Novel Cdc42-binding proteins Gic1 and Gic2 control cell polarity in yeast

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Cdc42p, a Rho-related GTP-binding protein, regulates cytoskeletal polarization and rearrangements in eukaryotic cells, but the effectors mediating this control remain unknown. Through the use of the complete yeast genomic sequence, we have identified two novel Cdc42p targets, Gic1p and Gic2p, which contain consensus Cdc42/Rac interactive–binding (CRIB) domains and bind specifically to Cdc42p–GTP. Gic1p and Gic2p colocalize with Cdc42p as cell polarity is established during the cell cycle and during mating in response to pheromones. Cells deleted for both *GIC* genes exhibit defects in actin and microtubule polarization similar to those observed in *cdc42* mutants. Finally, the interaction of the Gic proteins and Cdc42p is essential, as mutations in the CRIB domain of Gic2p that eliminate Cdc42p binding disrupt Gic2p localization and function. Thus, Gic1p and Gic2p define a novel class of Cdc42p targets that are specifically required for cytoskeletal polarization in vivo.

[*Key Words:* Polarization; cytoskeleton; Rho GTPases; *Saccharomyces cerevisiae*]

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In polarized cells, the actin and microtubule cytoskeletons are highly asymmetric and serve to spatially regulate various cellular functions, including targeted secretion, signaling, and nuclear migration (Glotzer and Hyman 1995; Chant 1996; Drubin and Nelson 1996). The actin cytoskeleton maintains cell shape and plays a pivotal role in cell motility, cytokinesis, and phagocytosis. Reorganization of the actin cytoskeleton is regulated both through the cell cycle and in response to extracellular signals. Members of the Rho family of small GTPases have emerged as key regulators of actin filament dynamics and the assembly of focal adhesion contacts (Hall 1994; Ridley 1995). Rho GTPases are also important for maintaining cellular transformation (Symons 1995). GTPases act as molecular switches cycling between GTP- and GDP-bound conformations. When in the GTP-bound conformation, GTPases are thought to interact with target proteins that mediate their effects.

Cdc42p, a highly conserved Rho-type GTPase, has been shown to control polarized axis formation in both yeast and mammalian cells (Johnson and Pringle 1990; Hall 1994; Stowers et al. 1995). In fibroblasts, microinjection of activated Cdc42p triggers formation of filopodia or microspikes at the cell periphery (Kozma et al. 1995; Nobes and Hall 1995). In yeast, Cdc42p controls the polarization of actin and microtubules during both the vegetative cell cycle and mating. Polarity is initiated by choosing a site on the surface of the cell, and then growth is directed toward this site. During vegetative growth by budding, polarization is directed by a cell-type-specific program, which is controlled by a group of nonessential genes (*BUD1-BUD9, AXL1,* and *BUD10/AXL2;* Chant 1996). During mating, polarization is directed toward the mating partner by a mechanism involving the *FAR1* gene (Dorer et al. 1995; Valtz et al. 1995). Remodeling of the actin cytoskeleton toward these sites during both budding and mating requires the products of multiple genes, including *BEM1, CDC24,* and *CDC42. CDC24* encodes a GDP–GTP exchange factor for Cdc42p (Sloat and Pringle 1979; Zheng et al. 1994). Bem1p contains two SH3 domains and is thought to provide a scaffold by interacting directly with Cdc42p, Cdc24p, Ste20p, and Ste5p (Peterson et al. 1994; Leeuw et al. 1995; Lyons et al. 1996; Park et al. 1997). Cdc42p and Bem1p localize to the sites of polarized growth: the bud site during the cell cycle and the shmoo tip in cells exposed to pheromones (Ziman et al. 1993; Pringle et al. 1995). Cells lacking Cdc42p or Cdc24p function are unable to polarize their cytoskeleton and, as a consequence, arrest as large unbudded cells (Adams et al. 1990). In contrast, cells deleted for *BEM1* are viable but exhibit morphological abnormalities (Chenevert et al. 1992).

In addition, Cdc42p has been shown to function upstream of mitogen-activated protein (MAP) kinase signal transduction pathways. In mammalian cells, Cdc42p triggers the Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) cascade (Bagrodia et al. 1995; Coso et al. 1995; Hill et al. 1995; Minden et al. 1995) and

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has also been implicated in the activation of the $p70^{S6}$ kinase (Chou and Blenis 1996). In yeast, Cdc42p has been suggested to play a role in MAP kinase signaling pathways during mating (Simon et al. 1995; Zhao et al. 1995) and during pseudohyphal growth (Mösch et al. 1997). Members of the p21-activated kinase family (PAK) appear to be important effectors, mediating at least part of the signaling role of Cdc42p (Manser et al. 1994; Zhang et al. 1995; Brown et al. 1996; Peter et al. 1996; Leberer et al. 1997). Binding of Cdc42p to PAK-like kinases occurs through a short segment that is conserved among several Cdc42p targets and has been termed the Cdc42/Rac-interactive-binding (CRIB) domain (Burbelo et al. 1995). Activation of PAK kinases, however, is neither necessary nor sufficient for cytoskeletal polarization mediated by Cdc42p (Joneson et al. 1996; Lamarche et al. 1996).

The effectors of Cdc42p that control the cytoskeleton remain unknown. Although a number of candidate effectors have recently been described in yeast and mammals, none of these proteins can fully account for the effects of Cdc42p on cell polarization. The protein altered in patients suffering from Wiskott-Aldrich Syndrome (WASP) is important for some aspects of actin organization (Aspenstrom et al. 1996; Kolluri et al. 1996; Symons et al. 1996), but experiments in yeast have shown that the WASP-related molecule Las17/Bee1 is dispensible for cell polarization (Li 1997; D. Mitchell and G. Sprague, pers. comm). The formin-related proteins Bni1p and Bnr1p bind to several Rho-related GTPases, and thus may not act as specific Cdc42p targets (Kohno et al. 1996; Evangelista et al. 1997; Imamura et al. 1997). IQGAP family molecules, which are related by sequence to GTPase activating proteins (GAP), represent a possible class of Cdc42p effectors (Brill et al. 1996; Hart et al. 1996; Kuroda et al. 1996; McCallum et al. 1996), but our recent work suggests that yeast cells deleted for the gene, *IQG1,* encoding an IQGAP-related molecule are able to polarize (Epp and J. Chant, unpubl.). Finally, the ACK tyrosine kinase remains a largely unexplored mammalian target of Cdc42p (Manser et al 1994); however, ACK is not present in yeast and, therefore, seems an unlikely candidate for an ubiquitous Cdc42p effector involved in cytoskeletal polarization.

To identify Cdc42p effectors important for cytoskeletal polarization, we searched the complete *Saccharomyces* genomic sequence for additional proteins containing a CRIB domain. In this paper, we describe two novel Cdc42p effectors, Gic1p and Gic2p, which bind specifically to Cdc42p-GTP through their conserved CRIB domain. Importantly, Gic1p and Gic2p are required for cell polarization in vivo during the cell cycle and in response to extracellular signals, but they are dispensable for MAP kinase signal transduction. Our data suggest that Gic1p and Gic2p specifically link Cdc42p to dynamic rearrangements of the actin and microtubule cytoskeletons.

Results

Identification of GIC1 *and* GIC2

To identify potential Cdc42p targets, the complete genomic sequence of *Saccharomyces cerevisiae* was searched for gene products that contain the CRIB domain, which is shared by a number of known Cdc42p effectors (Manser et al. 1994; Burbelo et al. 1995) (Fig. 1A). Five proteins were identified: three PAK-related serine/threonine kinases (Ste20p, Cla4p, and Skm1p; Leberer et al. 1992; Ramer and Davis 1993; Cvrckova et al. 1995) and two uncharacterized open reading frames, which we denoted *GIC1* and *GIC2* (GTPase interactive components 1 and 2). Gic1p and Gic2p are related proteins of similar size with a highly conserved amino-terminal region followed by the CRIB domain and a less conserved carboxyl terminus (Fig. 1B). Further database searches indicated that Gic1p and Gic2p define a new class of CRIB domain proteins, and that there are only two Gic family members in yeast.

Gic1p and Gic2p bind specifically to Cdc42p–GTP in vitro and in vivo

We tested whether the Gic proteins bind directly to Cdc42p by both biochemical and two-hybrid experiments. Columns containing immunoaffinity-purified Gic2 protein were assayed for their ability to retain recombinant Cdc42p. As illustrated in Figure 2A, Cdc42p preloaded with GTPγS readily bound to the Gic2p column, whereas Cdc42p–GDP exhibited no detectable affinity. Recombinant Cdc42p containing a *T35A* mutation in the GTPase effector domain, preloaded with GTPγS, failed to interact with Gic2p, suggesting that the interaction between Gic2p and Cdc42p occurs through the effector domain of Cdc42p.

The specificity of these interactions, particularly in relation to other Rho-type GTPases, was further examined by the two-hybrid system (Fields and Song 1989) (Table 1). Strong interactions were detected between the GTP-bound form of Cdc42p (G12V) and both Gic proteins, whereas no interaction was observed with the GDP-bound form of Cdc42p (D118A). Importantly, neither Bem1p nor any of the related Rho GTPases of yeast interacted with Gic2p, showing that Gic2p is a specific binding partner of Cdc42p. Mutating three conserved residues in the CRIB consensus sequence ($Gic2p^{crib-}$) to alanine residues abolished the interaction with Cdc42p, showing that the interaction between Gic2p and Cdc42p requires an intact CRIB domain.

Further evidence showing an in vivo interaction between the Gic proteins and Cdc42p is illustrated in Figure 2B. Overproduction of a Gic2p fragment ($Gic2p^{1-208}$), containing the CRIB domain, interferes in a dominant-negative fashion with cell growth. This growth defect was suppressed by overproduction of Cdc42p, but not by overproduction of $Cdc42p^{T35A}$, a related GTPase (Rho1p), proteins involved in cell polarity (Cdc24p or Bem1p), or the established Cdc42p target, Cla4p. The simplest interpretation of this result is that overproduction of the Gic2p fragment competes for active Cdc42p within the cell, thereby preventing proper regulation of endogenous Cdc42p targets. Additional Cdc42p overcomes this deficiency. The restoration of growth by

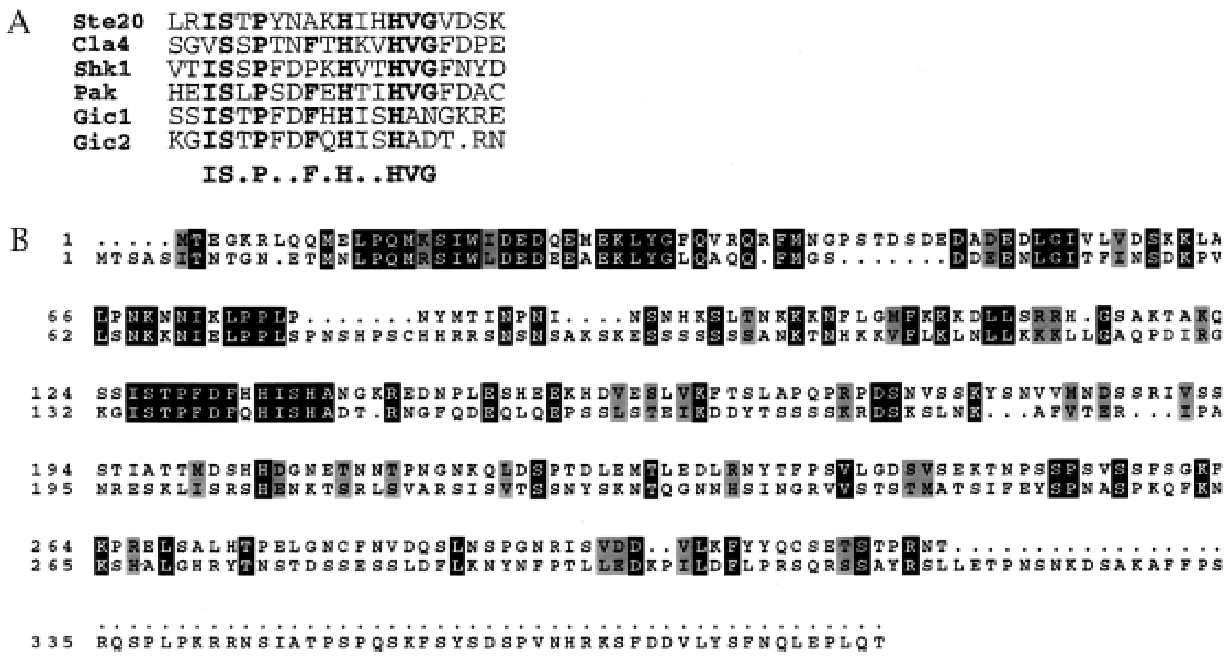

Figure 1. Gic1p and Gic2p, two novel Cdc42p binding proteins. (*A*) Alignment of the CRIB domains of Gic1p and Gic2p to several Ste20/PAK-related kinases. Consensus amino acid residues are shown in bold (Burbelo et al. 1995). Sequences are from the following sources: Ste20p and Cla4p (Leberer et al. 1992; Ramer and Davis 1993; Cvrckova et al. 1995), Shk1p (Markus et al. 1995), and PAK1 (Manser et al. 1994). (*B*) Alignment of Gic1p (*top*) and Gic2p. Identical amino acid residues are indicated with black boxes. Similar amino acids are shaded.

Cdc42p, but not by Cdc42p^{T35A}, supports this view and suggests that Gic2p binds the Cdc42p effector domain. Taken together, the in vitro binding data, two-hybrid analysis, and genetic suppression experiments show that Gic1p and Gic2p interact specifically with GTP-bound Cdc42p in vivo.

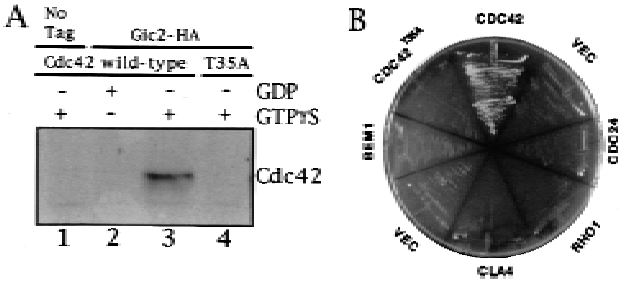

Figure 2. Gic1p and Gic2p bind Cdc42p-GTP. (*A*) Gic2p binds to Cdc42p-GTP. Sepharose beads containing purified yeast Gic2p were assayed for their ability to retain *E. coli* expressed Cdc42p preloaded with either GTPγS (lanes *1,3,4*), or GDP (lane *2*). (Lane *1*) Control beads. (Lanes *2–4*) Gic2-HAp beads. The following Cdc42 proteins were analyzed: (Lanes *1–3*) wild-type Cdc42p. (Lane *4*) Cdc42p^{T35A}. Cdc42p was detected by immunoblotting. (*B*) Overproduction of an amino-terminal fragment of Gic2p inhibits cell growth (overexpressed from the *GAL1* promoter). Cells accumulate with a large unbudded morphology. This growth defect could be suppressed by a high-copy-number plasmid carrying *CDC42*, but not by high-copy-number plasmids carrying no insert (VEC), *CDC24, RHO1, CLA4, BEM1,* or *CDC42^{T35A}*.

Gic2p is produced in a cell cycle-dependent manner

We examined whether the Gic proteins were present during the G_1 phase of the cell cycle when Cdc42p directs cell polarity. Temperature-sensitive *cdc15* cells were arrested in late mitosis and released from the cell cycle block by shifting the culture to the permissive temperature. As shown in Figure 3A, Gic2p accumulated throughout the G_1-phase and peaked at the time of polarity establishment (15 min prior to bud emergence; BE in Fig. 3A). Gic2p rapidly disappeared at the time of bud emergence, concomitant with the appearance of Cln2p (Fig. 3B). No Gic2 protein was detected in G_2 cells. In contrast, Cdc42p levels remained constant throughout the time course (data not shown; Ziman et al. 1993). We conclude that Gic2p is expressed in a cell cycle-dependent manner reaching maximal levels in late G_1, consistent with a role in the establishment of cell polarity.

Gic1p and Gic2p colocalize with Cdc42p to regions of polarized cell growth

Next, we determined the subcellular localization of the Gic proteins (Fig. 4). As axes of cell polarity are being established, Gic1p and Gic2p were found to be asymmetrically distributed in patterns similar to that of Cdc42p. During cell division, Gic1p and Gic2p localized

Table 1. *Two-hybrid interactions between the Gic proteins, Bem1p, and Rho-family GTPases*

DNA-binding domain fusion[a]	Activation domain fusion [b]	lacZ expression (Miller units)[c]
Cdc42p	vector	49
Cdc42p	Gic2p (full length)	1503
Cdc42p	Gic2p (1-208)	1616
Cdc42p	Gic1p (1-176)	1530
Cdc42p	Gic2p (Crib-)	50
Cdc42p (D118A) GDP bound	vector	9
Cdc42p (D118A)	Gic2p (full length)	11
Cdc42p (G12V) GTP bound	vector	151
Cdc42p (G12V)	Gic2p (full length)	1372
Rho1p	vector	22
Rho2p	vector	24
Rho3p	vector	18
Rho4p	vector	27
Rho1p	Gic2p (full length)	17
Rho2p	Gic2p (full length)	14
Rho3p	Gic2p (full length)	15
Rho4p	Gic2p (full length)	7
Bem1p	vector	8
Bem1p	Gic2p (full length)	12

[a]The LexA DNA binding domain fusions were carried on pEG202 and contain the carboxy-terminal Cys-Ser substitutions, which prevent prenylation (Stevenson et al. 1995).
[b]The activation domain fusions were carried on pJG4-5, and pJG4-5 provided the vector control.
[c]The average Miller units expressed of the *lexA-lacZ* reporter from three independent experiments are presented.

to the future bud site and later to the surfaces of small buds, as visualized by immunofluorescence microscopy (Fig. 4A). Cdc42p has been shown previously to localize to these same regions of polarized growth (Ziman et al. 1993). No localized Gic1p, Gic2p, or Cdc42p was observed in cells with medium to large buds; however, on occasion, some Cdc42p or Gic protein could be seen in the mother-bud neck very late in the cell cycle (data not shown). During mating, Gic1p, Gic2p, and Cdc42p colocalized to the tips of mating projections (shmoos) (Fig. 4B). Thus, Gic1p and Gic2p, together with Cdc42p, are located in regions that direct cytoskeletal polarization.

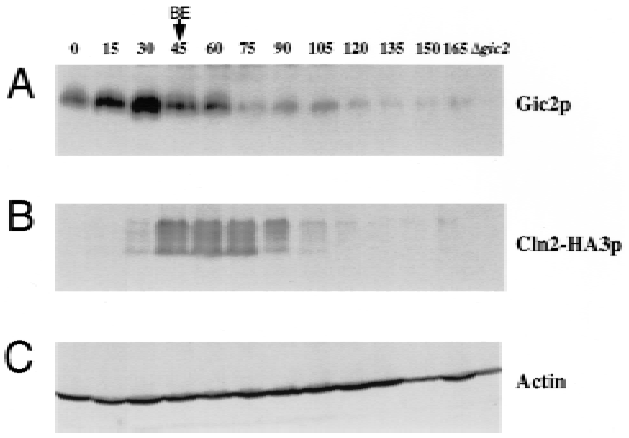

Figure 3. Gic2p is expressed in a cell cycle-dependent manner. Cells were synchronized by releasing temperature-sensitive *cdc15* cells from their block in late mitosis. Aliquots were collected every 15 min and analyzed by immunoblotting. The time of bud emergence is indicated as BE. (*A*) Gic2p; (*B*) Cln2p; (*C*) actin.

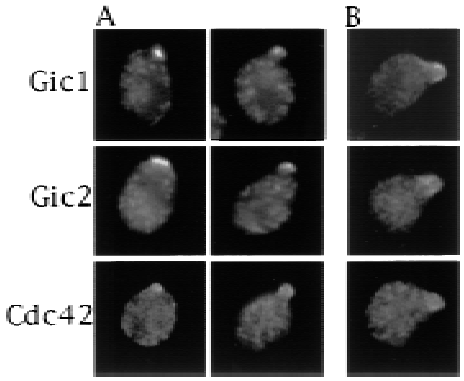

Figure 4. Gic1p and Gic2p colocalize with Cdc42p to regions of polarized growth. Epitope-tagged Gic1p, Gic2p, and Cdc42p, expressed from their native promoters on centromeric plasmids, were detected by indirect immunofluorescence. Gic1p, Gic2p, and Cdc42p proteins localize to the incipient bud site and the surfaces of small buds (*A*), and to the tips of polarized mating projections (*B*).

Gic1p and Gic2p are functionally redundant proteins necessary for cell polarization in vivo

To determine whether the Gic proteins play a role in cell polarization, we studied the effects of *GIC1* and *GIC2* null mutations. As Gic1p and Gic2p are closely related by sequence, it seemed likely that the two genes would be functionally redundant. Consistent with this prediction, strains carrying either the *GIC1* or *GIC2* deletion grew normally, but strains deleted for both *GIC1* and *GIC2* were slow growing at 23°C and 30°C, and were dead at 37°C (Fig. 5A, data not shown). In a population of double mutants at 30°C, >80% of the cells accumulated as large, unbudded, multinucleate cells (Fig. 5D,I). Examination of both actin and microtubules in these mutants showed that the morphological defects reflected an underlying deficiency in cytoskeletal polarization. Whereas wild-type cells and the single *gic* mutant strains budded and polarized actin normally (Fig. 5B,C; data not shown), *gic1Δ gic2Δ* double mutants were deficient in actin polarization with actin patches distributed randomly at the cell cortex and actin cables either absent or misaligned (Fig. 5E). Spindle alignment in *gic1Δ gic2Δ* cells was also aberrant (Fig. 5H). Thus, cells lacking Gic proteins exhibit defects in cell polarization similar to those observed in cells lacking Cdc42p function (Adams et al. 1990).

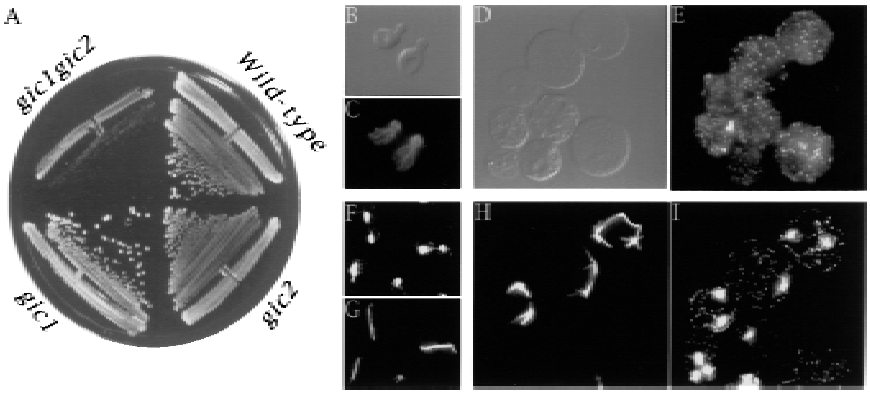

Figure 5. Gic1p and Gic2p encode redundant proteins required for efficient growth and cell polarization in vivo. (*A*) Strains harboring deletions in *GIC1* or *GIC2* grow at rates indistinguishable from the wild-type parental strain. Strains deleted for both *GIC1* and *GIC2* grow slowly at 30°C. (*B–I*) Wild-type (*B,C,F,G*) and *gic1Δ gic2Δ* cells (*D,E,H,I*) were observed by DIC (*B,D*) and epifluorescence microscopy (*C,E,F–I*). Actin (*C,E*), DNA (*F,I*), and microtubules (*G,H*) are shown.

Gic1p and Gic2p are required for polarized morphogenesis but not MAP kinase signal transduction during mating

Cdc42p has been implicated in both cell polarization and MAP kinase signal transduction during yeast mating (Zhao et al. 1995; Simon et al. 1995; Stevenson et al. 1995). To determine whether the Gic proteins play a role in signal transduction, we measured transcriptional induction and cell cycle arrest in response to mating pheromones, both readouts of MAP kinase signaling (Herskowitz 1995). When exposed to pheromone, the *gic1Δ gic2Δ* strains induced wild-type levels of the transcriptional reporter *FUS1*–LacZ (Trueheart et al. 1987) and were unaffected for cell cycle arrest (Fig. 6A,B). Thus, the Gic proteins are not required to activate the MAP kinase pathway during yeast mating. In contrast to wild-type cells, however, *gic1Δ gic2Δ* cells did not form polarized mating projections (shmoos) in response to mating pheromones (Fig. 6C) and, consequently, exhibited a 100-fold decrease in mating efficiency (Fig. 6D). Thus, Gic1p and Gic2p are unlikely to mediate any effect of Cdc42p on MAP kinase signal transduction, but they are important effectors of Cdc42p for the establishment of cell polarity during mating, as well as during vegetative growth.

Binding of Gic2p to Cdc42p is essential to Gic2p function in vivo

To assess the importance of Cdc42p binding for Gic2p function in vivo, we analyzed the function of a Gic2 protein containing CRIB domain mutations that abolish detectable Cdc42p interaction (Manser et al. 1994; Burbelo et al. 1995; Peter et al. 1996; Leberer et al. 1997) (Table 1). Although the mutant protein was produced at wild-type levels (Fig. 7B), the $gic2^{crib-}$ allele was unable to complement the growth defect of the *gic1Δ gic2Δ* double mutant (Fig. 7A), showing that binding of Gic2p to Cdc42p is essential for Gic2p function in vivo. Additionally, $Gic2^{crib-}$ protein failed to concentrate efficiently at sites of polarized growth and, instead, was distributed throughout the cytoplasm (Fig. 7C). Thus, the interaction between Cdc42p and Gic2p is required for proper Gic2p function and localization. Recently, it has been shown that Cdc42p also directs the localization of the protein kinase Ste20p (Peter et al. 1996; Leberer et al. 1997), suggesting that Cdc42p might generally target effectors to sites of polarized growth.

Genetic interactions between the GIC *genes and other factors involved in cell polarization*

In addition to Cdc42p, Bem1p and Bem2p are also in-

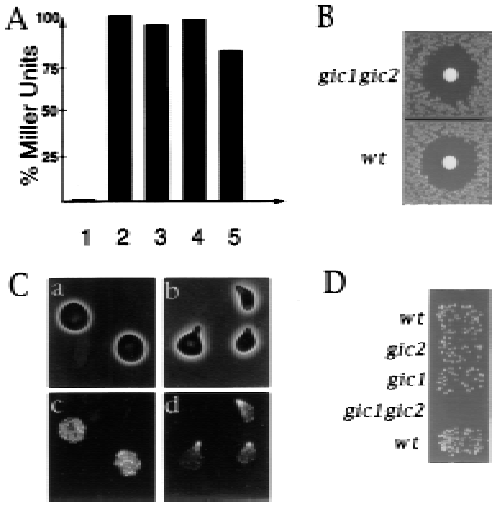

Figure 6. Gic1p and Gic2p are required for polarized morphogenesis but not for MAP kinase signaling in response to pheromones. (*A*) Activity of the MAP kinase signaling pathway triggered by pheromones was measured by induction of the *FUS1–LacZ* reporter, and plotted as percent Miller Units relative to wild-type controls. (Lane *1*) No α-factor added. (Lanes 2–5) α-factor added for 1 hr. The following strains were analyzed: (1 and 2) wild-type; (3) *gic1Δ;* (4) *gic2Δ;* 5: *gic1Δ gic2Δ*. (*B*) *gic1Δ gic2Δ* (*top* panel) and wild-type cells (*bottom* panel) are able to arrest their cell cycle in response to α-factor as assessed by halo assay. (*C*) *gic* double mutants (*a* and *c*), but not cells lacking *GIC2* (*b* and *d*), are defective for polarized morphogenesis in response to α-factor. (*a* and *b*) Phase contrast photographs; (*c* and *d*) actin visualized by rhodamine-phalloidin. (*D*) Mating efficiencies of wild-type and *gic* mutant strains to a *far1-c* tester (Valtz and Peter 1997).

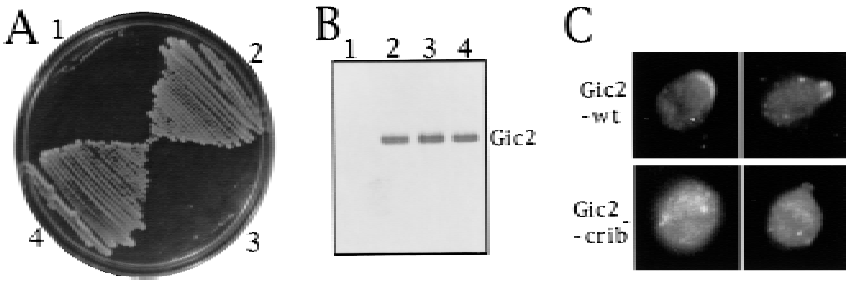

Figure 7. Interaction between Gic2p and Cdc42p is essential for Gic2p function and localization. (*A*) The growth defect of *gic1Δ gic2Δ* cells could be complemented by plasmids expressing wild-type *GIC2* but not *gic2*$^{crib-}$. (*1*) *gic1Δ gic2Δ* strain carrying vector. (*2*) *gic1Δ gic2Δ* carrying vector with a *GIC2* insert. (*3*) *gic1Δ gic2Δ* carrying vector with a gic2^{crib-} insert. (*4*) A *gic1* strain carrying no plasmid for comparison to sector *2*. (*B*) Wild-type and Gic2p^{crib-} proteins are present at equal levels. Lane numbers correspond to those of panel *A*. Gic2 protein was detected by immunoblotting with specific Gic2p-antibodies. (*C*) Asymmetric localization of Gic2p is dependent on a functional CRIB domain. Localization of epitope-tagged wild-type Gic2p (*top* panels) is contrasted with the localization of epitope-tagged Gic2p^{crib-} (*bottom* panels), both expressed from a high-copy-number plasmid.

volved in the establishment of cell polarity. Bem2p functions as a GAP for Rho-type GTPases (Zheng et al. 1993; Peterson et al. 1994), whereas Bem1p may provide a scaffold for several polarity establishment proteins. Because the phenotype of *gic1Δ gic2Δ* cells resembles that of cells lacking Bem1p and Bem2p, we tested whether overproduction of Gic1p and Gic2p could rescue the growth defect of these cells. We found that high-copy-number vectors carrying *GIC1*, but not *GIC2*, partially restored growth at 37°C to *bem1Δ* or *bem2Δ* cells (Fig. 8A, and data not shown). Conversely, a multicopy plasmid carrying *BEM1* was able to partially rescue the growth defect of *gic1Δ gic2Δ* cells (data not shown). These results further support the view that Gic1p and Gic2p are involved in polarity establishment.

Interestingly, the growth defect of *gic1Δ gic2Δ* cells could also be suppressed by overproduction of wild-type Cdc42p (Fig. 8B). Overproduction of Cdc42p in its GTP-bound form interfered with cell proliferation of *gic1Δ gic2Δ* cells, indicating that at least some of the lethal effects of Cdc42p-GTP are not dependent on the presence of the Gic proteins (Fig. 8B). Finally, multicopy plasmids carrying either *GIC1* or *GIC2* were not able to restore growth of a temperature-sensitive *cdc42* mutant (data not shown). The simplest interpretation of these genetic suppression results is that Cdc42p controls multiple targets that coordinate cytoskeletal polarization, and that overproduction of Cdc42p can compensate, at least partially, for the lack of some effectors.

Discussion

Gic1p and Gic2p are Cdc42p-specific effectors required for establishment of cell polarity

Our results identify two novel effectors of Cdc42p that are required for cellular polarization during polarized cell

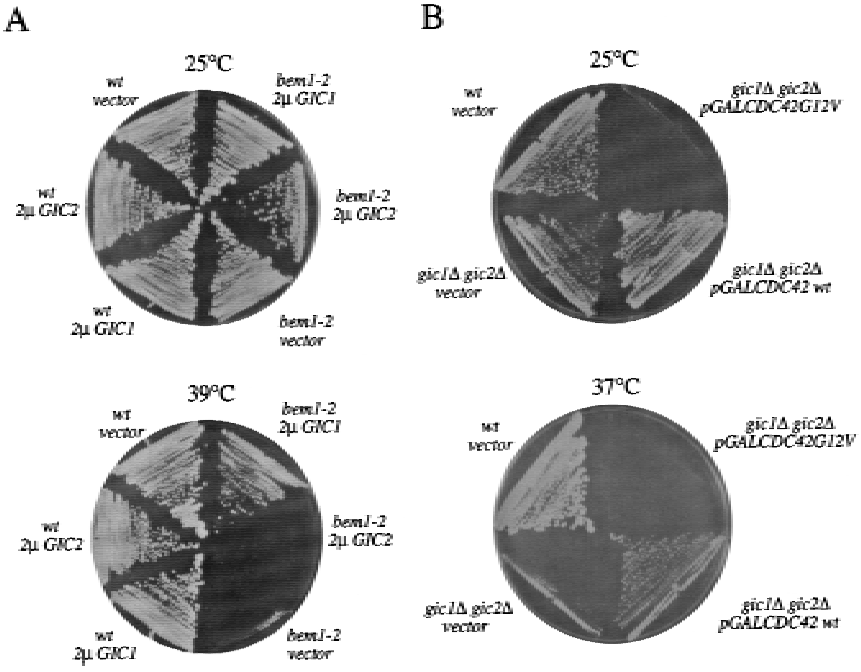

Figure 8. Genetic interaction between the Gic proteins, Bem1p, and Cdc42p. (*A*) Overexpression of Gic1p but not Gic2p is able to partially restore growth of cells lacking Bem1p function at 39°C. (*B*) Overexpression of wild-type Cdc42p but not Cdc42p in its active GTP-bound form (G12V) is able to suppress the growth defect of *gic1Δgic2* mutants at 37°C.

division and mating chemotropism. A number of other Cdc42p-interacting proteins have been identified previously, including PAK kinases, WASP-related factors, and formins (Symons 1996; Frazier and Field 1997). Although these molecules are suggested or shown to be Cdc42p targets, they do not appear to account for the effect of Cdc42p on cell polarization. Available evidence suggests that members of the PAK kinase family are involved in the activation of MAP kinase signaling cascades rather than affecting cytoskeleton polarization (Cvrckova et al. 1995; Joneson et al. 1996; Lamarche et al. 1996). WASP-related molecules are not necessary for actin polarization in yeast, although they do affect some aspects of cortical actin morphology (Li 1997; D. Mitchell and G.F. Sprague, pers. comm.). Finally, recent work has implicated Bni1p, a formin-related molecule, as a Cdc42p effector that contributes to actin polarization during mating (Evangelista et al. 1997). *BNI1*, however, is dispensable for polarization of actin during budding (Jansen et al. 1997), and furthermore, Bni1p does not bind specifically to Cdc42p but also interacts with the related GTPases Rho1p, Rho3p, and Rho4p (Evangelista et al. 1997; Imamura et al. 1997; Kohno et al. 1997).

In contrast, we have presented four lines of evidence that argue strongly that Gic1p and Gic2p are Cdc42p effectors critical for controlling cell polarity. First, Gic1p and Gic2p bind specifically to Cdc42p-GTP with no detectable affinity for Cdc42p–GDP or related Rho proteins. Second, Gic1p and Gic2p colocalize with Cdc42p to the future bud site or mating projection at times when cytoskeletal polarization is being established. Third, the cell polarization defects exhibited by *gic1Δ gic2Δ* cells are similar to those of *cdc42* mutants. Finally, and importantly, Gic2p function correlates with Cdc42p binding: Disruption of the Cdc42p–Gic2p interaction eliminates Gic protein function in vivo.

On the basis of these observations, we propose that during the cell cycle or in response to extracellular signals, Cdc42p is converted to the GTP-bound form in a spatially restricted manner. In turn, Cdc42p–GTP binds to Gic1p and Gic2p, which contribute toward cytoskeletal polarization (Fig. 9). It is unclear, at present, how Gic1p and Gic2p exert these effects, and the *GIC* sequences provide no obvious clues. Gic proteins may bind cytoskeletal elements directly, or they may link Cdc42p to key actin or microtubule-binding factors. Although no Gic homologs are yet known from other organisms, we consider it likely that Gic-like proteins link Cdc42p to cell polarity and cytoskeletal organization in higher eukaryotes, as, to date, all known Cdc42-binding proteins are conserved broadly.

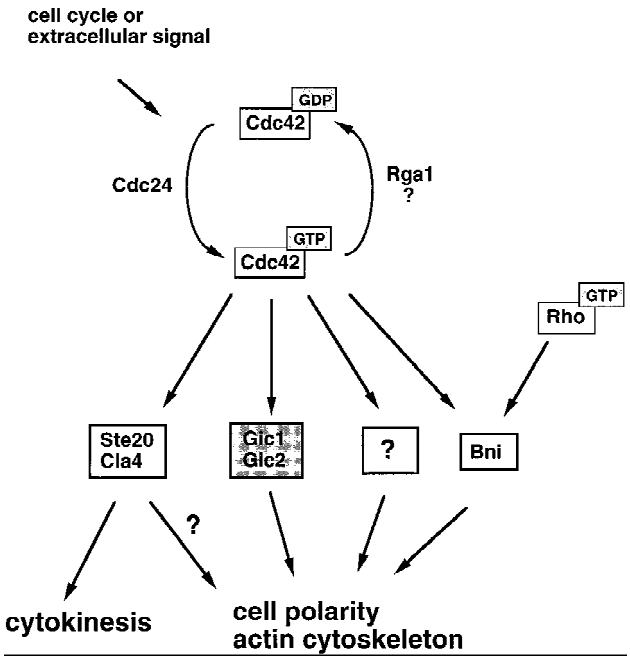

Figure 9. A pathway for cytoskeletal polarization mediated by Cdc42p in yeast. Cell cycle progression or extracellular signals lead to the conversion of GDP to GTP on Cdc42p, possibly by activation of the exchange factor Cdc24p. GTP–Cdc42p then binds and thereby localizes the effectors Gic1p and Gic2p to sites of polarized growth, which in turn contribute to polarization of the actin cytoskeleton. Cdc42p might exert some of its effects on the cytoskeleton not only through Gic1p and Gic2p but also through other targets including Bni1p, Ste20p, and Cla4p. Bni1p is known to interact also with other members of the Rho-GTPase family.

The effects of Cdc42p on the cytoskeletal organization and MAP kinase signaling are mediated through distinct effectors

Evidence presented here and elsewhere confirms the view that Cdc42p mediates its effects on the cytoskeleton and signal transduction pathways through distinct effectors. PAK-related kinases function upstream of MAP kinase signaling cascades (Leberer et al. 1992), and Cdc42p has been shown to bind and thereby activate these kinases in vitro (Manser et al. 1994; Martin et al. 1995; Simon et al. 1995). Available evidence, however, suggests that PAK-like kinases are not sufficient to account for cytoskeletal polarization mediated by Cdc42p, as a mutant form of Cdc42p that is unable to bind PAK kinases and fails to activate the JNK/SAPK kinase pathway fully promotes actin rearrangements when injected into fibroblasts (Joneson et al. 1996; Lamarche et al. 1996). Consistent with these observations, yeast cells lacking the two PAK-related kinases Ste20p and Cla4p are able to polarize their actin cytoskeleton (Cvrckova et al. 1995). In contrast, we show here that Gic1p and Gic2p are essential for cell polarization in yeast, but that Gic1p and Gic2p are dispensible for pheromone-induced signal transduction, indicating that these Cdc42p-targets are specific for mediating cytoskeletal rearrangements.

Cdc42p may regulate the cytoskeleton through multiple effectors

The evidence presented here firmly establishes that the Gic proteins are Cdc42p targets controlling cytoskeletal

polarization. The Gic proteins, however, are not likely to be the sole cytoskeletal effectors of Cdc42p. First, *cdc42* mutant cells are nonviable, whereas cells lacking both Gic1p and Gic2p are slow growing. Second, overexpression of either Cdc42p or Bem1p was able to at least partially restore the growth defect of *gic1Δ gic2Δ* cells, showing that increased levels of Cdc42p and Bem1p can reduce the need for the Gic proteins. In contrast, overexpression of either Gic protein failed to restore growth of cells lacking functional Cdc42p. Taken together, these results raise the possibility that Cdc42p and related GTPases direct cytoskeletal polarization, not through a single effector, but by signaling through a combination of effectors, each of which controls a parameter of actin or microtubule dynamics (Fig. 9). Together, Gic proteins and other factors would account for the essential role of Cdc42p in establishing axes of cell polarization.

Materials and methods

Genetic, recombinant, and database search methods

Standard yeast growth conditions and genetic manipulations were used (Rose and Fink 1990). Yeast transformations were performed by the lithium acetate method (Ito et al. 1983). Standard procedures were used for recombinant DNA manipulations (Sambrook et al. 1989; Ausubel et al. 1991). PCR reactions were performed by use of the Expand polymerase kit as recommended by the manufacturer (Boehringer Mannheim). Products were purified with the Wizard PCR purification kit according to the instructions of the manufacturer (Promega). Database searches were performed by use of the SGD (Stanford University) and the NCBI BLAST programs (National Institutes of Health).

GIC *cloning and deletions*

The *GIC1* and *GIC2* genes were cloned by PCR of genomic DNA from a haploid S288C-derived yeast strain (*MAT***a** *ade2-101, ura3-52, trp1-Δ1, his3Δ200, leu2Δ, lys2-801*) and the following oligonucleotides:

GIC1: oTP358 (5′-ATTTGCGGCCGCCGTCATCAGGAGTTCGAGGTCCAGAGGCATTGTTATCGG-3′) and oTP359 (5′-ATAAAGCTTCATTACGAGGAAACTATGGTGAAGATTACTGGG-3′); *GIC2:* oTP356 (5′-GCATTGCGGCCGCTTATAATTTTGGCGTTCAGCAAGCGCGCGG-3′) and oTP357 (5′-GTTAATTCGAAGATATAATAAACGAATGTATGGGATAACGCC-3′). An internal *Bam*H1–*Sph*I fragment of the *GIC1* gene was replaced with the *URA3* gene to generate the plasmid pMJ33. The *GIC2* deletion construct was produced by removing an internal *Spe*I–*Bam*HI fragment of the *GIC2* gene, and replacing it with the full length *LEU2* gene to generate the pBSK+ based plasmid, pMJ43. The resulting disruption constructs were excised from pMJ43 and pMJ33 and used to generate heterozygous diploid *gic2Δ::LEU2* and *gic1Δ::URA3* strains in the W303 background (K700: *MAT* **a**/α *ade2-1, trp1-1, leu2-3, 112, his3-11, 15, ura3, GAL, psi*$^{+}$) by single step gene replacement (Rothstein 1991). Haploid *gic1::URA3* and *gic2::LEU2* segregants of opposite mating type were obtained by sporulation and used to generate a diploid strain (YMP1053), which was then sporulated to isolate *gic1 gic2* double deletion mutants. Tetrads were dissected on YPD medium with 1 M sorbitol and grown at 25°C.

Construction of the crib^{-} *and truncation mutations of* GIC2

The *gic2*$^{crib-}$ allele was generated by mutating three consensus CRIB amino acids (I134, S135, and P137) to alanine residues by two-step PCR with the following oligonucleotide combinations: oTP356 and oTP434 (5′-GTGAAATATGTTGAAAATCAAATGCTGTGGCG-3′); oTP429 (5′-GGTGCCGCCACAGCATTTGATTTTCAACATATTTCAC-3′) and oTP357. The mutated Crib^{-} *GIC2* gene was sequenced, and subcloned into pJG4-5 for two hybrid experiments (generating pMJ177), as well as several other yeast vectors (pRS314, pRS424, YEp352; Sikorski and Hieter 1989).

The amino-terminal fragment of *GIC2* (amino acid residues 1–208) was amplified by PCR with oligonucleotides oTP409: (5′-ACTGGAATTCAATATGACTAGTGCAAGTATTACC-3′) and oTP423: (5′-ACGCTCGAGTCATAGTCTTGATGTCTTATTTTCGTGCG-3′), and subcloned into the two hybrid vector pJG4-5 (generating pMJ64) and a *GAL1* expression vector to generate pMJ127.

Construction of epitope-tagged Gic1p, Gic2p, and Cdc42p

Epitope-tagged versions of Gic1p and Gic2p containing the influenza hemagglutinin (HA) epitope (YPYDVPDYA) fused to the carboxyl terminus of each protein were constructed by use of PCR and the following oligonucleotide pairs: *GIC1:* (5′-CGCGAATTCGCGAAAAGACAACAAC-3′) and (5′-GCTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAGGTATTTCGAGGAGTACTAGTTTC-3′); *GIC2:* (5′-CGAGATCTAGATGTTGCCTATTTCTCG-3′), and (5′-GCTCTAGATTAAGCGTAGTCTGGGACGTCGTATGGGTAAGTTTGCAGGGGCTCGAGCTGG-3′). Isolated PCR products containing 300 bp of the endogenous *GIC1* and *GIC2* promoters were derived directly from SEY6210 genomic DNA and cloned into pRS313, pRS314, YEp351, and Yep352-based plasmids as *Eco*RI–*Xba*I or *Xba*I fragments. The integrity of the constructs was confirmed by sequencing and Western blotting. Plasmids expressing either HA–Gic1p or HA–Gic2p were found to fully complement both the growth and morphology defects associated with *gic1Δ gic2Δ* mutants. Construction of an amino-terminal epitope-tagged Cdc42p in a pRS315-based plasmid was generated by use of PCR and oligonucleotides (5′-CGGGATCCTATTAGCTCTTCCACAAAATGTACCCATACGACGTCCCAGACTACGCTCAAACGCTAAAGTGTGTTGTTGTCGG-3′) and (5′-GCTCTAGACGGGCATATACTAATATGACTACA-3′). PCR products were cloned into the pRS315 vector containing 500 bp of the endogenous *CDC42* promoter as *Bam*HI/*Xba*I fragments, and analyzed by Western blotting. Centromeric plasmids expressing HA–Cdc42p fully complemented a *cdc42::TRP1* disrupted strain in plasmid shuffle experiments.

Production of polyclonal Anti-Gic2p antisera

Polyclonal anti-Gic2p antibodies were generated against the full length Gic2p coding sequence cloned into pGEX-4T (Pharmacia) and expressed as a GST-fusion protein. Soluble GST–Gic2p, purified with glutathione Sepharose (Pharmacia) was used to immunize rabbits (Elevage Scientifique des Dombes, France). Antibodies were affinity-purified against GST–Gic2p as described (Harlow and Lane 1988). Standard procedures were used for yeast cell extract preparation and immunoblotting (Peter et al. 1993). Antibodies are specific to Gic2p as no signal is detected in extracts from *gic2Δ* cells (data not shown).

Cell synchronization

To release cells from a *cdc15-2* block, cells were grown to exponential phase in YPD medium and then shifted to 37°C for 2 hr. Cells were released by shifting the culture to 25°C, and

aliquots were taken at 15-min intervals. Protein extracts were prepared as described above. Cell cycle synchrony was monitored by fluorescence-activated cell sorting analysis and microscopic determination of the budding index.

Two-hybrid assays

Two-hybrid assays were performed in yeast strain EGY48 transformed with pEG202-based plasmids expressing LexA DNA-binding domain fusions, and pJG4-5-based plasmids containing transcriptional activation domain fusions (Gyuris et al. 1993). LexA–GTPase fusions all contain carboxy-terminal Cys to Ser substitutions that prevent prenylation. *LacZ* reporter activity was measured as described previously (Stern et al. 1984).

Cdc42p-binding assays

Gic2p affinity matrices were prepared as follows: Extracts of yeast expressing Gic2-HAp from the *GAL1* promoter were prepared in TNE450 buffer (450 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl at pH 7.5, 0.1% NP-40) and depleted of Gic2-HAp by use of 11HA monoclonal antibodies (Babco, Berkeley, CA) covalently coupled to protein G-Sepharose (Peter et al. 1996) (Pharmacia). Gic2-HAp columns were washed three times with TMT (10 mM Tris at pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 % Triton X-100) prior to Cdc42p binding. Cdc42p and Cdc42p^{T35A} proteins were produced in *Escherichia coli* strain NB42 as 6His fusion proteins by use of the pTrcHis vector (Invitrogen) and purified on Co^{2+} Sepharose-6B columns coupled with iminodiacetic acid (Sigma). Purified Cdc42p was preloaded with GTP-γS or GDP, as described (Park et al. 1993), and incubated for 1 hr at 4°C with affinity matrix in 200 µl of buffer B (10 mM Tris at pH 7.5, 85 mM NaCl, 6 mM $MgCl_2$, 10% glycerol) containing 0.6 mM GTP-γS, or 0.6 mM GDP. After four washes with TMT buffer, Cdc42p was eluted with 100 µl of gel sample buffer and detected by immunoblotting with Cdc42p-specific antisera (Peter et al. 1996).

Immunofluorescence

Actin and microtubule staining was performed on cells fixed and treated by standard methods (Pringle et al. 1991). Actin was visualized by use of rhodamine-conjugated phalloidin (Molecular Probes) at a concentration of 1 mg/ml, and microtubules were detected with the YOL1/34 monoclonal antibody (Accurate) and FITC-conjugated goat anti-rat secondary antibodies (Jackson Labs) at 1/1000 dilution. The DNA stain Hoechst (Sigma) was used at a final concentration of 0.01 mg/ml.

Immunofluorescence microscopy of the Gic proteins was performed in W303 cells on fully complementing carboxy-terminally HA epitope-tagged versions expressed under their own promoters in *gic1Δ gic2Δ* cells from the pRS313 or pRS314-based centromeric plasmids, respectively. Amino-terminally-tagged Cdc42p was expressed from its own promoter in the pRS315 plasmid in wild-type haploid strain 1241-2D (Chant and Hersowitz 1991). HA-tagged proteins were visualized (Pringle et al. 1991; Brown et al. 1994) with the 11 HA monoclonal (Babco) and CY3-conjugated goat anti-mouse secondary antibodies (Jackson Labs) at 1/1000 dilutions. Pheromone treated cells were exposed to 4 mg/ml of synthetic α-factor (Sigma) for 1.5 hr. For Figure 7C, HA-tagged Gic2p and Gic2p^{crib-} were produced from multicopy YEp351-based plasmids in a wild-type strain.

Mating assays

Mating assays were performed with the tester strains IH1793 (*MAT*α, *lys1*) and IH2625 (*MAT*α, *lys1, far1-c*) as described (Valtz and Peter 1997). Shmoo morphology was examined after addition of 10^{-6} M α-factor to 3 ml of log phase cultures for 3 hr at 25°C. For cell cycle arrest (halo) assays, 10^3–10^4 cells were plated on YPD–sorbitol plates (1M sorbitol). Ten micrograms of α-factor in 20 µl of 0.01 M HCl was spotted on a sterile filter disc (Schleicher and Schuell) and placed on plates, which were then incubated for 3 days at 25°C.

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