding in the previous cell cycle. In contrast, diploid **a**/α cells exhibit a bipolar pattern, in which mother and daughter cells bud at either pole of the cell (Hicks *et al.*, 1977; Chant and Herskowitz, 1991). Selection of a bud site hence determines an axis of cell polarity and ultimately determines the plane of cell division.

A GTPase module comprised of the Ras-family GTPase Rsr1p/Bud1p (hereafter Rsr1p), its guanine nucleotide exchange factor (GEF) Bud5p, and its GTPase activating protein Bud2p is necessary for selecting the proper site for polarized growth in both haploid and diploid cells (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Chant *et al.*, 1991; Bender, 1993; Park *et al.*, 1993). In the absence of the Rsr1p GTPase or its regulators, cortical cues such as Axl2p/Bud10p, which mark the proper site of polarized growth on the cell cortex, are no longer coupled to polarity establishment (Chant and Pringle, 1995; Park *et al.*, 1997; Kang *et al.*, 2001), resulting in random bud-site selection. The *rsr1*Δ, *bud2*Δ, or *bud5*Δ mutants still undergo polarized growth, indicating that the Rsr1p GTPase module plays an essential

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role not in establishing the axis of polarized growth, but in positioning it.

The establishment of cell polarity relies upon another GTPase, Cdc42p. First discovered in *S. cerevisiae* (Adams *et al.*, 1990; Johnson and Pringle, 1990), this multifunctional GTPase of the Rho family has evolutionarily conserved functions critical for polarized morphogenesis (reviewed in Johnson, 1999). Specific mutations in yeast *CDC42* and *CDC24*, which encodes the GEF of Cdc42p, prevent polarized organization of the actin structures and exocytotic landmarks (e.g., Sec3p) at the bud site (Sloat *et al.*, 1981; Adams *et al.*, 1990; Pringle *et al.*, 1995; Pruyne and Bretscher, 2000; Zhang *et al.*, 2001). Without this asymmetric distribution of the actin cytoskeleton and secretory apparatus to the bud site, an axis of polarized growth is not maintained and bud formation does not occur. Thus a key issue in understanding how yeast cells are committed to utilize a specific site for polarization is to identify functional linkages between the Cdc42p and Rsr1p GTPase modules.

Previous studies modeled Cdc24p as a link between the Rsr1p and Cdc42p GTPases (reviewed in Gulli and Peter, 2001). An interaction between Cdc24p and Rsr1p was first deduced from genetic analyses (Bender and Pringle, 1989) and was confirmed subsequently in vitro using recombinant proteins (Zheng *et al.*, 1995; Park *et al.*, 1997). The association of Cdc24p with Rsr1p is nucleotide-specific: GTP-bound Rsr1p preferentially interacts with Cdc24p in vitro (Zheng *et al.*, 1995; Park *et al.*, 1997). In contrast, GDP-bound Cdc42p preferentially interacts with Cdc24p (Zheng *et al.*, 1995; Davis *et al.*, 1998; Drees *et al.*, 2001), as expected for a GTPase interacting with its GEF. Combined with the observation that Rsr1p is required to localize Cdc24p at the proper bud site (Park *et al.*, 2002), these results favor a model in which the cycling of Rsr1p through its GTP bound state recruits Cdc24p to the proper bud site, where it activates Cdc42p for interaction with its downstream effectors (Park *et al.*, 1997). A similar bridging of Ras and Rho GTPases by a RhoGEF has been observed in *Schizosaccharomyces pombe* (Chang *et al.*, 1994).

Herein, we present both genetic and biochemical data indicating that Rsr1p directly interacts with Cdc42p, in addition to its association with Cdc24p. Our data support the idea that the mechanism that couples the selection of a polarized growth site to the establishment of cell polarity involves multiple cross-talks between the Ras and Rho GTPase modules, rather than a single communication across a GEF bridge as previously thought.

MATERIALS AND METHODS

Media and Transformations

Yeast strains were cultured in rich (YPD) medium (Sherman *et al.*, 1986) at 25°C unless stated otherwise. Strains used in this study are listed in Table 1. To track the segregation of auxotrophic markers and to selectively maintain plasmids, strains were cultured in complete synthetic (SC) medium (Sherman *et al.*, 1986) lacking the appropriate amino acid(s) (e.g., SC-Ura). Nourseothricin-resistant strains were selected on 100 μg/ml clonNAT (Werner Bioagents, Jena-Cospeda, Germany). All yeast transformations were performed according to Schiestl and Gietz (1989).

Identification and Analyses of Synthetic Genetic Interactions

Synthetic genetic arrays (SGA) were screened three times at 33°C with KKY281 and KKY283 by the method of Tong *et al.* (2001). Synthetic interactions were confirmed by random spore and/or tetrad analysis. The function of interacting genes was defined by reference to the Yeast Proteome Database (YPD; Incyte Genomics, Beverly, MA). KKY281 and KKY 283 were derived from Y3611, using pKK1467 and pKK1471 as sources of integrating DNA following the method of Kozminski *et al.* (2000) for linking *LEU2* to *cdc42*. Y3611 was created by crossing Y3598 to BY4742. In Y3598, *MFA1pr-HIS3-MFα1pr-LEU2* was integrated at the *CAN1* locus to generate *can1Δ::MFA1pr-HIS3-MFα1pr-LEU2*. The construction of Y3598 involved four steps. First, a 455-base pair fragment containing the upstream sequence of *CAN1* was amplified from yeast genomic DNA with primers (TAGGGCGAACTTGAAGAATAACC) and (GCCACGTTGCACACTATCCTGTGCTATGCCTTTTTTTTTTTTTTGTT), which contained a 21-base pair sequence (underlined) from the 5′ end of *MFA1pr-HIS3*. Second, *MFA1pr-HIS3* was amplified from genomic DNA obtained from Y2420 with primers (CAGGATAGTGTGCAACGTGGC) and (CTCATTGAATCTTTGCATTCAGAGCGTCTACATAAGAACACCTT-TGGTGG), which contained a 27-base pair sequence (underlined) from the 5′ end of the *MFα1-LEU2*. Third, *MFα1pr-LEU2* was amplified from genomic DNA obtained from Y2823 with primers (ACGCTCTGAATGCAAAGATTCAATGAG) and (ATCAAAGGTAATAAAACGTCATATTTAAGCAAGGATTTTCTTAACTTC), which contained a 24-base pair sequence (underlined) from the 5′ end of the *CAN1* upstream sequence. Fourth, a 300-base pair fragment containing the *CAN1* downstream sequence was amplified from yeast genomic DNA with primers (ATATGACGTTTTATTACCTTTGAT) and (ACGAAAAATGAGTAAAAATTATCTT). Finally, the set of PCR products above were used as templates to generate a fused product with primers (TAGGGCGAACTTGAAGAATAACC) and (ACGAAAAATGAGTAAAAATTATCTT). BY4741 transformants carrying *can1Δ::MFA1pr-HIS-MFα1pr-LEU2* were selected on synthetic medium lacking histidine.

Isogenic *rsr1Δ*, *cdc42*, *rsr1Δ cdc42* haploid strains were derived as meiotic products of a cross between KKY86 and KKY400, 404, 426, or 444. KKY86 is a meiotic product of KKY85, which was produced by the integration of an *HIS3*-containing PCR product (primers oKK47: CTCCACTGAACAATAATACTATTATTTAGTAACGATATAGAACATTTACATACAACGCAGATTGTACTGAGAGTGCACC and oKK48: CTTTAAAAACTTATACAACGTATGCTAATACTTTAAACTAAACCTTTTAGAACTATACTGTGCGGTATTTCACACCGC; *RSR1* flanking sequence underlined; template pRS303) at the *RSR1* locus of DDY1102, creating a complete deletion of *RSR1*.

Screen for cdc42 Dosage Suppressors

To identify dosage suppressors of the *cdc42-118* temperature-sensitive growth defect, DDY1326 was transformed with a 2-μm plasmid-based *S. cerevisiae* genomic library (Carlson and Botstein, 1982), plated on SC-Ura, and then incubated immediately at 37°C for 4 d, selecting simultaneously for transformants and dosage suppressors. Approximately 8300 transformants were screened. Fifty-three transformants grew at 37°C. To test whether survival at 37°C was plasmid dependent, transformants were streaked onto SC plates containing 0.5 mg/ml 5-fluoroorotic acid (FOA) to counterselect the library plasmid. After incubation for 4 d at 25°C, single colonies were picked from SC+FOA plates, struck onto SC plates, and incubated for 3 d at 37°C. Eleven transformants displayed plasmid-dependent growth. PCR analysis using primers KK27 (GTACATCACCATTGTCCAGGTG) and D (Kozminski *et al.*, 2000) eliminated transformants with plasmid-borne copies of *CDC42* from further consideration. Library plasmids were rescued from these transformants, amplified in *Escherichia coli*, and tested for redundancy using *Rsa*I restriction site mapping. To verify that each nonredundant library plasmid contained a plasmid-borne *cdc42* suppressor, library plasmids were retransformed into DDY1326. Seven transformants grew on SC-Ura at 37°C. The ends of each genomic fragment in the seven nonredundant library plasmids were partially sequenced from the flanking vector sequence, using primers YEp24F (CCGCTTTGGCCGCCG) and YEp24R (GCCTATATCGCCGACATC). The resulting sequences were then used in a WU-BLAST search of the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) to obtain the full sequence of each genomic fragment. Library plasmid pKK1369 contains a genomic fragment of chromosome VII (base pairs 794092–802090). The other six suppressors will be described elsewhere.

Identification of RSR1 as a Dosage Suppressor of cdc42-118

To determine whether *RSR1* is an allele-specific high copy suppressor of *cdc42-118* and the only suppressor of *cdc42-118* in YEp24 library clone pKK1369, isogenic *CDC42* (DDY1300), *cdc42-101* (DDY1304), *cdc42-118* (DDY1326), *cdc42-123* (DDY1336), *cdc42-129* (DDY1344), and *rho1–104* (KKY37) strains were transformed with pKK1369, pKK1369 with *RSR1* deleted (pKK980), or a 2-μm plasmid (YEplac195) with (pKK925) or without (pKK1365) a *RSR1* insert. Transformants were streaked onto SC-Ura plates, and scored for growth after incubation at 25 and 36°C for 3 d. To determine whether *CDC24* and alleles of *RSR1* are dosage suppressors of *cdc42-118*, a *cdc42-118* strain was transformed with 2-μm YEplac195 plasmids carrying $rsr1^{T35A}$ (pKK1095), $rsr1^{G12V}$ (pKK1240), $rsr1^{K16N}$ (pKK1243), $rsr1^{K260-264S}$ (pHP1123) (Park *et al.*, 2002), or YEp103 (*CDC24*) (Ziman and Johnson, 1994).

Construction of Plasmids

To delete *RSR1* from the YEp24 library clone, pKK1369 was digested with *Bss*HII and *Sac*I. The vector was then blunt-end ligated after Mung Bean

Table 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source
DDY1102	*MAT***a**/*MATα his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 ADE2/ade2-1 lys2-801am/LYS2*	Kozminski *et al.* (2000)
DDY1300	*CDC42:LEU2*	Kozminski *et al.* (2000)
DDY1304	*cdc42-101:LEU2*	Kozminski *et al.* (2000)
DDY1326	*cdc42-118:LEU2*	Kozminski *et al.* (2000)
DDY1336	*cdc42-123:LEU2*	Kozminski *et al.* (2000)
DDY1344	*cdc42-129:LEU2*	Kozminski *et al.* (2000)
KKY37	*MAT***a** *rho1-104 leu2-3,112 ura3-52 lys2-801am*	This study
KKY238	*CDC42:LEU2* [pKK1365, 2μ vector YEplac195]	This study
KKY239	*cdc42-101:LEU2* [pKK1365]	This study
KKY240	*cdc42-118:LEU2* [pKK1365]	This study
KKY286	*cdc42-123:LEU2* [pKK1365]	This study
KKY288	*cdc42-129:LEU2* [pKK1365]	This study
KKY290	*rho1-104* [pKK1365]	This study
KKY1010	*CDC42:LEU2* [pKK1369, 2μ YEp24 library clone]	This study
KKY1011	*cdc42-101:LEU2* [pKK1369]	This study
KKY1012	*cdc42-118:LEU2* [pKK1369]	This study
KKY1013	*CDC42:LEU2* [pKK980, 2μ YEp24 library clone *rsr1Δ*]	This study
KKY1014	*cdc42-118:LEU2* [pKK980]	This study
KKY190	*CDC42:LEU2* [pKK925, YEplac195 (*RSR1*)]	This study
KKY66	*cdc42-101:LEU2* [pKK925]	This study
KKY68	*cdc42-118:LEU2* [pKK925]	This study
KKY287	*cdc42-123:LEU2* [pKK925]	This study
KKY289	*cdc42-129:LEU2* [pKK925]	This study
KKY291	*rho1-104* [pKK925]	This study
KKY256	*CDC42:LEU2* [pKK1095, YEplac195 ($rsr1^{T35A}$)]	This study
KKY60	*cdc42-118:LEU2* [pKK1095]	This study
KKY193	*CDC42:LEU2* [pKK1240, YEplac195 ($rsr1^{G12V}$)]	This study
KKY200	*cdc42-118:LEU2* [pKK1240]	This study
KKY194	*CDC42:LEU2* [pKK1243, YEplac195 ($rsr1^{K16N}$)]	This study
KKY201	*cdc42-118:LEU2* [pKK1243]	This study
KKY69	*cdc42-118:LEU2* [pHP569, YEp103 (*CDC24)]*	This study
Y147	*MAT***a** *cdc24-4 ura3 leu2-3, 112 his3*	Bender and Pringle (1989)
KKY342	*CDC42:LEU2 rsr1::HIS3*	This study
KKY343	*cdc42-101:LEU2 rsr1::HIS3*	This study
KKY344	*cdc42-101:LEU2 RSR1*	This study
KKY345	*cdc42-118:LEU2 rsr1::HIS3*	This study
KKY346	*cdc42-118:LEU2 RSR1*	This study
KKY297	*cdc42-129:LEU2 rsr1::HIS3*	This study
KKY298	*cdc42-129:LEU2 RSR1*	This study
KKY85	*MAT***a**/*MATα rsr1::HIS3/RSR1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 ura3-52/ura3-52 ADE2/ade2-1 lys2-801am/LYS2*	This study
KKY86	*MATα rsr1::HIS3 ura3-52 leu2-3,112 his3Δ200 lys2-801 am*	This study
Y3611	*MAT***a**/*MATα can1Δ::MFA1pr-HIS3-MFα1pr-LEU2/CAN1 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15Δ0/MET15 lys2Δ0/LYS2*	This study
Y3598	*MAT***a** *can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	This study
Y2420	*MAT***a** *mfa1Δ::MFA1pr-HIS3 ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Tong *et al.* (2001)
Y2823	*MATα mfα1Δ::MFα1pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Tong *et al.* (2001)
BY4741	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Brachmann *et al.* (1998)
BY4742	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Brachmann *et al.* (1998)
KKY281	*MATα CDC42:natMX can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	This study
KKY283	*MATα cdc42-118:natMX can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	This study
YD00000-YD09999	*MAT***a** *orfΔ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* (1999)
HPY16	*MAT***a** *ura3-52 his3-Δ1 leu2 trp1Δ63 prb1-1122 pep4-3 prc1-407*	Park *et al.* (1993)

All *cdc42:LEU2* and *CDC42:LEU2* strains above are *MAT***a** *ura3-52 leu2-3,112 his3Δ200 lys2-801am* and are isogenic except at *CDC42*. The strain background is S288C. Ten back-crosses transferred *rho1-104* (Yamochi *et al.*, 1994) into the S288C DDY/KKY strain background. Plasmid bearing KKY strains were isolated as transformants of DDY1300, DDY1304, DDY1326, DDY1336, DDY1344 (Kozminski *et al.*, 2000), and KKY37, respectively.

nuclease digestion, forming pKK980. To clone *RSR1* into YEplac195, a *Sac*I-*Sal*I *RSR1*-containing fragment was subcloned into the *Sac*I and *Sal*I sites of pKK1365 (YEplac195) from pHP674-1 (Park *et al.*, 1997), forming pKK925. To clone $rsr1^{T35A}$ into YEplac195, a *Sac*I-*Bsu*36I fragment from pHP675 (Park *et al.*, 1997) was subcloned into the *Sac*I and *Bsu*36I sites of pKK925, forming pKK1095. To clone $rsr1^{G12V}$ and $rsr1^{K16N}$ into YEplac195, a *Sac*I-*Bsu*36I fragment from YEp13($rsr1^{G12V}$) and YEp13($rsr1^{K16N}$) (Ruggieri *et al.*, 1992), was subcloned into the *Sac*I and *Bsu*36I sites of pKK1095, forming pKK1240 and pKK1243, respectively. The *rsr1* constructs were sequenced to verify the identity of each *rsr1* allele. To clone *rsr1-7* ($rsr1^{K260-264S}$) into YEplac195, *Bsu*36I-*Sal*I fragment of pKK925 was replaced with the *Bsu*36I-*Sal*I fragment from pRS304-$rsr1^{K260-264S}$-*GFP* (pHP1065) (Park *et al.*, 2002).

For the integration of *CDC42:natMX* or *cdc42-118:natMX* into Y3611, a *Not*I-*Not*I *LEU2* fragment was removed, respectively, from pKK655 and pAC326 (Kozminski *et al.*, 2000) and replaced with a *Not*I-*Not*I fragment containing *natMX*, forming pKK1467 and pKK1471. The *natMX* fragment was amplified by PCR from p4339 (Tong *et al.*, 2001) using the primers (*Not*I sites underlined) oKK133 (AGTCTCTAGCGGCCGCACATGGAGGCCCAGAATACCC) and oKK134 (AGTCTCTAGCGGCCGCAGTATAGCGACCAGCATTCAC).

To fuse GST to the N-terminus of Cdc42p, *CDC42* was subcloned as a *Bsg*I-*Hin*dIII fragment from pKK944 into the *Bsg*I and *Hin*dIII sites of vector pKK1017, forming pKK1025. To construct pKK1017, GST coding sequence (*Bgl*II-*Bam*HI ends) was amplified by PCR from the template pGEX-2T (pKK858; Amersham Bioscience, Piscataway, NJ), using primers (restriction sites underlined) oKK42 (GCGCGAGATCTATGTCCCCTATACTAGGTTATTG) and oKK43 (ATATAAGGATCCACGCGGAACCAGATC) and subcloned into the *Bam*HI site of pFastBac-1 (pKK855; GIBCO-BRL). To construct pKK944, a *Bsg*I-*Hin*dIII fragment was subcloned from pKK661 into the *Bsg*I and *Hin*dIII sites of pKK1017. pKK661 was constructed by subcloning a PCR fragment (*Bam*HI-*Hin*dIII ends) that contains the *CDC42* coding region amplified from pKK177 (Kozminski *et al.*, 2000) into the same sites of vector pGAT2 (Peränen *et al.*, 1996). The fusion constructs were sequenced to verify an in-frame fusion and the sequence of the *GST* and *CDC42* coding sequences.

Preparation of Yeast Lysate and Immunoprecipitation

Yeast lysate preparation and immunoprecipitation were carried out at 4°C using an Eppendorf centrifuge. Yeast cell extracts were prepared from protease-deficient strain HPY16 carrying pRS425(*HA-RSR1*) (Park *et al.*, 1997) and YEp103(*CDC42*) using the lysis buffer G (4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM DTT, 10%, glycerol, pH 8.1) and various proteases as previously described (Park *et al.*, 1997) with slight modifications. After centrifugation for 4 min at 1900 rpm, the whole cell extracts were centrifuged for 15 min at 14,000 rpm to obtain a membrane fraction. The membrane fraction was resuspended in Buffer G containing 1% Triton X-100 and rocked for 30 min at 4°C. A soluble protein fraction was recovered by centrifugation for 15 min at 14,000 rpm, diluted approximately threefold with Buffer G (with no detergent; typically ~1 ml final), and precleared with anti-mouse IgG agarose beads (Sigma, St. Louis, MO) before incubating with 2 μl of anti-HA antibody (HA.11 from Covance Research Products, Denver, PA). After immunoprecipitation, association of Cdc42p with Rsr1p was determined by immunoblotting with affinity-purified rabbit polyclonal antibodies against Cdc42p (prepared according to the method of Ziman *et al.*, 1991), and recovery of Rsr1p was determined with polyclonal antibodies against Rsr1p (a kind gift of A. Bender).

Protein Expression and Purification

The Bac-To-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA) was used to express GST-Cdc42 fusion proteins. Plasmid constructions are described above. GST-Cdc42p was purified from HIGH FIVE cells (Invitrogen) 48 h postinfection at an MOI of 10, using glutathione agarose beads as described by Cerione *et al.* (1995).

GST-Rsr1p, GST-Rsr1p^{T35A}, MBP-Cdc24^{C}, and His_6-tagged Cdc42p were expressed and purified from a protease-deficient *E. coli* strain (BL21) essentially as previously described (Kellogg *et al.*, 1995; Park *et al.*, 1997). After harvesting cells, the cell pellet was frozen and ground under liquid nitrogen in a mortar. The fine powder was then rapidly resuspended in PBS containing 0.1% Triton X-100, 1 mM DTT and a cocktail of protease inhibitors. His_6-tagged Cdc42p was purified using a column containing iminodiacetic acid immobilized on Sepharose 6B (Sigma) coupled to Co^{2+}. When necessary, Rsr1p was released from the affinity matrix by cleaving GST-Rsr1p with thrombin (~2000 NIH units/mg protein, Sigma) as follows: After resuspension of the Sepharose in PBS buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$, the protein concentration was adjusted to 0.3 mg/ml before thrombin was added to the bead slurry at a ratio of 1% (wt/wt). After incubation for 3.5 h at 25°C, the GST moiety that remained bound to the beads was removed by centrifugation and the supernatant was incubated 1 h at 4°C in presence of *p*-aminobenzamidine coupled to agarose beads (Sigma) to remove the thrombin.

In Vitro Binding Assays

In vitro binding assays were performed as described (Park *et al.*, 1997) with slight modification. GST-fusion proteins, His_6- Cdc42p, and Rsr1p (after the GST moiety was cleaved off) were dialyzed overnight at 4°C against a buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 10% glycerol) containing 2.5 μM GDP after purification. Approximately 1 μg of protein was diluted to a final volume of 50 μl with 50% glutathione Sepharose bead slurry and incubated for 1 h at 4°C. The beads were collected by centrifugation and resuspended in Buffer I (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM EDTA, 10% glycerol, 0.1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin). After incubation for 1 h at room temperature, Buffer I was substituted with Buffer I containing 5 mM $MgCl_2$ plus 0.5 mM GTPγS or 0.5 mM GDP (Roche Diagnostics, Indianapolis, IN). After 30 min incubation at room temperature, the beads were resuspended in Buffer I containing 10 mM $MgCl_2$ plus 0.5 mM GTPγS or 0.5 mM GDP instead of 10 mM EDTA and then incubated 20 min at room temperature to stabilize the nucleotide bound state of the GTPase.

Filter Binding Assays

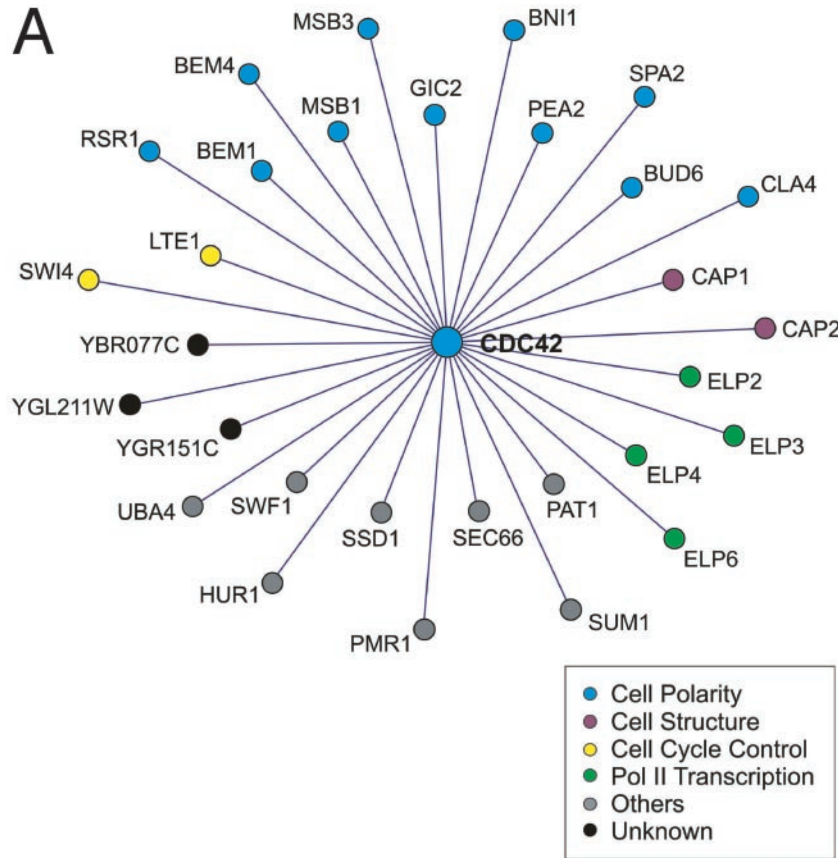
Filter binding assays were carried out as described previously (Diekmann *et al.*, 1994). The purified recombinant proteins were preloaded with nucleotides as described above except [8,5′-^{3}H]GTP or [8,5′-^{3}H]GDP (38.2 Ci/mmol, NEN Life Science Products) was loaded onto one of the GTPases. After in vitro binding reaction, the reaction mix was diluted to 1 ml with wash buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol) and then filtered through a nitrocellulose filter (0.2-μm pore diameter, Millipore). After washing with 10 ml of Buffer I, the radioactivity retained on the filters was quantified by scintillation counting.

RESULTS

A CDC42 Allele Defective in Polarity Establishment Identifies a RSR1-CDC42 Interaction

To identify genes that interact with *CDC42* during the establishment of cell polarity in late G1, we applied synthetic genetic array (SGA) analysis (Tong *et al.*, 2001) to screen for genes that confer a growth defect when deleted in combination with *cdc42-118*, an allele that is specifically and conditionally defective in polarity establishment (Kozminski *et al.*, 2000). At restrictive temperatures, *cdc42-118* cells arrest as large, round, unbudded, multinucleate cells, most likely due to an inability to organize the actin cytoskeleton at the bud site (Kozminski *et al.*, 2000). We identified 30 *CDC42*-interacting genes (Figure 1A). Ten of these genes have a previously defined role in polarized bud growth (see the "Cell Polarity" category in Figure 1A), validating the screen. Of these, only six have shown either a synthetic genetic interaction (*MSB3*, *GIC2*, *BNI1*, *CLA4*; Caviston *et al.*, 2002; Hofken and Schiebel, 2002) with *CDC42* and/or a physical interaction (*GIC2*, *BNI1*, *CLA4*, *BEM1*, and *BEM4*) between Cdc42p and their gene product (Chen *et al.*, 1997; Mack *et al.*, 1996; Brown *et al.*, 1997; Evangelista *et al.*, 1997; Uetz *et al.*, 2000; Bose *et al.*, 2001; Drees *et al.*, 2001). For the 20 remaining genes, especially those placed into functional categories other than "Cell Polarity," their identification in this screen suggested that they have a function associated with the Cdc42p-dependent development of cell polarity in late G_1. For the open reading frames classified as "Unknown," this interaction with *CDC42* was the first assignment of function.

The identification of *RSR1* as a *CDC42*-interacting gene was particularly intriguing. To date, Rsr1p has been thought only to position the axis of polarized growth before bud emergence and not to affect bud formation per se. To confirm that *RSR1* affects the polarity establishment function of Cdc42p, we compared the growth of *rsr1*Δ cells containing different separation-of-function alleles of *CDC42* (Kozminski *et al.*, 2000). As expected, alleles of *CDC42* that exhibit a growth defect due to a loss of polarity (*cdc42-101*, *cdc42-118*) in late G_1 conferred synthetic sickness at 30°C and synthetic lethality at 34°C when combined with *rsr1*Δ (Figure 1B). In



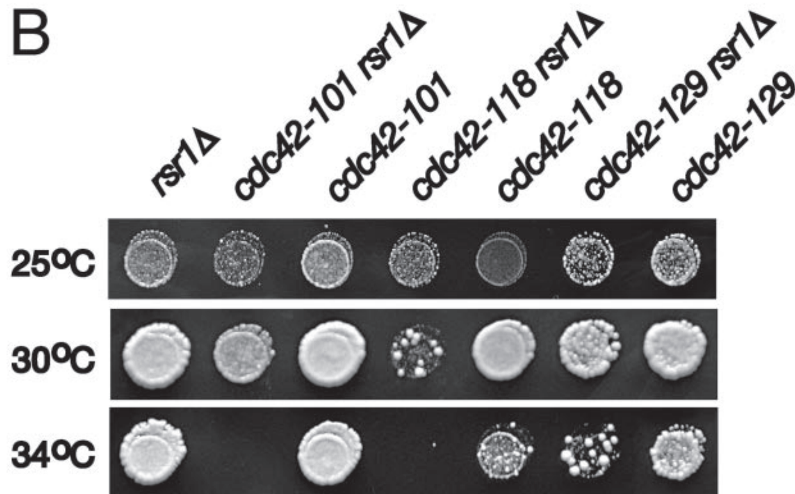


Figure 1. Synthetic genetic analyses identified *RSR1* as a *CDC42*-interacting gene. (A) Genetic interaction network representing synthetic lethal/sick interactions of *cdc42-118* determined by SGA analysis. Genes are colored according to their YPD-defined cellular function. (B) Equivalent dilutions of *rsr1*Δ, *cdc42*, and *rsr1*Δ *cdc42* mutants grown at 25, 30, and 34°C for 2d on SC-URA. The strains shown (left to right) are KKY 342, 343, 344, 345, 346, 297, and 298.

contrast, an allele of *CDC42* that is defective in the switch from apical to isotropic bud growth in G_2/M (*cdc42-129*) conferred only synthetic sickness at 34°C when combined with *rsr1*Δ (Figure 1B). Because the *cdc42* alleles used in this study are temperature-sensitive for growth, it was important to verify that the sick or lethal phenotype displayed by a *rsr1*Δ *cdc42* double mutant was due to the absence of *RSR1*. As shown in Figure 1B, the restrictive temperature range of the *rsr1*Δ *cdc42* mutants did not correlate with the restrictive temperature range of each *cdc42* allele, indicating a *bona fide* synthetic interaction between *cdc42* and *rsr1*Δ in particular double mutants. These results suggest that *RSR1* stimulates the polarity establishment function of *CDC42*.

Although *RSR1* has a known role in bud-site selection (Bender and Pringle, 1989; Chant and Herskowitz, 1991), a second independent line of evidence supported a role for *RSR1* in *CDC42*-dependent polarity establishment. Concomitant with our synthetic lethality studies, *RSR1* was identified in a second screen as a multicopy dosage suppressor of the *cdc42-118* temperature-sensitive growth defect. In contrast to *cdc42-118* cells containing a multicopy vector plasmid, *cdc42-118* cells bearing a multicopy *RSR1* plasmid did

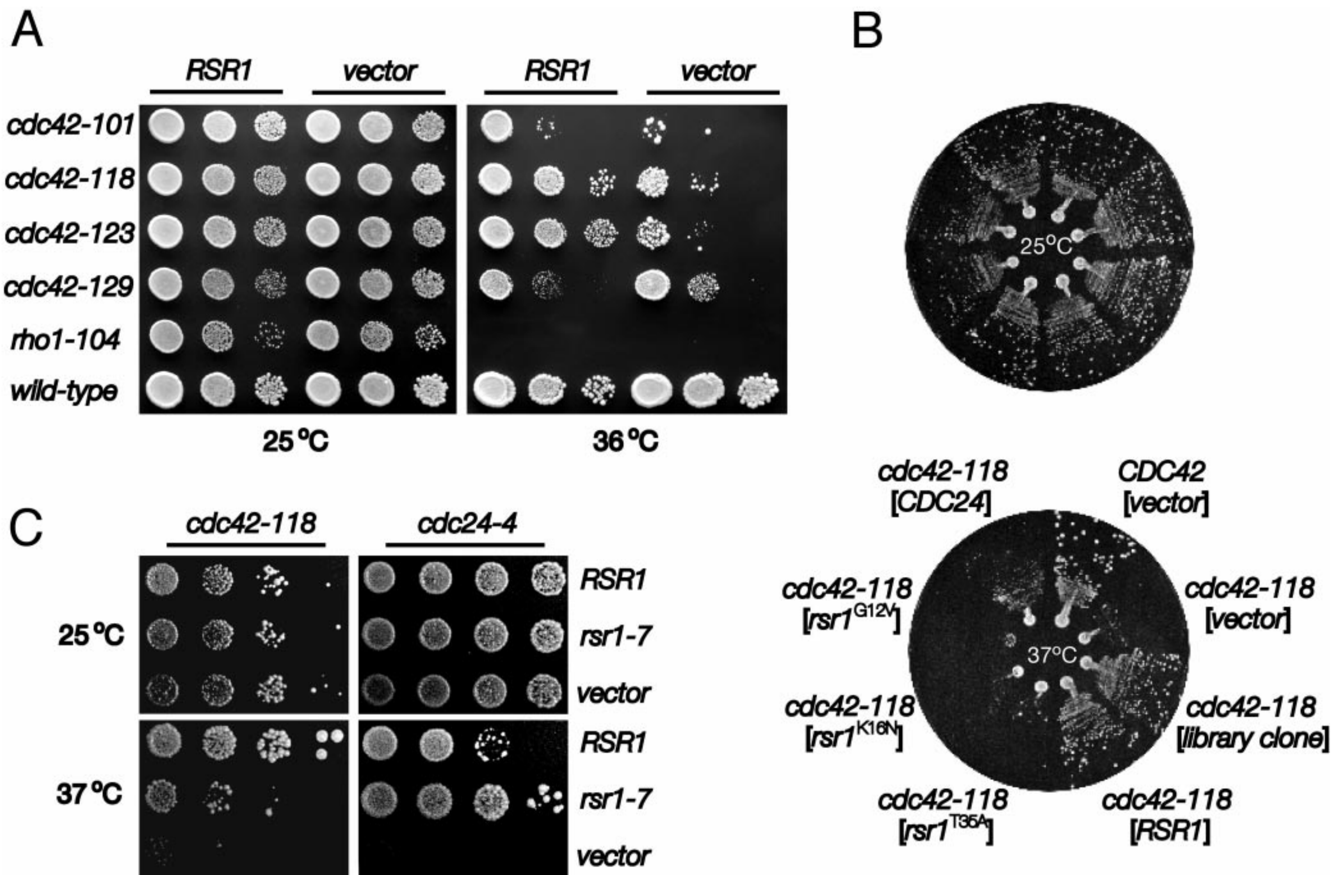


Figure 2. A multicopy *RSR1* plasmid, but not plasmids carrying other *rsr1* alleles, suppressed the growth defect of *cdc42-118*. (A) Dilution series compared growth at 25 and 36°C (SC-URA, 4d) of wild-type and mutant yeast transformed with a high-copy (YEplac195) 2-μm plasmid containing *RSR1* or no insert. Each spot of cells represented a 10-fold serial dilution from left to right. From top to bottom, *RSR1*, 2 μm-plasmid containing strains are KKY 66, 68, 287, 289, 291, and 190; strains containing only 2 μm-plasmid are KKY 239, 240, 286, 288, 290, and 238. (B) Wild-type and *cdc42-118* strains were transformed with 2-μm plasmids (shown in brackets) containing no insert, a genomic fragment containing *RSR1*, alleles of *RSR1*, or *CDC24* and grown at 25 (top panel) or 37°C (bottom panel) for 3 d on SC-URA. The strains shown (clockwise from 1 o'clock) are KKY 238, 240, 1012, 68, 60, 201, 200, and 69. Note: *RSR1* suppressed *cdc24-118* better than *CDC24*. (C) The *cdc42-118* and *cdc24-4* mutants, transformed with 2-μm plasmids containing *RSR1*, *rsr1-7*, or no insert, were grown at 25°C (for 3 d) or 37°C (for 4 d) on SC-URA (for *cdc42-118*) or SC-URA + 1 M sorbitol (for *cdc24-4*). Tenfold serial dilutions of each *cdc42-118* transformant and 2.5-fold serial dilutions of each *cdc24-4* transformant are shown.

not undergo growth arrest at 36°C (Figure 2A), and appeared for the most part, even at 37°C, morphologically wild-type (≤4% of the population exhibited a large, unbudded morphology; our unpublished results). Multicopy *RSR1* did not suppress the minor budding pattern defect found in the *cdc42-118* mutant at 25°C (our unpublished results). As observed with the synthetic genetic interactions described above, the multicopy dosage suppression by *RSR1* was also specific to the *cdc42* alleles defective in polarity establishment. Overexpression of *RSR1* neither suppressed a temperature-sensitive allele of *RHO1* (Figure 2A), which encodes a Rho-family GTPase essential for bud formation (Yamochi *et al.*, 1994), nor every *CDC42* separation-of-function allele tested (Figure 2A). Like *cdc42-118*, these separation-of-function alleles are temperature-sensitive for growth at 36°C, even though they are expressed at the wild-type levels (Kozminski *et al.*, 2000). At 36°C, multicopy *RSR1* suppressed the growth defect of the three alleles defective in polarity establishment: *cdc42-118* and *cdc42-123*, and to a moderate extent, *cdc42-101* (Figure 2A). Suppression of *cdc42-129*, which is not defective in polarized growth, did not exceed that of the vector control (Figure 2A), indicating that *RSR1* is not a general bypass suppressor of *cdc42* mutations. Consistent with the synthetic lethality data, these results suggest that *RSR1* specifically interacts with *CDC42* to affect its polarity establishment function before bud emergence.

Alleles of RSR1 Suggest a Direct Interaction between Rsr1p and Cdc42p In Vivo

RSR1 was previously identified as a multicopy dosage suppressor of a temperature-sensitive *cdc24-4* polarized growth defect (Bender and Pringle, 1989). This interaction requires Rsr1p to be in the GTP-bound state and to possess an intact effector domain (Ruggieri *et al.*, 1992; Park *et al.*, 1997). To determine whether similar requirements exist for the *RSR1*-*CDC42* interaction, we tested whether particular alleles of *RSR1*, expressed at about wild-type levels (our unpublished results), could suppress the *cdc42-118* growth defect. As in the case of *cdc24*, we found that an effector domain mutation in *RSR1* (*rsr1*^{T35A}) inhibited the multicopy suppression of

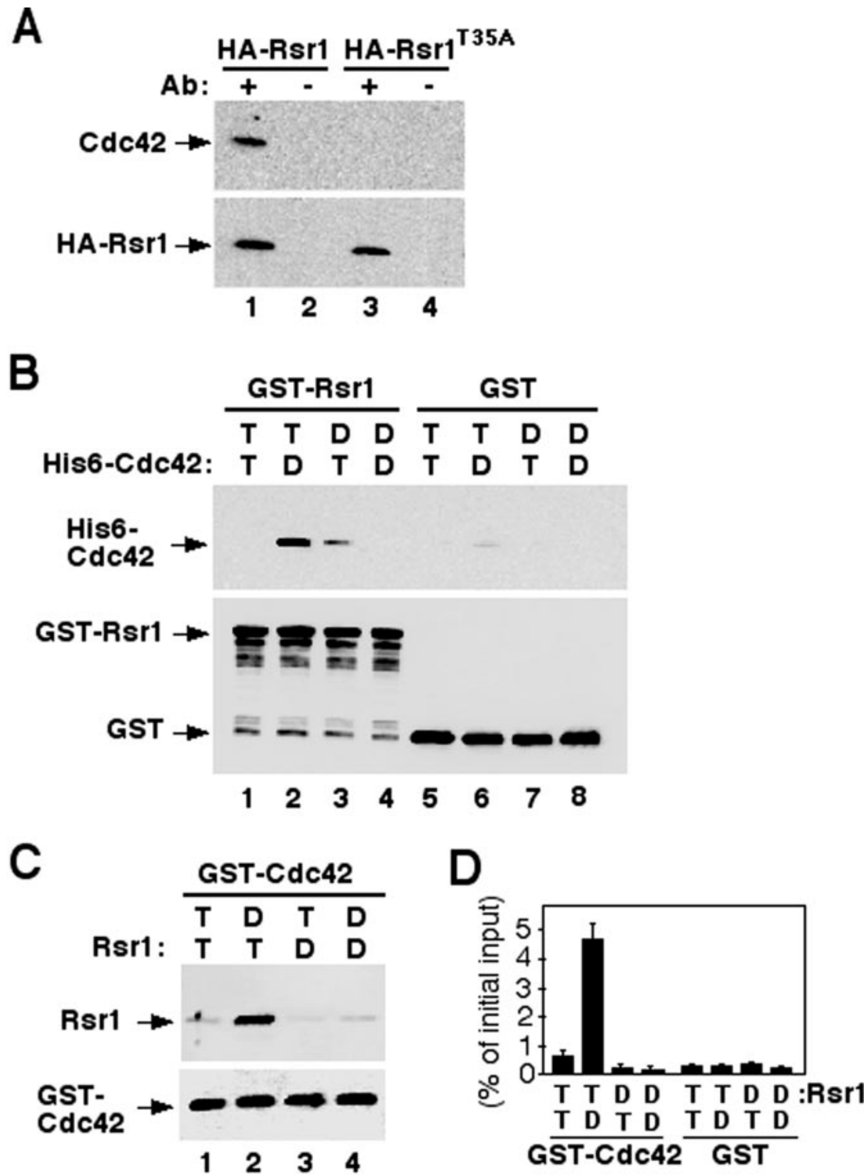


Figure 3. Rsr1p interacts with Cdc42p. (A) Cdc42p coimmunoprecipitated with HA-Rsr1p from yeast extracts. *CDC42*, *HA-RSR1*, and *HA-rsr1^{T35A}* were expressed from multicopy plasmids in yeast. Association of Cdc42p and HA-Rsr1p was not detectable when HA-Rsr1p and Cdc42p were expressed at the endogenous level. After immunoprecipitation of HA-Rsr1p (lane 1) or HA-Rsr1^{T35A}p (lane 3) with an antibody against the HA epitope, association of Cdc42p was determined by immunoblotting with polyclonal antibodies against Cdc42p (top panel). Approximately equal amounts of HA-Rsr1p and HA-Rsr1^{T35A}p were recovered for each reaction as judged by immunoblotting with polyclonal antibodies against Rsr1p (bottom panel). Control reactions without HA antibody were shown in lanes 2 and 4. (B) Association of GST-Rsr1p and His$_6$-Cdc42p in vitro. GST-Rsr1p (~400 nM) preloaded with GTPγS (T) or GDP (D) was incubated with His$_6$-tagged Cdc42p (~400 nM) preloaded with GTPγS or GDP. Rsr1p-GTPγS + Cdc42p-GTPγS (lane 1); Rsr1p-GTPγS + Cdc42p-GDP (lane 2); Rsr1p-GDP + Cdc42p-GTPγS (lane 3); Rsr1p-GDP + Cdc42p-GDP (lane 4). GST that had been preincubated with GTPγS (lanes 5 and 6) or GDP (lanes 7 and 8) was used as a control. Association of Cdc42p with Rsr1p was determined by immunoblotting with antibodies against Cdc42p (top panel). Approximately equal amounts of GST-Rsr1 and GST proteins were recovered for each reaction as judged by immunoblotting with antibodies against GST (bottom panel). (C) Association of Rsr1p and GST-Cdc42p in vitro. Rsr1p (~400 nM) purified after removal of GST was preloaded with GTPγS (T) or GDP (D) and incubated with GST-Cdc42p (~400 nM) preloaded with GTPγS or GDP. Rsr1p-GTPγS + Cdc42p-GTPγS (lane 1); Rsr1p-GTPγS + Cdc42p-GDP (lane 2); Rsr1p-GDP + Cdc42p-GTPγS (lane 3); Rsr1p-GDP + Cdc42p-GDP (lane 4). Association of Rsr1p with GST-Cdc42p was determined by immunoblotting with antibodies against Rsr1p (top panel). Approximately equal amounts of GST-Cdc42p were recovered for each reaction as judged by immunoblotting with antibodies against Cdc42p (lower panel). (D) Quantification of the amounts of Rsr1p associated with GST-Cdc42p. Rsr1p preloaded with [^{3}H]GTPγS (T) or [^{3}H]GDP (D) was incubated with GST-Cdc42p preloaded with GTPγS or GDP. The amount of [^{3}H]GTPγS- or [^{3}H]GDP-bound Rsr1p that associated with GST-Cdc42p was determined by a filter binding assay. An average of five independent assays is shown as percentage of counts (cpm) divided by the initial input to each reaction.

the temperature-sensitive *cdc42-118* growth defect (Figure 2B), indicating that the effector domain of Rsr1p is necessary for efficient suppression. In contrast to the *RSR1-CDC24* interaction, neither multicopy *rsr1^{G12V}* nor *rsr1^{K16N}*, which encode Rsr1p locked in the GTP- or GDP-bound state respectively (Ruggieri *et al.*, 1992), could suppress the *cdc42-118* growth defect at 37°C (Figure 2B), suggesting that cycling of Rsr1p between GTP- and GDP-bound states is important for suppression. Interestingly, *CDC24* was a weaker multicopy suppressor of *cdc42-118* than *RSR1* (Figure 2B). Thus, the suppression of the *cdc42* growth defect by multicopy *RSR1* was not likely to be due to the enhancement of an interaction between crippled Cdc42p and Cdc24p by Rsr1p. Consistent with the idea, we found that multicopy *rsr1-7* (*rsr1$^{K260-264S}$*) (Park *et al.*, 2002), expressed at about the same level as wild-type *RSR1* (our unpublished results), poorly suppressed the *cdc42-118* growth defect, though in contrast it suppressed the *cdc24-4* growth defect similarly to (or slightly better than) wild-type *RSR1* (Figure 2C). Taken together, this allele-specific suppression of *cdc42-118* by *RSR1* suggests that the interaction between Rsr1p and Cdc42p is direct in vivo and that the interaction is not bridged by Cdc24p.

Rsr1p Interacts with Cdc42p In Vivo and In Vitro

To test whether Rsr1p interacts with Cdc42p in vivo, we carried out immunoprecipitation experiments. We prepared a Triton X-100 soluble fraction of membrane-associated pro-

teins from a yeast strain in which Cdc42p and a functional HA epitope-tagged Rsr1p were expressed from multicopy plasmids. As shown in Figure 3A, Cdc42p coimmunoprecipitated with HA-Rsr1p, but not with HA-Rsr1^{T35A}p. These data indicate that Cdc42p associates with Rsr1p in vivo and that the effector domain of Rsr1p is important for the association.

To test whether Rsr1p can bind to Cdc42p in the absence of Cdc24p, we carried out in vitro binding experiments using Rsr1p fused to glutathione *S*-transferase (GST) and six-histidine-tagged (His$_6$-) Cdc42p purified from *E. coli*. After preloading GST-Rsr1p and His$_6$-Cdc42p with GTPγS, a nonhydrolyzable GTP analogue, or with GDP, the GTPases were incubated together. In control experiments, GST preincubated with GTPγS or GDP was mixed with nucleotide-bound His$_6$-Cdc42p. As shown in Figure 3B, Cdc42p-GDP associated preferentially with Rsr1p-GTPγS. In a complementary analysis, Rsr1p (GST removed) was incubated with GST-Cdc42p purified from baculovirus-infected cells. As in the previous experiment, Rsr1p-GTPγS interacted preferentially with Cdc42p-GDP (Figure 3C) but not with the GST control (unpublished data).

To quantify the amount of Rsr1p associated with Cdc42p and to confirm the nucleotide-bound state of each associated GTPase, we performed a filter-binding assay (Park *et al.*, 1993; Diekmann *et al.*, 1994) after the in vitro binding reaction using Rsr1p preloaded with [^{3}H]GTP or [^{3}H]GDP. Approximately 5% of the total [^{3}H]GTP-Rsr1p added to the reaction associated with Cdc42p-GDP (Figure 3D). A smaller amount of [^{3}H]GTP-Rsr1p also associated with Cdc42p-GTPγS. Similar results were obtained in a complementary experiment using His$_6$-Cdc42p preloaded with [^{3}H]GDP or [^{3}H]GTP and GST-Rsr1p. Taken together, these results indicate that Rsr1p interacts directly with Cdc42p in a nucleotide-dependent manner.

The nucleotide-specific association of Rsr1p and Cdc42p was observed at a relatively low concentration ($<$430 nM) and equimolar ratio of Rsr1p and Cdc42p. At the higher concentrations we tested ($0.8 \sim 2$ μM of each GTPase), the interaction between the two GTPases was not specific to each nucleotide-bound state. For example, we observed a strong interaction between Rsr1p-GTP and Cdc42p-GTP (our unpublished results), which might be due to the formation of heterodimers—similar to the formation of homodimers in vitro by these (Beven and Park, unpublished data) and mammalian Rho GTPases at high concentrations (Zhang and Zheng, 1998; Inouye *et al.*, 2000). The physiological significance of homodimer formation of small GTPases is not clear at the present time.

The Cdc24p Fragment Associated with Rsr1p-GTP Enhances Interaction Between Rsr1p-GTP and Cdc42p-GDP In Vitro

Because both Cdc24p (Zheng *et al.*, 1995; Park *et al.*, 1997) and Cdc42p (this study) appear to interact specifically with the GTP-bound form of Rsr1p, we wanted to know whether these associations were cooperative or competitive. To address this question, we performed in vitro binding reactions using GST-Rsr1p-GTPγS, His$_6$-Cdc42p-GDP, and the C-terminal half (residues 472–854) of Cdc24p (Cdc24^C) fused to a maltose-binding protein (MBP). This truncated form of Cdc24p interacts with Rsr1p (Park *et al.*, 1997), but lacks the GEF domain that would exchange the nucleotide on Cdc42p. The amount of Cdc42p associated with Rsr1p increased proportionally with an increase of Cdc24^C in the reaction (Figure 4A, lanes 1–4). When MBP-Cdc24^C was cosedimented with amylose resin, Cdc42p was not associated with Cdc24^C in the absence of Rsr1p (Figure 4A, lanes 5–7), confirming that Cdc42p binds to Rsr1p and not to Cdc24^C. Similarly, when we used GST-Cdc42p and Rsr1p (GST removed), a proportionally higher amount of Rsr1p was associated with GST-Cdc42p with an increase of Cdc24^C (Figure 4B; lanes 1–4). In the absence of Rsr1p, no Cdc24^C was recovered with GST-Cdc42p (Figure 4B; lanes 5–7), as expected. To quantify the enhancement of the Rsr1p-Cdc42p interaction by Cdc24^C, the binding reaction was carried out using His$_6$-Cdc42p preloaded with [^{3}H]GTP or [^{3}H]GDP and GST-Rsr1p preloaded with GTPγS in the presence of Cdc24^C, and the amount of Cdc42p associated with GST-Rsr1p was determined by a filter-binding assay. Association of [^{3}H]GDP-Cdc42p with Rsr1p-GTPγS increased about threefold when 20 nM Cdc24^C ($\sim$ 5% of the concentration of Rsr1p or Cdc42p) was added to the reaction (Figure 4C). Taken together, these data show that Rsr1p interacts with both Cdc24^C and Cdc42p, and that Cdc24^C enhances the interaction between Rsr1p-GTP and Cdc42p-GDP.

The association of Cdc24p and Cdc42p with Rsr1p led to the idea that Cdc24p and Cdc42p bind to different regions of Rsr1p. Consistent with this prediction, we found that the effector domain mutant Rsr1^{T35A}p, although defective in interaction with Cdc24p (Park *et al.*, 1997), interacted with Cdc42p similarly to the wild-type Rsr1p in vitro (Figure 5A). Thus the effector domain of Rsr1p is unlikely to be directly involved in the Rsr1p-Cdc42p interaction. To address an apparent contradiction with our coimmunoprecipitation data, which indicated that the effector domain of Rsr1p is necessary for a Rsr1p-Cdc42p interaction, we examined the association of Rsr1^{T35A}p with Cdc42p in the presence of Cdc24^C in vitro. Unlike Rsr1p, which displayed a proportional increase in Cdc42p binding as the amount of Cdc24^C was increased (Figure 4), GST-Rsr1^{T35A}p associated with approximately equal amounts of Cdc42p in the presence or absence of MBP-Cdc24^C (Figure 5B, lanes 1–4; top panel). MBP-Cdc24^C did not associate with GST-Rsr1^{T35A}p (Figure 5B, lanes 1–4; middle panel), and thus was unlikely to enhance GST-Rsr1^{T35A}p binding to His$_6$-Cdc42p. Thus, the effector domain of Rsr1p is required for enhanced association of Rsr1p with Cdc42p. Taken with our coimmunoprecipitation and multicopy suppression data, the efficient association between Cdc42p and Rsr1p in vivo is likely to require interaction between Cdc24p and Rsr1p through the effector domain of Rsr1p.

DISCUSSION

Two different genetic screens for genes important for Cdc42p-dependent polarity establishment identified *RSR1*, a gene known to be necessary for proper bud-site selection in *S. cerevisiae* (Bender and Pringle, 1989; Chant and Herskowitz, 1991). Rsr1p interacted with Cdc42p in vivo and in vitro: Cdc42p coimmunoprecipitated with Rsr1p from yeast cell extract and also directly interacted with Rsr1p in vitro. Previous work suggested that bud site selection is coupled to polarity establishment through direct interaction between Rsr1p-GTP and Cdc24p, a GEF for Cdc42p (Zheng *et al.*, 1995; Park *et al.*, 1997). Based on our new data, we propose that the GTPase modules that govern bud site selection and polarity establishment are bridged not only by the GEF Cdc24p but also through direct interactions of GTPases. The interaction between Rsr1p and Cdc42p may contribute to guiding Cdc42p to the proper bud site as well as to stabilizing a bud-site assembly complex.

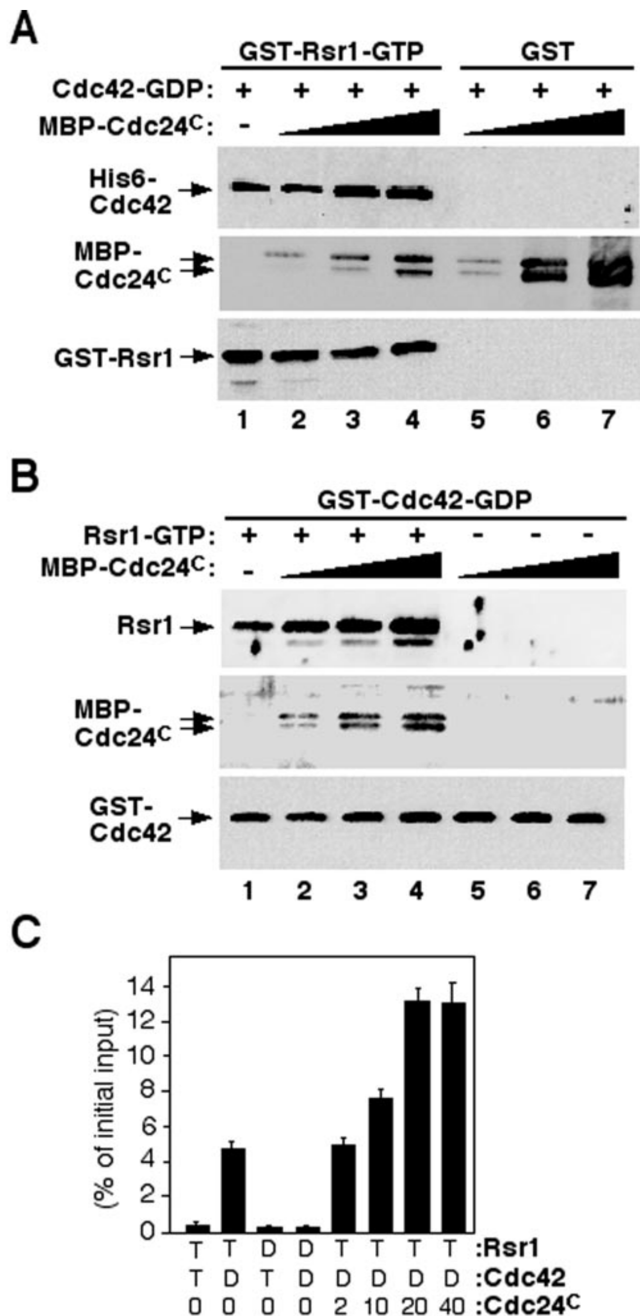


Figure 4. The C-terminal half of Cdc24p enhances interaction between Rsr1p-GTP and Cdc42p-GDP. (A) GST-Rsr1p (~400 nM) preloaded with GTPγS was incubated with His_6-tagged Cdc42p (~400 nM) preloaded with GDP in the absence (lane 1) or in the presence of MBP-$Cdc24^C$ (lane 2: 2 nM; lane 3: 10 nM; lane 4: 20 nM). GST-Rsr1p was collected with glutathione Sepharose. As a control, GST was incubated with MBP-$Cdc24^C$ and Cdc42p-GDP, and MBP-$Cdc24^C$ was brought down with amylose resin (lanes 5–7). Approximately equal amounts of GST-Rsr1p were recovered for each reaction as judged by immunoblotting with Rsr1p-specific antibodies (bottom panel: lanes 1–4). Association of Cdc42p and MBP-$Cdc24^C$ with GST-Rsr1p or GST was determined by immunoblotting with Cdc42p-specific antibodies (top panel) and with polyclonal antibodies against Cdc24p (middle panel), respectively. (B) GST-Cdc42p (~400 nM) preloaded with GDP was incubated with Rsr1p (after cleavage of GST; ~400 nM) preloaded with GTPγS in the absence (lane 1) or in the presence of MBP-$Cdc24^C$ (lane 2: 2 nM; lane 3: 10 nM; lane 4: 20 nM). As a control, GST-Cdc42p preloaded with GDP was incubated with MBP-$Cdc24^C$ (2–20 nM) without Rsr1p (lanes 5–7). Approximately equal amounts of GST-Cdc42p were recovered for each reaction as judged by immunoblotting with GST-specific antibodies (bottom panel). Association of Rsr1p with Cdc42p was determined by immunoblotting with Rsr1p-specific antibodies (top panel). Presence of MBP-$Cdc24^C$ in the eluents was determined by immunoblotting with Cdc24p-specific antibodies (middle panel). (C) His_6-Cdc42p preloaded with [^{3}H]GTP (T) or [^{3}H]GDP (D) was used in a filter binding assay with GST-Rsr1p preloaded with GTPγS (T) or GDP (D) in the presence of various amount of $Cdc24^C$ (0–40 nM). The data are averages of three independent assays.

Association of the Ras-family GTPase Rsr1p with the Rho Family GTPase Cdc42p

Three lines of evidence presented herein suggest that Rsr1p directly interacts with Cdc42p rather than being bridged by other binding partners such as Cdc24p or Bem1p in vivo. First, *RSR1* displayed specific genetic interactions with *CDC42*, similarly to genes that encode proteins known to interact directly with Cdc42p such as *GIC2* and *CLA4*. These interactions were allele-specific: *rsr1Δ* exhibited synthetic lethality with *cdc42* mutants specifically defective in polarity establishment but not with another *cdc42* mutant or *rho1*. In addition, *RSR1* on a multicopy plasmid suppressed only *cdc42* mutants with a polarity establishment defect. Furthermore, an allele of *RSR1*, *rsr1*$^{K260-264S}$, that could suppress *cdc24-4* similarly to the wild-type *RSR1,* poorly suppressed the *cdc42* polarized growth defect. The allele specificity of these interactions suggested strongly that *RSR1* affects a specific *CDC42* function, the establishment of cell polarity in late G1. Second, Cdc42p coimmunoprecipitated with Rsr1p from yeast cell extract in a condition where we did not detect Cdc24p associated with Rsr1p, suggesting in vivo association of the two GTPases. However, we cannot rule out the possibility that Cdc24p was subject to detection limits in our experiments due to low avidity of our antibodies against Cdc24p and/or very transient association of Cdc24p with Rsr1p or Cdc42p. Third, recombinant Rsr1p and Cdc42p associated in vitro in the absence of other proteins. This interaction was nucleotide-dependent and was enhanced in the presence of a truncated form of Cdc24p that contains the Rsr1p-binding domain. One caveat of these in vitro binding assays is that the truncated form of Cdc24p rather than the full length Cdc24p was used. This truncated Cdc24p, which lacks the GEF domain, was used to avoid the nucleotide exchange of Cdc42p and also to determine direct association of Cdc42p with Rsr1p. It remains to be tested whether the full length Cdc24p functions similarly and if so, how association of Cdc24p with Rsr1p affects the interaction between Rsr1p and Cdc42p.

Although both Cdc24p and Cdc42p appear to interact with Rsr1p, the binding regions are likely to be different. It was shown previously that *rsr1*^{T35A} on a multicopy plasmid failed to suppress *cdc24-4,* unlike wild-type *RSR1,* and that $Rsr1^{T35A}$p also failed to interact with Cdc24p in vitro (Park *et al.,* 1997). In contrast, $Rsr1^{T35A}$p was not defective in interaction with Cdc42p in vitro, even though *rsr1*^{T35A} on a multicopy plasmid failed to suppress *cdc42-118* (this study). Interestingly, the fragment of Cdc24p that was shown to interact with Rsr1p failed to enhance the interaction between $Rsr1^{T35A}$p and Cdc42p. The same Cdc24p fragment does not bind to $Rsr1^{T35A}$p (Park *et al.,* 1997) and thus likely fails to enhance the interaction between $Rsr1^{T35A}$p and Cdc42p. A similar failure of binding enhancement may explain why

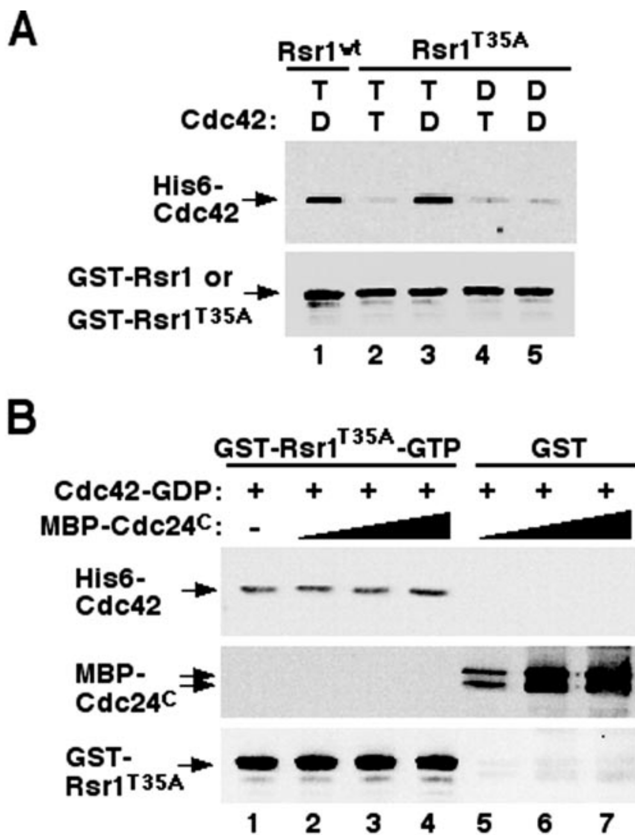


Figure 5. The effector domain of Rsr1p is not directly involved in interaction with Cdc42p. (A) The Rsr1p^{T35A} protein interacts with His$_6$-tagged Cdc42p similarly to the wild-type Rsr1p in vitro. Rsr1p-GTPγS + Cdc42p-GDP (lane 1); Rsr1p^{T35A}-GTPγS + Cdc42p-GTPγS (lane 2); Rsr1p^{T35A}-GTPγS + Cdc42p-GDP (lane 3); Rsr1p^{T35A}-GDP + Cdc42p-GTPγS (lane 4); Rsr1p^{T35A}-GDP + Cdc42p-GDP (lane 5). Proteins were analyzed as in Figure 3B. (B) Interaction between GST-Rsr1p^{T35A}-GTP and His$_6$-Cdc42p-GDP in the presence of C-terminal half of Cdc24p. GST-Rsr1p^{T35A}p (~400 nM) preloaded with GTPγS was incubated with His$_6$-Cdc42p (~400 nM) preloaded with GDP in the absence (lane 1) or in the presence of MBP-Cdc24^C (lane 2: 2 nM; lane 3: 10 nM; lane 4: 20 nM). GST was used as a control instead of GST-Rsr1p in the presence of MBP-Cdc24 ^{C} and Cdc42p-GDP, and MBP-Cdc24 ^{C} was brought down with amylose resin (lanes 5–7). Proteins were analyzed as in Figure 4A.

rsr1^{T35A} failed to suppress *cdc42-118*. Thus, it is likely that the association of Cdc24p and Rsr1p facilitates the interaction between Rsr1p and Cdc42p in vivo.

Recent pieces of data hint at the possibility that Rsr1p may associate with Cdc42p to enhance the association of Cdc42p with its effectors. In vitro studies with Cdc42p^{D76A}, which is encoded by *cdc42-118*, exhibited little defect in binding to GTP-bound Rsr1p, in comparison to the wild-type Cdc42p (Beven and Park, unpublished observations). Thus the *cdc42-118* growth defect might be due to a defective interaction of Cdc42p^{D76A} with a downstream target that can be ameliorated by high levels of Rsr1p. It has been proposed that D76 of Cdc42p forms an intramolecular hydrogen bond with K187 to stabilize the interaction of the C-terminus of Cdc42p with regulators or effectors of Cdc42p (Kozminski *et al.*, 2000), consistent with the idea that distant mutations within small GTPases have allosteric effects on the selectivity of effector binding (Heo and Meyer, 2003). By interacting with residues of Cdc42p other than D76, Rsr1p may help stabilize of the C-terminus of Cdc42p to improve the selectivity of effector binding. As suggested by genetic data, Gic1p and Gic2p may be two such effectors. *cdc42-118* exhibits synthetic lethality with *gic2*Δ (this study) and *rsr1*Δ is synthetically lethal with *gic1 gic2* mutations (Kawasaki *et al.*, 2003). This web of genetic interactions further supports the idea that Rsr1p has a role both in bud site selection and in polarity establishment.

A Model for Bud Site Assembly at a Proper Bud Site

We previously proposed a scheme by which the Rsr1p GTPase cycle plays a role in localizing proteins necessary for cell polarity to a cellular landmark (Park *et al.*, 1997). Based on the model, multiple proteins interact with each other and with the bud-site landmark to establish a site for new assembly of the actin cytoskeleton. Subsequently, we showed that the regulators of Rsr1p, Bud2p, and Bud5p, localize independently to the presumptive bud site (Park *et al.*, 1999; Kang *et al.*, 2001), although the maintenance of each at the bud site depends upon the presence of the other proteins of the GTPase module. Moreover, localization of Cdc24p to the presumptive bud site in late G1 is dependent on Rsr1p (Park *et al.*, 2002). Here we present a revised model of how the Rsr1p and Cdc42p GTPase modules couple the identification of a specific growth site to the establishment of polarized growth at that site (Figure 6). We have incorporated the following new information into this scheme: Rsr1p-GTP binds to Cdc42p-GDP, the interaction is enhanced by the association of Rsr1p with Cdc24p (this study), and Cdc42p-GTP binds to Bem1p (Butty *et al.*, 2002). Bem1p is thought to be a scaffold that interacts with Cdc24p, Cdc42p, Rsr1p, and Cla4p (Bender *et al.*, 1996; Park *et al.*, 1997; Butty *et al.*, 1998, 2002; Gulli *et al.*, 2000; Bose *et al.*, 2001), all of which have been shown to localize to the proper bud site (Ziman *et al.*, 1993; Ayscough *et al.*, 1997; Toenjes *et al.*, 1999; Nern and Arkowitz, 2000; Shimada *et al.*, 2000; Richman *et al.*, 2002). A recent study also indicates that localization of Bem1p to the incipient bud site requires activated Cdc42p (Butty *et al.*, 2002).

In Step 1 of our model (Figure 6), Bud5p, which localizes to the proper bud site through the interaction with a cortical cue (Kang *et al.*, 2001), exchanges GDP bound to Rsr1p for GTP. In Step 2, association of Rsr1p-GTP and its binding partners yields a complex of Rsr1p-GTP, Cdc24p, and Cdc42p-GDP at the presumptive bud site where Bud2p also localizes (Park *et al.*, 1999). We propose that association of Rsr1p-GTP with Cdc24p enhances the interaction between Rsr1p-GTP and Cdc42p-GDP. We further speculate that this ordered assembly of the complex allows the activation of Cdc42p by Cdc24p only at the proper bud site. In Step 3, Rsr1p-GTP is converted to Rsr1p-GDP through the action of its GAP, Bud2p (Park *et al.*, 1993). Conversion of Rsr1p-GTP to Rsr1p-GDP by Bud2p may allow targeted release of bud-site assembly proteins at the proper bud site (Benton *et al.*, 1993; Park *et al.*, 1997). The final critical event occurs in Step 4 where dissociation of Rsr1p from Cdc24p may activate the GEF activity of Cdc24p, converting Cdc42p-GDP to Cdc42-GTP. Bem1p may then join and maintain the bud-site assembly complex through the interaction with Cdc42p-GTP (Butty *et al.*, 2002) or with Rsr1p-GDP (Park *et al.*, 1997). Alternatively, activation of Cdc24p, without a concomitant stimulation of its GEF activity (Zheng *et al.*, 1995), may result from an interaction with Rsr1p. Conversion of Rsr1p-GDP to Rsr1p-GTP by Bud5p would then allow recycling of Rsr1p and further shuttling of Cdc24p, Cdc42p, and Bem1p to the

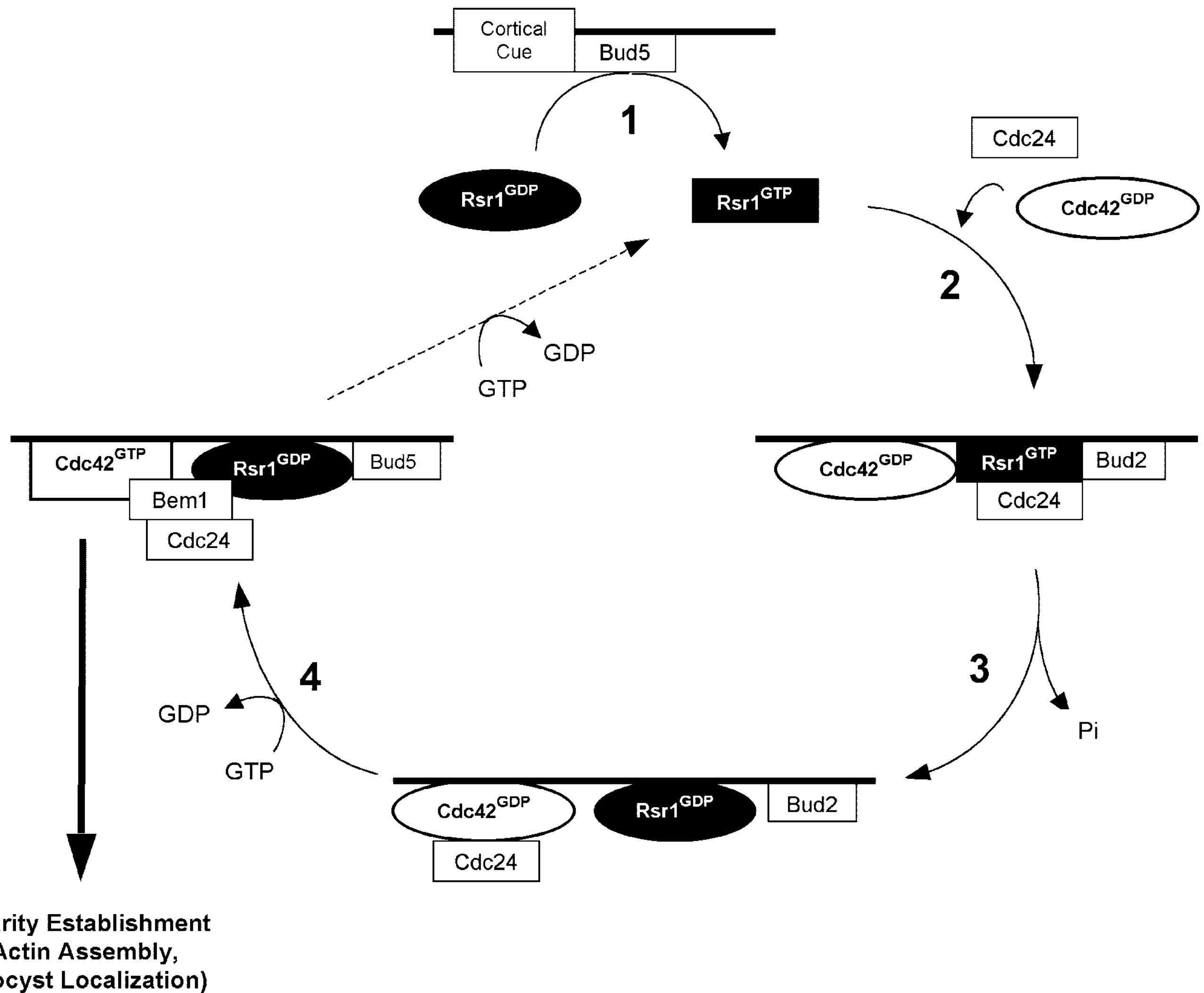


Figure 6. A model of how bud site selection couples to polarity establishment in late G1. Step 1: Bud5p exchanges GDP for GTP on Rsr1p. Step 2: Cdc24p and Cdc42p associate with GTP-bound Rsr1p at the bud site. Step 3: Bud2p activates Rsr1p to hydrolyze GTP bound to Rsr1p. Step 4: Dissociation of GDP-bound Rsr1p from Cdc24p may activate Cdc24p. Activated Cdc24p exchanges GDP for GTP on Cdc42p. GTP-bound Cdc42 then triggers actin assembly and exocyst localization to establish an axis of polarity. Bud5p at the bud site would then return Rsr1p to a GTP bound state (dashed line), allowing for another cycle of signal transduction. See DISCUSSION for details of the model.

proper bud site. The cycle is thus proposed to result in localization of a critical level of Cdc42p-GTP and polarity establishment proteins (e.g., Bem1p) to the bud site, triggering polarized assembly/organization of the actin cytoskeleton and secretory apparatus.

This model provides a simplified view for selection of a proper growth site, and the details of the model remain to be tested. Nonetheless, we think that the ordered assembly of a complex through multiple protein-protein interactions proposed here may ensure the establishment of polarity at a correct location. In the absence of the Rsr1p GTPase module, localization of Cdc24p and Cdc42p to a random bud site may occur through a distinct default pathway yet to be identified or by the stochastic accumulation of bud-site assembly proteins on the plasma membrane.

This study leads us to consider whether other Ras-family proteins are interacting directly with Cdc42p to foster the establishment of cell polarity. Ras2p, for example, appears to have a Cdc42p-dependent role in polarized growth. Ras2p is required for the polarized distribution of Cdc42p and maintenance of cytoskeletal polarity during mild temperature stresses (Ho and Bretscher, 2001). Furthermore, Ras2p signals via a Cdc42p/MAP kinase module to induce the polarized morphogenesis required for filamentous growth (Mosch *et al.,* 1996, 1999). In either case, the mechanism by which Ras2p signals to Cdc42p is unknown. It is tempting to speculate that Ras2p may interact directly with Cdc42p in a manner similar to that observed with Rsr1p. Similar interactions between Ras and Rho proteins may also occur in other organisms. A prime candidate is *S. pombe*, in which polarized morphogenesis is regulated by an interaction of Ras1p with Scd1p, a GEF of Cdc42p (Papadaki *et al.,* 2002). It will be interesting to see whether this morphogenetic pathway, analogous to the Rsr1p-Cdc24p-Cdc42p pathway in budding yeast, also contains a direct Ras-Rho interaction.

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