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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Since the inception of this project, a number of reports have highlighted different new and exciting aspects of the molecular biology of Bem1p, the scaffold protein in the yeast complex similar to the mammalian PIX complex. These include the discovery that the PX domain is a novel phosphoinositide binding domain, and the structure and characterization of the Cdc24p-interacting PB1 domain. These reports provided new insights into the molecular features of Bem1p. As a result, it became more urgent to explore previously unforeseen aspects of Bem1p function in the context of cell polarization. In this report I describe a series of very productive studies aimed at increasing our understanding of the actual molecular mechanism of the establishment of cell polarity. We demonstrate that the cytoskeleton (both actin and microtubules) is dispensable for the establishment of cell polarization, a surprising result that has broad implications for other cell types. Furthermore, we show that Bem1p is essential for polarity establishment in the absence of cortical cues. Capitalizing on our previous discoveries and work by others, we show that the SH3-2, PX, and PB1 domains are essential for this role. We also have preliminary evidence that PI binding may play a significant role in polarity establishment.

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Introduction.

We use the budding yeast *Saccharomyces cerevisiae* as an excellent model system to study actin cytoskeleton control, in particular the establishment of cell polarity, which is essential to understand cell motility and metastasis. In yeast, we have identified the existence of a multiprotein complex that is essential for the establishment of cell polarity, and which comprises exquisitely conserved proteins such as the GTPase Cdc42p, its exchange factor and activator Cdc24p (homologous to proto-oncogenes such as Dbl and Vav), its downstream effector Cla4p (homologous to p21-activated kinases, or PAKs), and the scaffold protein Bem1p (with regions of homology to many mammalian protein scaffolds). This protein complex, referred to here as the Bem1p multiprotein complex (BMC), is particularly interesting from the point of view of functional equivalents in mammalian cells. We realized that the PIX multiprotein complex described in the reports (Feng et al., 2002; Manser et al., 1998), containing Rac1, PIX and PAK, was analogous to the Bem1p-Cdc24p-Cdc42p-Cla4p complex in yeast (Bose et al., 2001; Gulli et al., 2000). We therefore hypothesized that by studying the latter complex in yeast we may be able to learn valuable lessons to guide a more directed study of the role of the PIX complex in mammalian cell motility.

Since the inception of this project, a number of reports have highlighted different new and exciting aspects of the molecular biology of Bem1p, the scaffold protein in the yeast complex. These include the discovery that the PX domain is a novel phosphoinositide (PI)-binding domain (Ago et al., 2001; Ellson et al., 2002; Ellson et al., 2001; Lu et al., 2002; Sato et al., 2001; Wishart et al., 2001; Xu et al., 2001; Yu and Lemmon, 2001), and the solution structure and characterization of the Cdc24p-interacting PB1 domain (Ito et al., 2001; Ponting et al., 2002; Terasawa et al., 2001). These reports provided new insights into the molecular features of Bem1p. As a result, it became more urgent to explore previously unforeseen aspects of Bem1p function in the context of cell polarization. In this report I describe a series of very productive studies aimed at understanding the actual molecular mechanism of the establishment of cell polarity. We demonstrate that the cytoskeleton (both actin and microtubules) is dispensable for the establishment of cell polarization, a surprising result that has broad implications for other cell types. Furthermore, we show that Bem1p is essential for polarity establishment in the absence of cortical cues. Capitalizing on our previous discoveries and work by others, we show that the SH3-2, PX, and PB1 domains are essential for this role. We also have preliminary evidence that PI binding may play a significant role in polarity establishment.

The studies reported here have enhanced our pursuit of Specific Aim #1 in the grant proposal: to characterize the role and regulation of the BMC. They provide a clear picture of the importance and role of the BMC in polarity establishment. The experiments proposed in the original proposal are complementary to these studies, and will be executed as a natural next step in our exploration of the role of this critical complex in polarity establishment and maintenance.

Experimental Results and Discussion.

Yeast cells divide following a well-defined spatial budding pattern (Pruyne and Bretscher, 2000). This budding pattern, different for diploid and haploid cells, requires a RAS-like GTPase, Rsr1p (Figure 1A). Rsr1p binds to GTP in a reaction catalyzed by its exchange factor Bud5p (Bender, 1993). In its GTP form, Rsr1p binds to Cdc24p, the exchange factor for Cdc42p (Park et al., 1997; Park et al., 2002). In this way, it is thought that Rsr1p recruits Cdc24p to the pre-bud site, which in turn leads to the generation of GTP-Cdc42p at that location on the cortex. This pool of GTP-Cdc42p is polarized, and leads to the polarization of actin. This polarized actin participates in polarized secretion, and bud emergence occurs.

If Rsr1p is responsible for recruiting Cdc24p to the cortex as the initial step in cell polarization, it would follow that in the absence of Rsr1p the cell would be unable to recruit Cdc24p, generate polarized GTP-Cdc42p, and make a bud. In other words, *rsr1*Δ cells should be inviable.

In fact, *rsr1*Δ cells are perfectly viable, and have no discernible phenotype other than the loss of the spatial budding pattern both in haploids and diploids (Ruggieri et al., 1992). Rsr1p appears to be important for regulating the spatial position of the bud site, but not its actual establishment and formation. The necessary conclusion is that Rsr1p is not essential for polarity and budding *per se*.

This left us with the question, 'How are Cdc24p and Cdc42p polarized in the absence of the cortical cues provided by the Rsr1p-dependent signaling module?' We hypothesized that the cytoskeleton, composed of actin filaments and microtubules, could in theory act as a Cdc42p-recruiting structure. The cytoskeleton establishes contacts with the cell cortex, and it was possible that in the absence of Rsr1p these sites acted as sites for Cdc24p or Cdc42p polarization by an unknown mechanism. To test this hypothesis I synchronized *rsr1*Δ *bud8*Δ cells in G1 by elutriation (so they were unbudded and depolarized), as shown in Figure 1B. The cells were released into media containing the actin-depolymerizing drug Latrunculin A and the microtubule-depolymerizing drug Nocodazole, or DMSO as a control. This way, the cytoskeletal structures in these cells were eliminated, and after different times samples were taken and processed for anti-Cdc42p indirect immunofluorescence, to measure polarization. Surprisingly, the cells polarized equally well in the drug-containing medium and the DMSO-containing control (Figure 1C). In parallel, a wild type strain exhibited the same behavior. From this experiment, we concluded that the cytoskeleton is not required for the establishment of cell polarity in the absence of bud site selection.

Next, we decided to take a genetic approach to identify factors that may be involved in the establishment of cell polarity in the absence of Rsr1p. We hypothesized that there may be a second pathway for polarity establishment when Rsr1p is not able to recruit Cdc24p. When *RSR1* is deleted, this pathway may become essential for polarity establishment. As a prediction, we would expect a mutation that inactivates this second pathway to cause lethality when combined with an *rsr1*Δ mutation, in other words, those two mutations should exhibit synthetic lethality.

In an attempt to identify factors involved in the second polarity establishment pathway, I evaluated genetic interactions between *rsr1*Δ and mutations in genes involved in polarity. The

method used to create these mutants was to delete one copy of the *BEM1* ORF in a homozygous diploid strain deleted for *RSR1*, *BUD5* or *BUD2*. Next, the strain was induced to sporulate and generate haploid cells bearing either one copy of wild-type *BEM1* or the deletion together with the second-site deletion. None of the double mutants was viable, indicating that Bem1p is essential when Rsr1p signaling is disrupted.

Of the panel of mutations tested, only one (*bem1*Δ), was synthetic lethal with *rsr1*Δ (Table 1). To confirm this result, the same test was performed with the regulators of Rsr1p, i.e. *bud2*Δ and *bud5*Δ, which also were synthetic lethal with *bem1*Δ. These results pointed to Bem1p as an important factor in the polarity establishment pathway. This is consistent with the severe morphological defects, arising from severe problems in polarity establishment, of *bem1*Δ cells.

cdc42-1	*cdc12-6*
cdc24-4	*spa2*Δ
*bem1*Δ	*msb1*Δ
*bem2*Δ	*cla4*Δ
*bem3*Δ	*bni1*Δ

Table 1. Mutations tested in combination with *rsr1*Δ to evaluate synthetic interactions.

We hypothesized that, since the BMC has a major role in cell polarization, it may have an essential role for the recruitment of Cdc24p and Cdc42p in the absence of Rsr1p. In order to test this hypothesis, I generated a series of conditional alleles of *BEM1* by random mutagenesis. This was necessary because placing the *BEM1* ORF under control of the regulatable GAL1 promoter did not provide a shutoff phenotype that was tight enough to evaluate the final phenotype, presumably owing to a prolonged half-life of the Bem1p polypeptide.

On the other hand, I was successful in generating a panel of temperature-sensitive alleles of *BEM1*. The *bem1-8* allele had a restrictive temperature of 39°C, at which *rsr1*Δ *bem1*Δ cells carrying the *bem1-8* allele on an episomal plasmid were inviable (Figure 2A). At the permissive temperature of 30°C, the cells were viable.

With these tools in hand, we decided to evaluate the terminal phenotype of these *bem1 rsr1* double mutants. The cells were grown in selective medium at the permissive temperature and synchronized in G1 to isolate an unbudded, depolarized population (Figure 2B). Half these cells were shifted to the restrictive temperature, keeping the other half at the permissive temperature. The cells carrying the wild-type allele of *BEM1* were able to polarize Cdc42p at both temperatures (Figure 2C). In contrast, the cells carrying the ts allele were unable to polarize at the restrictive temperature. At the permissive temperature, they were able to polarize with equal efficiency as the wild-type. This experiment suggests that the inability of *bem1 rsr1* double mutants to polarize Cdc42p is likely the reason for their inviability.

These data strongly suggest that Bem1p is required for the polarization of Cdc42p for budding. In *bem1*Δ cells, Rsr1p is presumably able to recruit Cdc24p in such a way that polarity still occurs; however, this is an inefficient mechanism and the cells have severe polarity defects.

In an *rsr1*Δ mutant, Bem1p is able to participate in the polarity mechanism, in spite of lacking the Rsr1p input to govern the spatial pattern of polarization.

Given these data, we were interested in characterizing which domains in the Bem1p structure are necessary for its role in polarization (Figure 3A). To test this, I generated a series of point mutants by site-directed mutagenesis. These mutations are predicted (and in some cases, have been shown) to disrupt the function of the SH3-2, PX and PB1 domains respectively (Bravo et al., 2001; Ito et al., 2001; Lu et al., 2002; Matsui et al., 1996; Terasawa et al., 2001).These mutant alleles were introduced into *bem1*Δ *rsr1*Δ cells that were kept alive with an URA3-marked episome carrying the wild-type *BEM1*. The mutant proteins were expressed at approximately the same levels as wild type Bem1p driven from the same vector, as assayed by western blotting (data not shown).

The cells were then forced to lose the URA3 plasmid by growth on media containing 5-FOA, to assay the viability of cells carrying the different point mutant alleles of *bem1*. We observed that P208L, which disrupts SH3-2, R316A, which disrupts PX, and K482A, which disrupts PB1, all caused the loss of viability of *bem1 rsr1* double mutants (Figure 3B). These results suggest that some ligand of the SH3-2 domain is required for the function of Bem1p. Known ligands include the Boi1p and Boi2p proteins (of unknown function) and the PAKs Ste20p (involved in mating pheromone signaling) and Cla4p (involved in morphogenesis, septin assembly, cytokinesis and cell polarity). Cla4p is a component of the BMC, which we characterized in a previous publication (Bose et al., 2001). Thus, we propose that PAKs may have a critical role in polarity establishment as part of the BMC. This is consistent with our finding that Cdc24p is a substrate for the kinase activity of Cla4p when present in the BMC. Other relevant substrates of Cla4p remain to be identified.

These results also suggest that some ligand of the PX domain may be important for polarity establishment. PX domains are known to bind to PI lipids and to SH3 domains. P355A is predicted to disrupt SH3 domain binding, and R369A is predicted to disrupt PI lipid binding. To our surprise, P355A was viable and R369A inviable in our test, indicating that SH3 domain binding is dispensable for polarity establishment but PI binding may be essential.

Finally, the results also indicate that binding to Cdc24p through the PB1 domain is essential for polarity establishment, thus suggesting that the integrity of the BMC is a critical requirement for polarity establishment in the absence of bud site selection.

We decided to reproduce previous reports of Bem1p binding to PI lipids. We expressed Bem1p as a recombinant protein fusion with the myc tag at the N-terminus in *E. coli*, and incubate PIP strips with the resultant protein extracts. Bem1p did indeed bind to PI(3)P as previously reported (Figure 3C). However, it appeared to bind equally well to PI(5)P and, perhaps with higher affinity, to $PI(3,5)P_2$. We concluded from this experiment that previous reports of Bem1p binding to PI(3)P were correct, though incomplete, and that the list of lipid binding partners should be enlarged to include PI(5)P and $PI(3,5)P_2$. We are currently working on generating the $Bem1p^{R369A}$ mutant protein to test our prediction that it may lack lipid binding activity.

Conclusions.

Based on the results presented above, we propose that the components of the BMC become polarized at the end of G1 phase in the cell cycle by a symmetry breaking mechanism. This model proposes the existence of local stochastic fluctuations in the assembly of subpopulations of BMCs, over the entire cortex of the cell (Figure 4A). When CDK/G1 cyclins start signaling at the end of G1, these local fluctuations become amplified by biochemical feedback loops and a single site on the cortex prevails on all others. In wild type cells, this process is aided by Rsr1p signaling, which effectively recruits the symmetry breaking mechanism to the right spot according to the budding pattern. In *rsr1*Δ cells, the process occurs at random on the cell cortex.

Data presented in the accompanying paper show that within the BMC there is a biochemical feedback loop (Figure 4B). Here, we show that Bem1p, a protein scaffold, is essential to the symmetry breaking mechanism (Figure 4C). Three domains in Bem1p are necessary for this function, the SH3-2, PX and PB1 domains.

Key research accomplishments:

- Publication showing the existence of multiprotein complexes containing Cdc42p, Cdc24p, Bem1p and Cla4p.
- Demonstration of direct phosphorylation of Cdc24p by Cla4p (see attached publication).
- Demonstration of synthetic lethality between *bem1*Δ and *rsr1*Δ, as well as its regulators, *bud5*Δ and *bud2*Δ.
- Isolation of a panel of *bem1*^{ts} mutations.
- Demonstration that the death phenotype of *rsr1*Δ *bem1*^{ts} double mutants is lack of polarity, indicating an essential role for Bem1p in the establishment of cell polarity in the absence of cortical markers.
- Generation and evaluation of functionality of a panel of single point mutants in Bem1p that affect residues that are extremely conserved throughout evolution, demonstrating a role in polarity establishment for the critical SH3-2, PX and PB1 domains.
- Demonstration of novel Bem1p lipid-binding properties.

Reportable outcomes:

- Publication of results in the Journal of Biological Chemistry (see attached article).
- Publication of results as a poster at the 2000 American Association for Cell Biology Meeting in San Francisco, California.
- Publication of results as a poster at the 2002 Yeast Genetics and Molecular Biology Meeting in Madison, Wisconsin.
- Presentation of abstract for poster/oral presentation at the 2002 American Association for Cell Biology Meeting in San Francisco, California.

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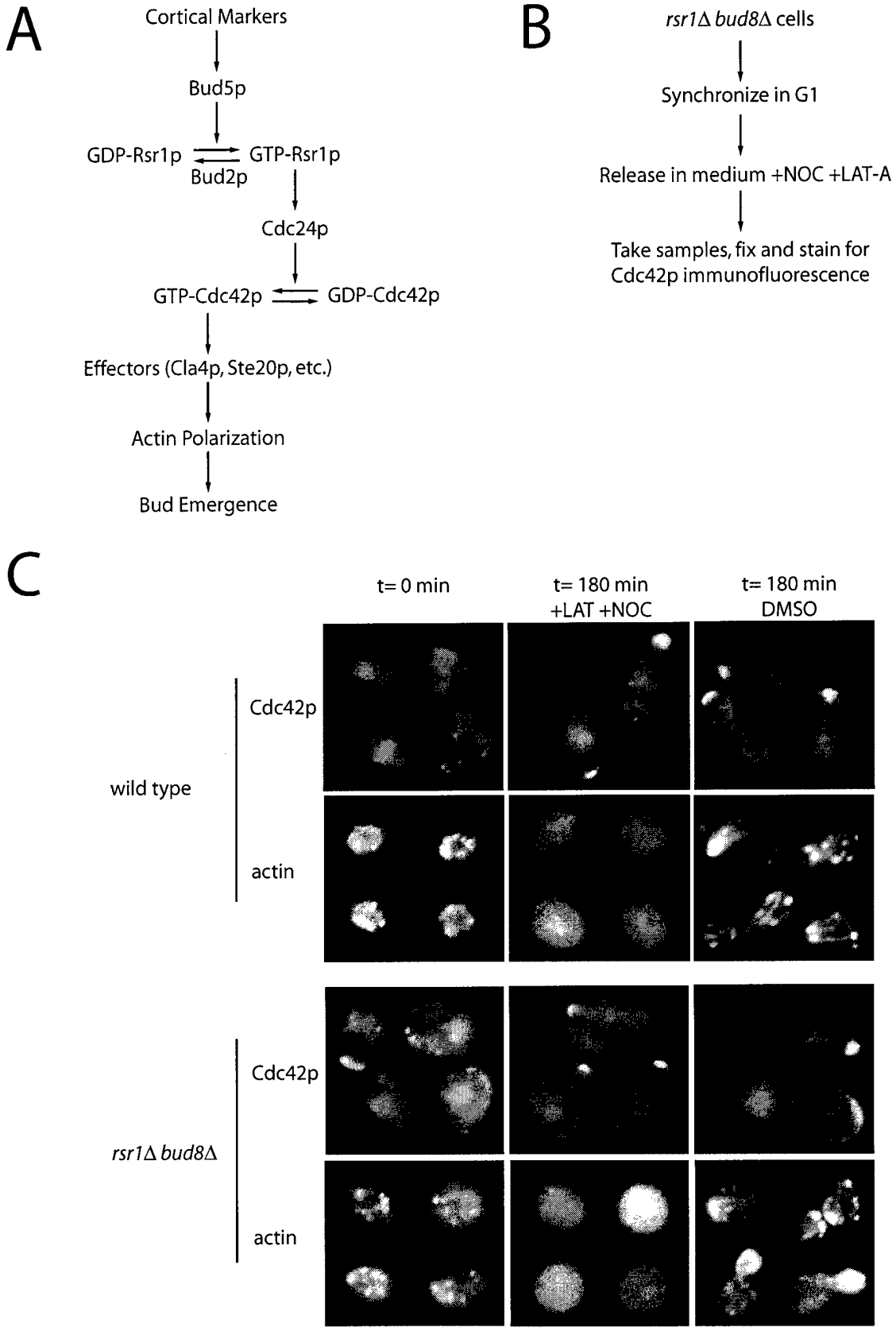

Figure 1. A. Schematic representation of the commonly held view of the series of events that lead to bud emergence in budding yeast. **B.** Schematic of the protocol followed to evaluate the role of the cytoskeleton in Cdc42p polarization in the absence of bud site selection. **C.** The *rsr1*Δ *bud8*Δ double mutants polarized Cdc42p in the presence of latrunculin and nocodazole as well as the wild type control. Approx. 45% polarized in both cases. LAT-A, latrunculin A. NOC, nocodazole.

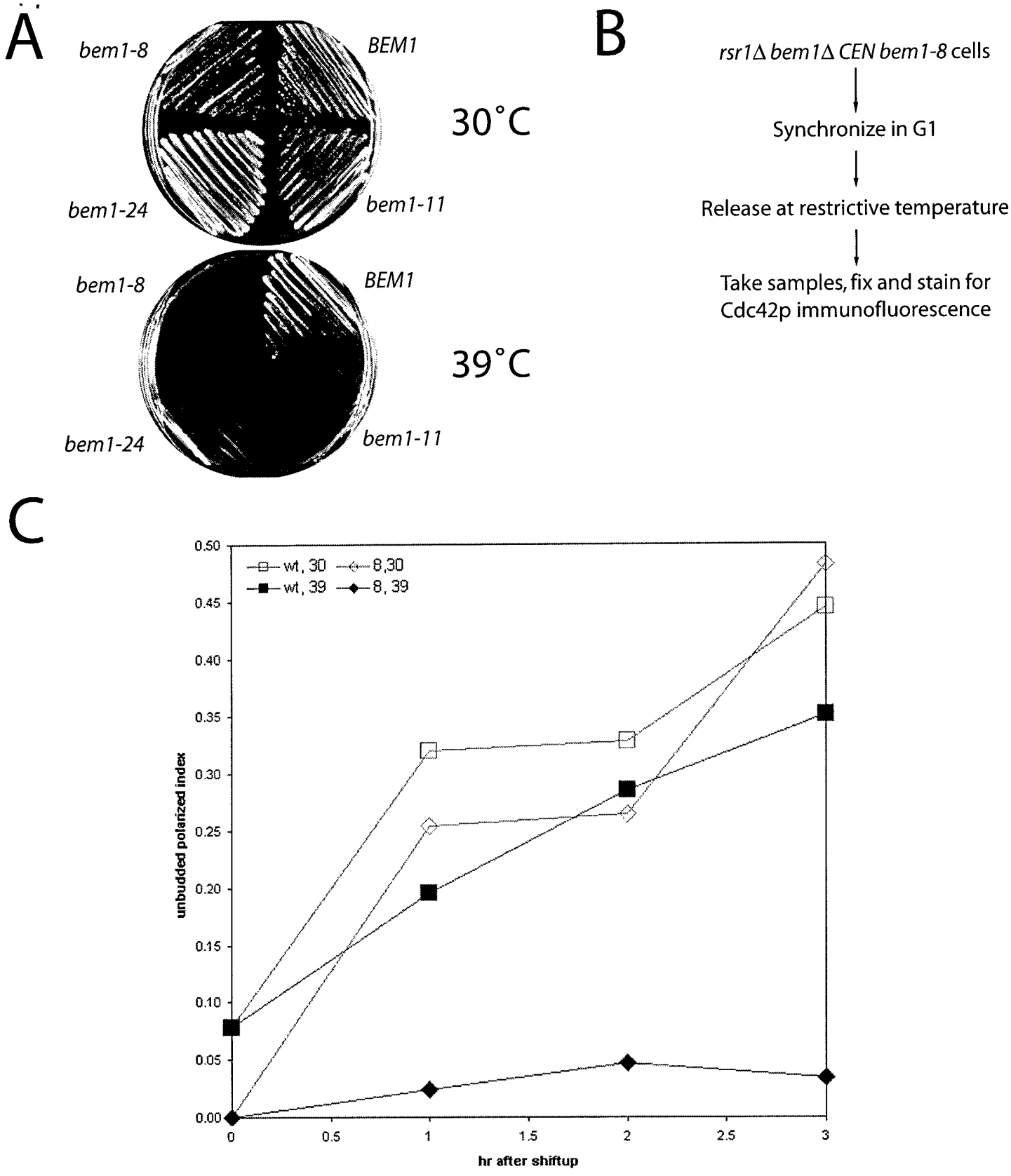

Figure 2. Bem1p is required for polarization in the absence of bud site selection. **A.** *bem1-8, bem1-11* and *bem1-24* are temperature-sensitive alleles of *BEM1*. *rsr1Δ bem1Δ* cells carrying the different alleles of *BEM1* on a *CEN* plasmid were plated on -LEU medium to select for the plasmid, and incubated at the permissive temperature of 30°C and the restrictive temperature of 39°C. **B.** Schematic diagram of the protocol followed to evaluate the terminal phenotype of *bem1 rsr1* double mutants. **C.** Cells carrying the ts allele of *BEM1* were unable to polarize Cdc42p at the restrictive temperature. Squares, wild type *BEM1*. Diamonds, *bem1-8*. Open symbols, 30°C. Filled symbols, 39°C.

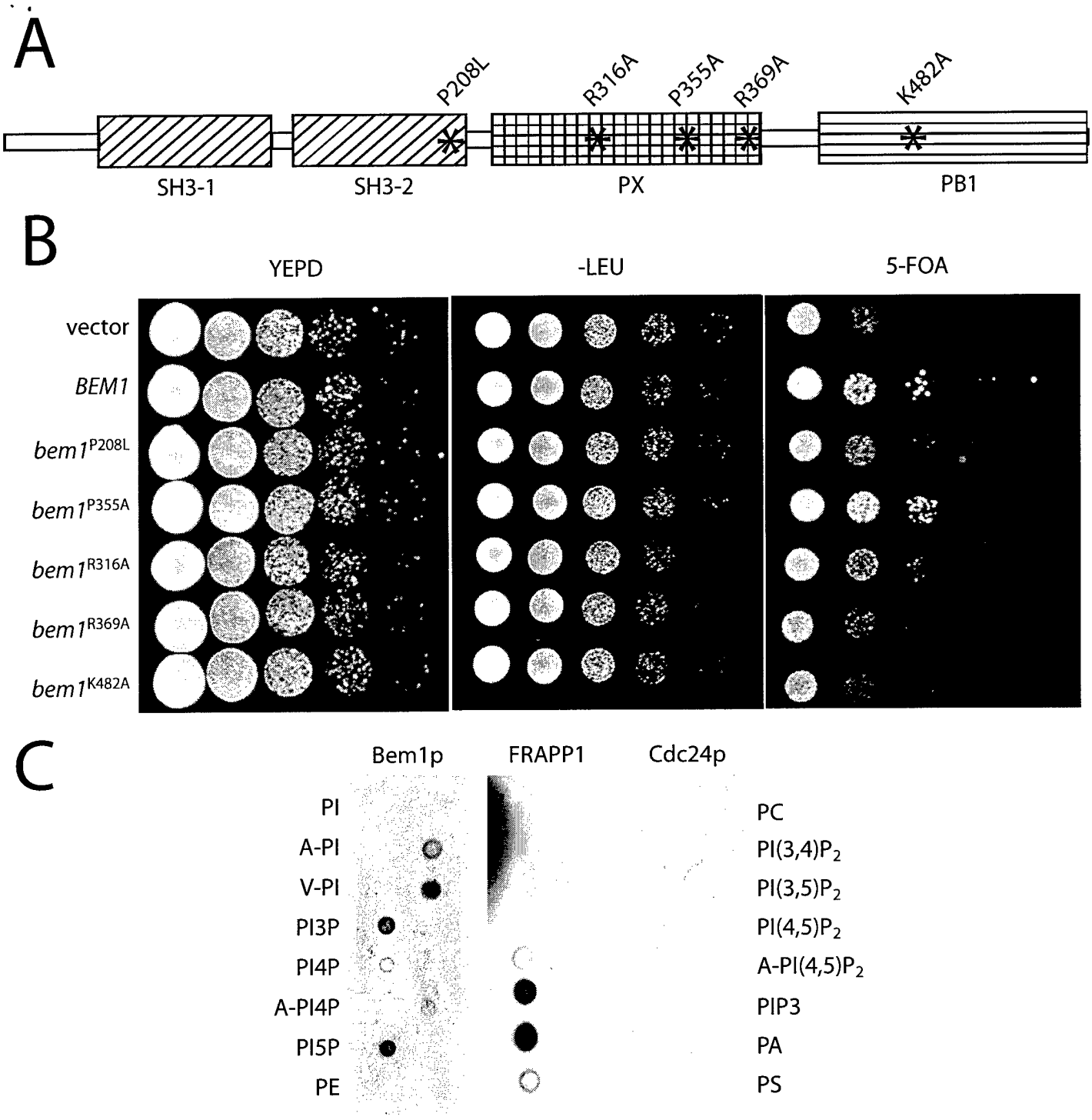

Figure 3. The SH3-2, PX and PB1 domains are necessary for polarization in absence of bud site selection. **A.** Domain structure of Bem1p. Indicated with asterisks are the point mutations created to evaluate domain function. **B.** Spot assays showing the domain requirements for viability of *rsr1 bem1* double mutant cells. Cells deleted for both *RSR1* and *BEM1*, and kept viable with a *URA3*-marked plasmid, were transformed with *LEU2*- marked plasmids bearing the different point mutant forms of *BEM1*. They were grown in -LEU liquid medium until log phase, diluted serially to plate known amounts of cells in each dot, and grown on different media for 3 days to evaluate viability. YEPD, rich medium. -LEU, medium lacking leucine. 5-FOA, medium to select for loss of the *URA3* plasmid bearing the wild type allele of BEM1. **C.** Bem1p binds to PI(3)P, PI(5)P and PI(3,5)P_2. As positive control, the PH domain from human FRAPP1 was known to bind PI(4)P and PI(5)P. As a negative control, Cdc24p did not bind to any lipid in this assay. PI, phosphatidylinositol, PE, phosphatidylethanolamine, PC, phosphatidylcholine, PA, phosphatidic acid, PS, phosphatidylserine. A- indicates animal origin, V- indicates plant origin.

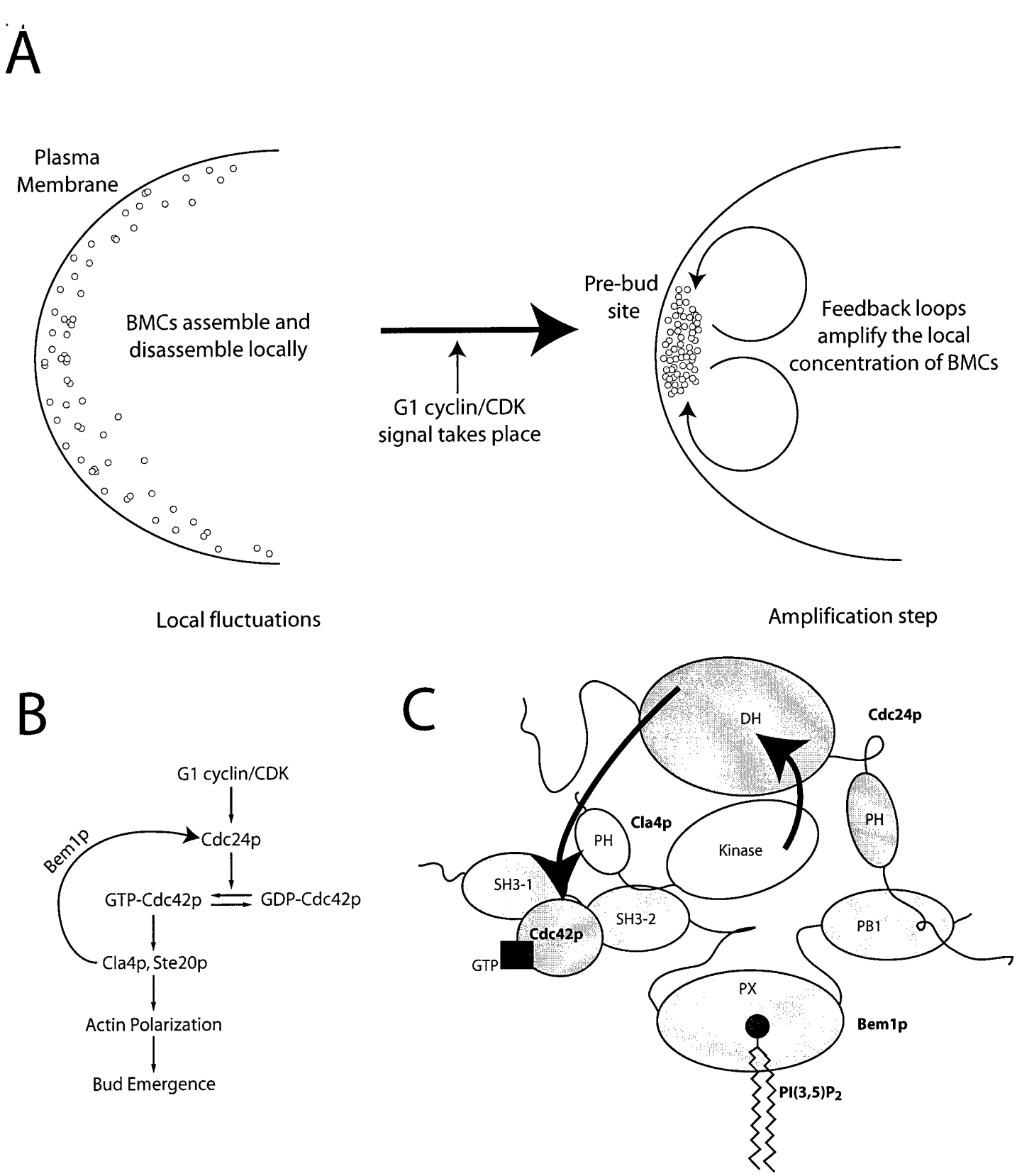

Figure 4. A. Schematic representation of the symmetry breaking model. In early G1 phase, BMCs assemble dynamically all over the cell cortex. These fluctuations become amplified when G1 cyclin/CDK signals the onset of polarization at the end of G1 phase., and lead to a single polarized spot by feedback mechanisms. **B.** The pathway leading to cell polarization, indicating the existence of a feedback loop from PAKs to Cdc24p, which depends on Bem1p. **C.** Schematic representation of the BMC, indicating the different domains that participate in each interaction. PH, pleckstrin homology domain. DH, Dbl homology domain. The role of lipid binding, although unknown, is important for symmetry breaking.

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Assembly of Scaffold-mediated Complexes Containing Cdc42p, the Exchange Factor Cdc24p, and the Effector Cla4p Required for Cell Cycle-regulated Phosphorylation of Cdc24p*

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In budding yeast cells, the cytoskeletal polarization and depolarization events that shape the bud are triggered at specific times during the cell cycle by the cyclin-dependent kinase Cdc28p. Polarity establishment also requires the small GTPase Cdc42p and its exchange factor, Cdc24p, but the mechanism whereby Cdc28p induces Cdc42p-dependent polarization is unknown. Here we show that Cdc24p becomes phosphorylated in a cell cycle-dependent manner, triggered by Cdc28p. However, the role of Cdc28p is indirect, and the phosphorylation appears to be catalyzed by the p21-activated kinase family member Cla4p and also depends on Cdc42p and the scaffold protein Bem1p. Expression of GTP-Cdc42p, the product of Cdc24p-mediated GDP/GTP exchange, stimulated Cdc24p phosphorylation independent of cell cycle cues, raising the possibility that the phosphorylation is part of a feedback regulatory pathway. Bem1p binds directly to Cdc24p, to Cla4p, and to GTP-bound Cdc42p and can mediate complex formation between these proteins *in vitro*. We suggest that Bem1p acts to concentrate polarity establishment proteins at a discrete site, facilitating polarization and promoting Cdc24p phosphorylation at specific times during the cell cycle.

The regulated reorganization of the actin cytoskeleton is a crucial element in the cell division cycle of proliferating cells and in the determination of cell shape and cell motility of nonproliferating cells. In recent years, it has become apparent that many signaling pathways that trigger actin reorganizations involve small GTPases of the Rho family (1). This family includes about 20% of the known Ras-related G proteins and can be subdivided into Rho, Rac, and Cdc42 subfamilies. *CDC42* was first identified as a gene required for the establishment of cell polarity in *Saccharomyces cerevisiae* (2), and subsequent studies showed that Cdc42p is also a key regulator of cell shape in a variety of other eukaryotes (1, 3–5). A prominent role of Cdc42p in all cases appears to be the polarization of cells toward a variety of signals (*e.g.* polarization of haploid yeast toward mating partners (6), of T cells toward antigen-presenting cells (7), or of fibroblasts toward wound sites (8)). Human and yeast *CDC42* are 80% identical and functionally interchangeable, suggesting that key functions of Cdc42p have been highly conserved (9, 10).

S. cerevisiae cells grow by budding, a process in which the rigid cell wall is locally expanded as a result of polarized secretion. Vectorial secretion involves delivery of secretory vesicles along polarized actin cables (11), which in turn are reorganized several times during the cell cycle (12), relocating growth zones to form a properly shaped bud. The actin reorganizations are triggered sequentially by changes in the activity of the cyclin-dependent kinase Cdc28p (13), which together with several cyclins acts as the master regulator of cell cycle progression in yeast (14). Bud emergence is a tightly controlled process that normally occurs at specific sites dictated by positional markers that are inherited by newborn cells (15, 16). After a period of uniform growth during G_1, activation of Cdc28p by G_1 cyclins triggers a dramatic polarization of many proteins as well as cytoskeletal elements toward the cortical site demarcated by the positional markers. Thus, a temporal signal (Cdc28p) allows a pre-existing cellular asymmetry (the positional markers) to be translated into a more general cytoskeletal asymmetry.

The isolation by Hartwell and co-workers (17) and Pringle and co-workers (2,18) of temperature-sensitive mutants that could not initiate bud formation at the restrictive temperature led to the identification of Cdc42p, along with Cdc24p and Bem1p, as critical regulators of cell polarization. Like most Ras superfamily members, Cdc42p exhibits slow intrinsic rates of GTP hydrolysis and GDP/GTP exchange that are accelerated by GTPase-activating proteins and GDP/GTP exchange factors, respectively. Cdc24p is a GDP/GTP exchange factor for Cdc42p (19), and the similarity between the phenotypes of *cdc24* and *cdc42* mutants suggests that Cdc24p is both responsible for and dedicated to the GTP loading of Cdc42p. Bem1p is a putative scaffold protein containing two SH3 domains, and *bem1Δ* mutants exhibit a less severe loss of cell polarity than *cdc42* or *cdc24* mutants, so that cells can proliferate (albeit poorly) in its absence (18, 20). Three putative GTPase-activating proteins for Cdc42p have also been identified, Bem3p, Rga1p, and Rga2p (21, 22) (see *Saccharomyces* genome data base for Rga2p), but their role in cell polarity remains unclear.

During early G_1, Cdc42p is distributed throughout the plasma membrane (and possibly internal membranes), but following Cdc28p activation a significant fraction of the Cdc42p in the cell is localized to a small patch of plasma membrane where

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TABLE I
Yeast strains used in this study

All strains are in the BF264–15Du (71) background (*ade1 his2 leu2–3, 112 trp1–1 ura3Δns*) unless indicated otherwise.

Strain	Relevant genotype
DLY1	a *bar1*
DLY5	a/α
DLY104	a/α *cdc28–4/cdc28–4*
DLY222	a *bar1 cln1 cln2 cln3 GAL1p-CLN3::TRP1*
DLY681	a/α *cdc42–1/cdc42–1*
DLY2727	a/α *leu2::GAL1p-CDC42$^{Q61L/Y40C}$::LEU2/leu2*
DLY2728	a/α *leu2::GAL1p-CDC42^{D57Y}::LEU2/leu2*
DLY2752[a]	a *bar1 HA-CDC24::URA3 GAL1p-CDC42^{Q61L}::LEU2*
DLY2767[a]	a/α *HA-CDC24::URA3 MYC-CLA4::LEU2*
DLY2768[a]	a *bar1 HA-CDC24::URA3 MYC-CLA4::LEU2 GAL1p-FLAG-CDC42::TRP1*
DLY2769[a]	a *bar1 HA-CDC24::URA3 MYC-CLA4::LEU2 GAL1p-FLAG-CDC42^{Q61L}::TRP1*
DLY2770[a]	a *bar1 HA-CDC24::URA3 MYC-CLA4::LEU2 GAL1p-FLAG-CDC42^{D57Y}::TRP1*
DLY2771[a]	a *bar1 HA-CDC24::URA3 GAL1p-FLAG-CDC42::TRP1*
DLY2772[a]	a *bar1 HA-CDC24::URA3 GAL1p-FLAG-CDC42^{Q61L}::TRP1*
DLY2773[a]	a *bar1 HA-CDC24::URA3 GAL1p-FLAG-CDC42^{D57Y}::TRP1*
DLY2781	a *bar1 bem1::URA3::BEM1::TRP1 GAL1p-CDC42^{Q61L}::LEU2*
DLY2782	a *bar1 bem1::URA3::bem1-CΔ35::TRP1 GAL1p-CDC42^{Q61L}::LEU2*
DLY2825	a *bar1 cla4::TRP1 GAL1p-CDC42^{Q61L}::LEU2*
DLY2826	a *bar1 ste20::TRP1 GAL1p-CDC42^{Q61L}::LEU2*
DLY2827	α bem1::URA3 GAL1p-CDC42^{Q61L}::LEU2
DLY2844	a *bar1 bem1::URA3::bem1-Δ142–199::TRP1 GAL1p-CDC42^{Q61L}::LEU2*
DLY3003	a *bar1 GAL-CDC42^{Q61L}::LEU2*
DLY3004	a/α *cdc28–4/cdc28–4 GAL1p-CDC42^{Q61L}::LEU2/leu2*
DLY3006	a/α *GAL1p-CDC42^{Q61L}::LEU2/leu2*
DLY4000[a]	a *bar1 BEM1-MYC::HIS3*
DLY4246[a]	a *bar1 HA-CDC24::URA3*
DLY4248[a]	a *bar1 HA-CDC24::URA3 BEM1-MYC::HIS3*
DLY4294[a]	a *bar1 BEM1-MYC::HIS3 GAL1p-CDC42::LEU2*
DLY4298[a]	a *bar1 HA-CDC24::URA3 BEM1-MYC::HIS3 GAL1p-CLB1Δ152::LEU2*
DLY4299[a]	a *bar1 HA-CDC24::URA3 BEM1-MYC::HIS3 GAL1p-CDC42::LEU2*
DLY4300[a]	a *bar1 HA-CDC24::URA3 BEM1-MYC::HIS3 GAL1p-CDC42^{Q61L}::LEU2*
DLY4301[a]	a *bar1 HA-CDC24::URA3 BEM1-MYC::HIS3 GAL1p-CDC42^{D57Y}::LEU2*
DLY4308[a]	a *bar1 HA-CDC24::URA3 BEM1-MYC::HIS3 GAL1p-SWE1::LEU2*
DLY4554	a *bar1 GAL1p-FLAG-CDC42::TRP1*
DLY4556	a *bar1 GAL1p-FLAG-CDC42^{Q61L}::TRP1*
DLY4738	a *bar1 HA-CLA4::LEU2*
DLY4739	a *bar1 MYC-CLA4::LEU2*
DLY4741[a]	a *bar1 BEM1-MYC::HIS3 HA-CLA4::LEU2*
DLY4781[a]	a *bar1 BEM1-MYC::HIS3 GAL1p-FLAG-CDC42::TRP1*
JMY1–12	a *bar1 ade1 his3 leu2–3,112 trp1–1 ura3Δns*
Y147[b]	a *cdc24–4*

[a] Indicated strains are in the JMY1–12 background, made by backcrossing *his3* and *HIS2* into BF264–15Du at least five times.
[b] Gift from J. Pringle (72).

the bud will form, together with Cdc24p and Bem1p (23–25). Cdc42p then appears to recruit a number of effectors to that site, promoting polarization of the actin cytoskeleton, assembly of a ring of septins, and targeting of secretion toward that site (see Refs. 24 and 26 for reviews). These observations suggested that Cdc28p promotes cell polarity through modulation of Cdc42p and its regulators. To elucidate these events, we undertook an analysis of the regulation of Cdc24p through the cell cycle. We found that Cdc24p underwent a cell cycle-dependent phosphorylation that appeared to occur in complexes containing Bem1p, GTP-Cdc42p, and the p21-activated kinase (PAK)[1] Cla4p.

EXPERIMENTAL PROCEDURES

Yeast Media and Cell Synchrony—Yeast media (YEPD-rich medium, synthetic complete medium (SC) lacking specific nutrients, and sporulation medium) have been described previously (27). YEPG and YEPS are as YEPD but with 2% galactose or sucrose instead of dextrose. Centrifugal elutriation to isolate early G_1 cells (13) and G_1 cyclin deprivation arrest/release (28) were performed as described previously.

Strains, Plasmids, and PCR Manipulations—Standard media and methods were used for plasmid manipulations (29) and yeast genetic manipulations (27). The *S. cerevisiae* strains used in this study are listed in Table I, and the plasmids are listed in Table II.

For expression of wild-type or GTP-locked mutant Cdc42p in yeast, *GAL1p-CDC42* and *GAL1p-CDC42^{Q61L}* constructs (30) (kind gift of D. Johnson) were cloned from pRS315 into pRS305 (31), yielding pDLB377 and pDLB378, and digested at the unique *Bst*EII site to target integration at *LEU2*. An identical strategy was used to express the GDP-locked (or nucleotide-free) *CDC42^{D57Y}* allele (7)[2] using pMOSB29 and to express the double mutant *CDC42$^{Y40C/Q61L}$* (32) allele using pMOSB61.

Various plasmids containing *BEM1* sequences were derived from the two-hybrid plasmid pDLB1010, which contains a *BEM1* PCR product amplified from yeast genomic DNA by PCR using MapPairs oligonucleotides for YBR200w (Research Genetics, Inc., Huntsville, AL) followed by a second PCR using oligonucleotides BD70F and BD70R (33), allowing gap repair into pOBD.CYH.

To create the *BEM1Myc::HIS3* allele, a 0.3-kb fragment encoding the last 100 residues of the *BEM1* open reading frame was amplified by PCR using pDLB1010 as template and the oligonucleotides 5′-GCTCTAGACGATACAGCAACCTTTGCAACAGC-3′ (*Xba*I site underlined) and 5′-GCGCGTCGACAATATCGTGAACGGAAATTTTCAG-3′ (*Sal*I site underlined). This PCR product was digested with *Xba*I and *Sal*I and inserted into *Xba*I/*Sal*I-digested pRS306-*GAL:SWE1Myc* (34), thus replacing the entire *SWE1* open reading frame and upstream sequences with the C-terminal *BEM1* fragment, fusing the end of *BEM1* with the 12 Myc tags in the plasmid, followed by the 3′-untranslated region of *SWE1*. The *Xba*I/*Bam*HI fragment containing *BEM1*, Myc, and 3′-untranslated sequences was then cloned into the corre-

[1] The abbreviations used are: PAK, p21-activated kinase; PCR, polymerase chain reaction; kb, kilobase pair; HA, hemagglutinin; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

[2] Gladfelter, A. S. Moskow, J. J. Zyla, T. R., and Lew, D. J. (2001) *Mol. Biol. Cell*, in press

TABLE II
Plasmids used in this study

Plasmid name	Vector	Insert	Source or ref.
pBB130ΔCK		*CLA4*	37
pCR42–3/5		*CDC42*	32
pDLB377	pRS305	*GAL1p-CDC42*	This study
pDLB378	pRS305	*GAL1p-CDC42^{Q61L}*	This study
pDLB678	2 μm *URA3*	*BEM1*	Alan Bender
pDLB722	2 μm *URA3*	*CLA4*	Erfei Bi
pDLB1004	pRS303	*BEM1 C-term-myc*	This study
pDLB1010	pOBD.CYH	*BEM1*	This study
pDLB1033	pUNI10	*CDC42*	This study
pDLB1034	pUNI10	*CDC42^{Q61L}*	Footnote 2
pDLB1035	pUNI10	*CDC42^{D57Y}*	Footnote 2
pDLB1189	pUNI10	*BEM1*	This study
pDLB1190	pUNI10	*bem1-NΔ234*	This study
pDLB1191	pUNI10	*CDC24*	This study
pDLB1193	pHB2-GST	*GST-BEM1*	This study
pDLB1234	pHB1-myc3	*myc-CDC42^{D57Y}*	Footnote 2
pDLB1238	pHB1-myc3	*myc-CDC42^{Q61L}*	Footnote 2
pDLB1309	pHB2-GST	*GST-CDC42^{Q61L}*	Footnote 2
pDLB1311	pHB2-GST	*GST-CDC42^{D57Y}*	Footnote 2
pDLB1325	pUNI10	*bem1-NΔ140*	This study
pDLB1473	YIplac204	*GAL1p*	This study
pDLB1485	pDLB1473	*GAL1p-FLAG-CDC42*	This study
pDLB1486	pDLB1473	*GAL1p-FLAG-CDC42^{Q61L}*	This study
pDLB1487	pDLB1473	*GAL1p-FLAG-CDC42^{D57Y}*	This study
pDLB1531	YIpLac128	*CDC42p-HA*	This study
pDLB1535	YIpLac128	*CDC42p-myc*	This study
pDLB1569	pUNI10	*CLA4*	This study
pDLB1576	pUNI10	*bem1-CΔ35*	This study
pDLB1627	pHY186	*CDC42p-HA-CDC24*	This study
pDLB1748	pDLB1531	*CDC42p-HA-CLA4*	This study
pDLB1749	pDLB1535	*CDC42p-myc-CLA4*	This study
pDLB1861	pHB1-myc3	*CLA4 CRIB*	Footnote 2
pDLB1862	YIplac204	*BEM1*	This study
pDLB1863	YIplac204	*bem1-CΔ35*	This study
pDLB1881	YIplac204	*bem1-Δ142–199*	This study
pEG-CDC24	pEG-KT	*CDC24*	24
pHB1-myc3			36
pHB2-GST			36
pHY171	pRS304	*GAL1p-FLAG-LoxP*	This study
pHY186	pRS306	*CDC42p-HA-LoxP*	This study
pMOSB29	pRS305	*GAL1p-CDC42^{D57Y}*	This study
pOBD.CYH			Becky Drees
pRS303			31
pRS305			31
pUNI-10			36
YIpLac128			35
YIpLac204			35

sponding sites of pRS303 (31), yielding pDLB1004. Digestion at the unique *Sty*I site within the *BEM1* sequence was used to target integration at the *BEM1* locus, appending 12 Myc epitopes to the full-length Bem1p. *BEM1Myc::HIS3* cells were similar to wild-type cells in terms of growth rate, cell morphology, and actin organization, indicating that Bem1p-Myc is fully functional.

For N-terminal epitope tagging of Cla4p, we first constructed the vectors pDLB1531 (HA tag) and pDLB1535 (Myc tag). These vectors have a YIplac128 (35) backbone, a 336-base pair *CDC42* promoter fragment from pCR42–3/5 (32), a start codon with a good Kozak consensus sequence, 12 tandem Myc or HA epitope tags, and *Nco*I-, *Nde*I-, and *Sac*I-cloning sites (detailed map available on request). A 540-base pair fragment encoding the Cla4p N-terminal 180 residues was amplified by PCR using yeast genomic DNA as template and the oligonucleotides 5′-CCGGCCATGGAAATGTCTCTTTCAGCTGCAGCG-3′ (*Nco*I site underlined) and 5′-AAAGAGCTCCCTTAGCAAAAATGGCG-3′ (*Sac*I site underlined). This fragment was digested with *Nco*I and *Sac*I and cloned into the corresponding sites of pDLB1531 and pDLB1535, generating pDLB1748 and pDLB1749, respectively. These plasmids were digested at the unique *Spe*I site within the *CLA4*-coding sequences to target integration at the *CLA4* locus, thereby generating a *CDC42* promoter-driven N-terminally tagged full-length *CLA4*.

For expression of other epitope-tagged genes and gene fragments in yeast or bacteria, we first cloned the relevant genes into the "univector" pUNI-10 (36). *CDC24* was excised from plasmid pEGKT-CDC24 (24) as a 2.6-kb *Bam*HI-*Sal*I fragment with the *Bam*HI site filled in with Klenow DNA polymerase and was cloned into pUNI-10 digested with *Nde*I and *Sal*I (with the *Nde*I site filled in with Klenow DNA polymerase), yielding pDLB1191. *CLA4* was excised from plasmid pBB130ΔCK (37) as a 2-kb *Eco*RV fragment and cloned into pUNI-10 digested with *Nde*I and *Eco*RV (with the *Nde*I site blunted with mung bean nuclease), yielding pDLB1569. This lacks the N-terminal 10 amino acids of *CLA4*. *BEM1* was excised from plasmid pDLB1010 as a 1.7-kb *Eco*RI-*Sma*I fragment with the *Eco*RI site filled in with Klenow DNA polymerase and was cloned into pUNI-10 digested with *Nde*I and filled in with Klenow DNA polymerase, yielding pDLB1189. pDLB1325 (*bem1-NΔ140*) contains C-terminal sequences starting at the *Bam*HI site of *BEM1* cloned into pUNI-10 (this construct lacks the N-terminal 140 residues, including the first SH3 domain). pDLB1190 (*bem1-NΔ234*) contains C-terminal sequences starting at the *Hpa*I site of *BEM1* cloned in pUNI-10 (this construct lacks the N-terminal 234 residues, encompassing the first and second SH3 domains). pDLB1576 (*bem1-CΔ35*) contains N-terminal sequences extending to the *Dra*I site of *BEM1* cloned in pUNI-10 (this construct lacks the C-terminal 35 residues, encompassing the Cdc24p-binding domain). All constructs were sequenced to confirm in-frame fusion with vector sequences and absence of additional mutations resulting from PCR manipulations.

For expression of HA-tagged Cdc24p in yeast, pDLB1191 was recombined with the host vector pHY186 *in vitro* with Cre recombinase (36). This vector was generated by first cloning the *CDC42* promoter (from

pCR42–3/5 (32)) into pRS306 (31) and then cloning sequences encoding three tandem HA tags and the lox recombinase target site (from pTag-lox (36)) downstream of the promoter (detailed map available on request). The recombined plasmid, pDLB1627, was digested at the unique *Swa*I site within the *CDC24* sequence to target integration at the *CDC24* locus.

For expression of FLAG-tagged Cdc42p in yeast, univector plasmids pDLB1033 (wild type), pDLB1034 (Q61L), and pDLB1035 (D57Y) (32)[2] were recombined with the host vector pHY171 *in vitro* with Cre recombinase (36). pHY171 was generated by first cloning the *GAL1* promoter into pRS304 (31) and then cloning sequences encoding the FLAG tag and the lox recombinase target site (from pTag-lox (36)) downstream of the promoter (detailed map available on request). The recombined plasmids were then digested with *Nde*I, and the vector band was gel-purified and religated to remove the lox sequences between the FLAG tag and the beginning of *CDC42*. The FLAG-*CDC42* sequences were then excised with *Nco*I and *Sac*I and cloned into the corresponding sites in pDLB1473. This vector has a YIplac204 (35) backbone, a *GAL1* promoter, a start codon with a good Kozak consensus sequence, and *Nco*I- and *Sac*I-cloning sites (detailed map available on request). The resulting plasmids, pDLB1485 (wild type), pDLB1486 (Q61L), and pDLB1487 (D57Y) were digested at the unique *Bst*XI site within the *TRP1* sequence to target integration at the *TRP1* locus.

For expression of recombinant proteins in bacteria, univector clones were recombined with either pHB1-Myc3 (36) (directing synthesis of proteins with three Myc tags fused to the N terminus) or pHB2-GST (directing synthesis of proteins with glutathione *S*-transferase (GST) fused to the N terminus). The recombined plasmids are listed in Table II.

To create the *bem1-CΔ35* allele, a 1.6-kb *Sac*I/*Xho*I fragment from pDLB1576 (with the *Sac*I site filled in with T4 DNA polymerase) was cloned into *Eco*RI/*Sal*I-digested YIplac204 (35) (with the *Eco*RI site filled in with Klenow DNA polymerase), yielding pDLB1863. This contains Bem1p-coding sequences from the start codon to the *Dra*I site (35 residues from the C terminus) cloned into the YIplac204 polylinker. Digestion at the unique *Kpn*I site near the N terminus of *BEM1* was used to target integration at the *bem1::URA3* locus (the disrupted allele retains N-terminal *BEM1* sequences (20), so homologous recombination generates a *bem1-CΔ35* allele driven from its own promoter at its normal chromosomal location and an adjacent promoterless *bem1::URA3* allele).

To create the *bem1-Δ142-199* allele, a 1.7-kb *Nco*I/*Xho*I fragment from pDLB1189 (with the *Nco*I site filled in with T4 DNA polymerase) was first cloned into *Eco*RI/*Sal*I-digested YIplac204 (35) (with the *Eco*RI site filled in with T4 DNA polymerase), yielding pDLB1862. This contains the full Bem1p open reading frame. The second SH3 domain was then deleted by digesting with *Bam*HI and *Sal*I, filling in with Klenow DNA polymerase, and religating the vector to yield an in-frame fusion lacking residues 142–199 of Bem1p, which was confirmed by sequencing and designated pDLB1881. This plasmid (and also pDLB1862, containing the full-length *BEM1*, to control for possible effects of the targeted integration strategy) was then digested with *Kpn*I to target integration at *bem1::URA3* as described for *bem1-CΔ35* above.

Yeast Lysates, Protein Analysis, and Phosphatase Treatment—Yeast cells were harvested by centrifugation, and cell pellets were stored frozen at −80 °C. Pellets were resuspended in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (or 0.1% when indicated) Nonidet P-40, 1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 2 μg/ml each of pepstatin A (Sigma) and leupeptin (Roche Molecular Biochemicals)), and the cells were lysed by vortexing with acid-washed glass beads. Lysates were clarified by centrifugation for 10 min at 14,000 rpm in an eppendorf centrifuge at 4 °C, and protein concentration was determined by the Bradford method (Bio-Rad).

For electrophoresis and immunoblotting, 20 μg of lysate protein per gel lane were mixed with hot (95 °C) 2× sample loading buffer (final concentrations, 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 25% glycerol, 355 mM β-mercaptoethanol, 0.01% bromphenol blue) and incubated at 95 °C for 5 min and separated by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell) that were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS/Tween) and incubated for 1 h at 4 °C with primary antibodies against Cdc24p (rabbit polyclonal anti-Cdc24p antibody kindly supplied by E. Bi and J. Pringle, used at 1:2000 dilution), c-Myc (mouse monoclonal anti-Myc 9E10 antibody, Santa Cruz Biotechnology, Santa Cruz, CA, used at 1:1000 dilution), or hemagglutinin (mouse monoclonal anti-HA 12CA5 antibody, Roche Molecular Biochemicals, used at 1:5000 dilution) in PBS/Tween containing 1% BSA. Following three washes with PBS/Tween, blots were incubated for 1–2 h at 4 °C with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG used at a 1:2500 dilution and goat anti-rabbit IgG used at 1:5000 dilution) from Jackson ImmunoResearch Laboratories, West Grove, PA. After three more washes, blots were developed with Renaissance Western blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

For immunoprecipitation, 0.5–1 mg of lysate protein was incubated for 1 h with 1 μl of the relevant antibody and then for a further 1 h with 30 μl of a 50% slurry of protein A-Sepharose (Sigma) at 4 °C with gentle rocking. Bead-bound proteins were washed four times with Nonidet P-40 lysis buffer prior to analysis by immunoblotting as described above. For phosphatase treatment, washed HA-Cdc24p immunoprecipitates were resuspended in 120 μl of phosphatase buffer (40 mM PIPES, pH 6.0,, 50 mM NaCl, 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin A and leupeptin) and split into 3 equal aliquots. One set was used as a no-treatment control, one set received 2 μl (0.1 units) of potato acid phosphatase (Sigma), and one set received phosphatase together with the phosphatase inhibitors sodium fluoride (50 mM final concentration) and sodium orthovanadate (10 mM final concentration). Following 30 min at 30 °C, the beads were washed with Nonidet P-40 lysis buffer prior to analysis by immunoblotting as described above.

Production of Recombinant Proteins, Binding Assays, and Phosphorylation in Vitro—GST fusion proteins were expressed in the protease-deficient *Escherichia coli* BL21, and Myc-tagged proteins were expressed in *E. coli* BL21(DE3) (Stratagene). Extracts were prepared in bacterial lysis buffer (750 mM sucrose, 100 mM NaCl, 100 mM Tris-Cl, pH 8.0, 5 mM EDTA) containing 7.5 μg/ml aprotinin, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride. Cells were treated with 2 mg/ml lysozyme (Sigma) for 20 min on ice and lysed by incubation with 2 mg/ml deoxycholic acid for 20 min at 4 °C. DNA was digested by addition of $MgCl_2$ to 15 mM and DNase I (Sigma) to 50 μg/ml and insoluble material was removed by centrifugation at 14,000 rpm at 4 °C for 10 min. GST fusion proteins were purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as specified by the manufacturer.

Binding assays were performed by incubating the bacterial extracts containing Myc-tagged proteins with the GST fusion proteins immobilized on glutathione beads in 200 μl of binding buffer (10 mM Tris-Cl, pH 7.5, 85 mM NaCl, 6 mM $MgCl_2$, 10% glycerol) at 4 °C for 1 h. Binding reactions were washed four times with 500 μl of binding buffer. Bead-bound proteins were resolved by SDS-PAGE and immunoblotted as described above. To confirm equal loading, the amount of GST fusion proteins in each lane was visualized by staining the membrane with india ink (38).

For analysis of Cdc24p phosphorylation *in vitro*, the indicated complexes were washed three times with 1 ml of Nonidet P-40 lysis buffer and once with 1 ml of reaction buffer (20 mM Tris, pH 7.5, 7.5 mM $MgCl_2$) and incubated in 10 μl of reaction mix (20 mM Tris, pH 7.5, 7.5 mM $MgCl_2$, 10 μM ATP, and 10 μCi of 3000 Ci/mmol [γ-^{32}P]ATP (PerkinElmer Life Sciences)) for 30 min at 30 °C. The reaction was stopped by addition of 10 μl of 2× SDS sample buffer and incubation at 95 °C for 5 min; proteins were resolved by SDS-PAGE, and ^{32}P incorporation was visualized by autoradiography.

Analysis of Cell Viability—For spot assays to monitor colony formation, cell cultures were grown at 20 °C to stationary phase in dextrose medium lacking uracil (to select for plasmid maintenance) and supplemented with 1 M sorbitol to preserve cell viability. Cell number was determined using a hemocytometer, and diluted stocks were generated to spot 5 μl containing ~1250, ~250, ~50, and ~10 cells onto the indicated plates, and plates were incubated at the indicated temperature for 3–4 days.

RESULTS

Cell Cycle-regulated Phosphorylation of Cdc24p—To investigate the potential for regulation of Cdc24p through the cell cycle, we first monitored Cdc24p abundance and modification in synchronized cells. Synchronous cell cultures were obtained by a G_1 cyclin deprivation/restoration protocol (28) (Fig. 1*A*) or by centrifugal elutriation (Fig. 1*C*), and Cdc24p was detected using a polyclonal anti-Cdc24p antibody (see "Experimental Procedures" for details). Western blot analysis revealed that Cdc24p abundance varied little during the cell cycle but that a subpopulation of cellular Cdc24p migrated with slower mobil-

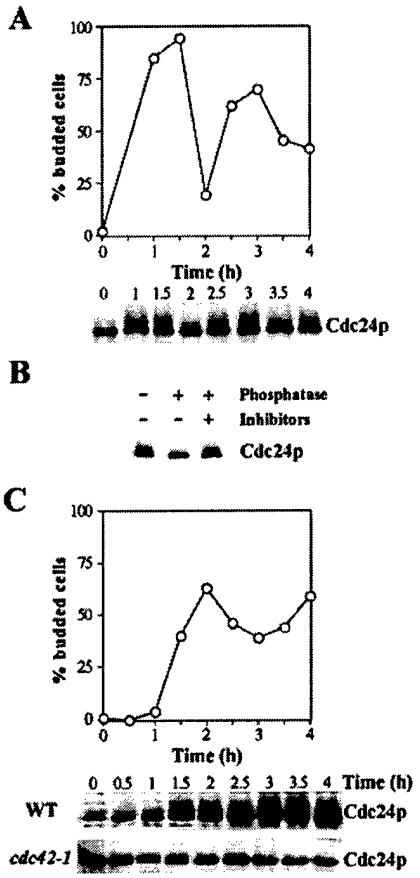

FIG. 1. **Cell cycle and Cdc42p-dependent phosphorylation of Cdc24p.** *A*, Cdc24p is modified to slower mobility species as cells progress through the cell cycle. Haploid *cln1 cln2 cln3 GAL1p-CLN3* (DLY222) cells were arrested in G_1 by incubation in YEPS (noninducing for the *GAL1* promoter) for 5 h and then stimulated to re-enter the cell cycle by addition of galactose (2% final concentration). Samples were taken at the indicated times following galactose addition for quantitation of bud formation and for Western blot analysis of Cdc24p. *B*, the Cdc24p mobility shift is due to phosphorylation. HA-tagged Cdc24p was immunoprecipitated from a lysate of yeast strain DLY4246, and separate aliquots were treated with potato acid phosphatase with or without phosphatase inhibitors as indicated, after which the Cdc24p species were resolved and detected as described under "Experimental Procedures." *C*, Cdc42p is required for Cdc24p phosphorylation. Wild-type (DLY5) and *cdc42-1*(DLY681) diploid G_1 cells were isolated by centrifugal elutriation and inoculated into YEPD at 38.5 °C to inactivate Cdc42–1p. Samples were taken at 30-min intervals for quantitation of bud formation and for Western blot analysis of Cdc24p. Note that the degree of synchrony attainable by this procedure is not as great as with the arrest-release protocol of *A*, so that the decrease in budding index and Cdc24p phosphorylation between the first and second cycles is not as evident.

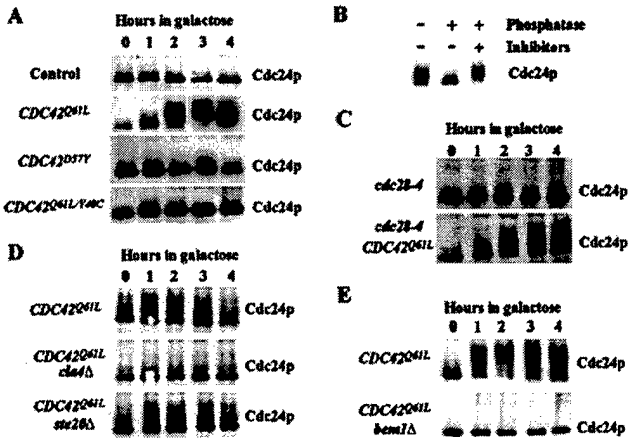

FIG. 2. **Role of GTP-Cdc42p, Cla4p, and Bem1p in Cdc24p phosphorylation.** *A*, GTP-Cdc42p stimulates Cdc24p hypermodification in a manner dependent on the effector domain. Strains expressing the indicated alleles of *CDC42* (control, DLY5; $CDC42^{Q61L}$, DLY3006; $CDC42^{D57Y}$, DLY2728; $CDC42^{Q61L/Y40C}$, DLY2727) under control of the *GAL1* promoter were grown in YEPS to exponential phase, and galactose was added (2% final concentration) at $t = 0$ to induce Cdc42p expression. Samples were taken at 1-h intervals for Western blot analysis of Cdc24p. *B*, the GTP-Cdc42p-induced hypermodification is phosphorylation. HA-Cdc24p isolated from cells of strain DLY2752 induced to overexpress $CDC42^{Q61L}$ as above was analyzed exactly as described in Fig. 1*B*. *C*, GTP-Cdc42p stimulates Cdc24p hyperphosphorylation independent of Cdc28p function. *cdc28-4* (DLY104) or *cdc28-4 GAL1p-*$CDC42^{Q61L}$ (DLY3004) strains were grown in YEPS at 23 °C to exponential phase, and G_1 cells were isolated by centrifugal elutriation, resuspended in YEPG to induce Cdc42p expression, and shifted to 37 °C to inactivate Cdc28p. Samples were collected and processed as above. *D*, Cdc24p hyperphosphorylation requires Cla4p but not Ste20p. Strains (*top*, DLY3003; *cla4*Δ, DLY2825; *ste20*Δ, DLY2826) expressing $CDC4^{Q61L}$ under control of the *GAL1* promoter were treated and processed as in *A* above. *E*, Cdc24p hyperphosphorylation requires Bem1p. Strains (*top*, DLY3003; *bem1*Δ, DLY2827) expressing $CDC42^{Q61L}$ under control of the *GAL1* promoter were treated and processed as above.

ity during SDS-PAGE, and the relative abundance of modified and unmodified forms changed as cells progressed through the cell cycle (Fig. 1, *A* and *C*). Immunoprecipitation and phosphatase treatment of HA-tagged Cdc24p showed that the slower migrating forms arose as a result of phosphorylation (Fig. 1*B*). Cdc24p phosphorylation was low in early G_1 cells isolated by centrifugal elutriation (Fig. 1*C*) and remained low in cells arrested by G_1 cyclin deprivation (Fig. 1*A*), suggesting that phosphorylation requires G_1 cyclin-Cdc28p activity. Following G_1 cyclin restoration, Cdc24p phosphorylation increased concomitant with bud formation (Fig. 1*A*, *1 h*) and subsequently decreased at around the time of cytokinesis (Fig. 1*A*, *2 h*). Thus, Cdc24p undergoes Cdc28p-dependent phosphorylation during the cell cycle.

Recent studies have shown that Cdc24p is sequestered within the nucleus of haploid (but not of diploid) cells during G_1 phase, as a stratagem that presumably contributes to readiness for mating (39–41). This finding raised the possibility that the regulated phosphorylation of Cdc24p was linked to its nuclear sequestration. However, we observed similar cell cycle-dependent phosphorylation of Cdc24p in both haploid (*e.g.* Fig. 1*A*) and diploid (*e.g.* Fig. 1*C*) cells, suggesting that the regulated phosphorylation is unconnected to the haploid-specific nuclear accumulation of Cdc24p.

In contrast to wild-type cells, no detectable Cdc24p phosphorylation occurred in the *cdc42-1* cells progressing through the cell cycle, indicating that Cdc42p is required for Cdc24p phosphorylation (Fig. 1*C*). This requirement could be explained in two very different ways. First, GTP-bound Cdc42p might be required, via some effector, to promote Cdc24p phosphorylation. Alternatively, GDP-bound Cdc42p, the substrate of Cdc24p, might be required; in this model an upstream Cdc24p-directed kinase (Cdc28p?) might specifically recognize a Cdc24p-Cdc42p complex. To distinguish between these possibilities, we overproduced mutants of *CDC42* locked in the GTP-bound ($Cdc42p^{Q61L}$) or GDP-bound ($Cdc42p^{D57Y}$ (7)) forms using the *GAL1* promoter. As shown in Fig. 2*A*, $Cdc42p^{Q61L}$ expression induced massive hyperphosphorylation of Cdc24p, as judged by the mobility shift upon analysis by SDS-PAGE (phosphatase treatment revealed that this enhanced mobility shift was also due to phosphorylation; see Fig. 2*B*). In contrast, $Cdc42p^{D57Y}$ did not induce Cdc24p phosphorylation. Expression of $Cdc42p^{Q61L}$ promoted Cdc24p phosphorylation even in *cdc28-4* mutants arrested in G_1 at the restrictive temperature (Fig. 2*C*), suggesting that Cdc28p is not required for

$Cdc42p^{Q61L}$ to trigger Cdc24p phosphorylation. Thus, Cdc28p stimulates Cdc24p phosphorylation in a manner that requires Cdc42p, whereas GTP-Cdc42p stimulates Cdc24p phosphorylation independent of Cdc28p.

Requirement for Cla4p and Bem1p in Cdc24p Phosphorylation—The finding that $Cdc42p^{Q61L}$ (presumably mimicking GTP-Cdc42p) specifically stimulated Cdc24p phosphorylation suggested that one or more Cdc42p effectors might be involved. In the course of other studies concerning downstream actions of Cdc42p, we had generated a panel of mutants containing alterations in the Cdc42p "effector loop."[2] In particular, mutation of Tyr-40 to Cys created a version of Cdc42p largely unable to bind the Cdc42/Rac-interactive binding motif (42) common to several effectors (43). As shown in Fig. 2A, when the Y40C mutation was introduced into $Cdc42p^{Q61L}$ (generating $Cdc42p^{Q61L/Y40C}$), expression of this protein was no longer effective in promoting Cdc24p hyperphosphorylation. This suggested that one or more of the Cdc42/Rac-interactive binding domain effectors might be involved. In *S. cerevisiae*, this group consists of three p21-activated kinases (PAKs: Cla4p, Ste20p, and Skm1p) and two related proteins with no known catalytic activity (Gic1p and Gic2p) (43). We found that Cla4p was required for a majority of the Cdc24p phosphorylation, whereas Ste20p was not (Fig. 2*D*). However, we consistently observed some residual Cdc24p phosphorylation in *cla4*Δ mutants (Fig. 2*D*); given the previously described functional redundancy between Cla4p and Ste20p (44, 45), this may indicate that both kinases can contribute to Cdc24p phosphorylation, with Cla4p playing the major role. As Cla4p is a GTP-Cdc42p-activated protein kinase (37), the simplest (although by no means the only) hypothesis consistent with our data would be that GTP-Cdc42p stimulates Cla4p to phosphorylate Cdc24p directly.

The *BEM1* gene was discovered through its genetic interactions with other polarity establishment genes (18). Bem1p is thought to be a scaffold protein and contains two SH3 domains as well as a C-terminal region that promotes direct binding to Cdc24p (20, 46). *In vitro*, the Bem1p-Cdc24p interaction does not seem to affect Cdc24p activity (47), and the functional role of this interaction remains unclear. We speculated that perhaps Bem1p binding might render Cdc24p susceptible to GTP-Cdc42p-induced phosphorylation. Indeed, *bem1*Δ mutants showed no detectable Cdc24p phosphorylation (as judged by mobility on SDS-PAGE), and expression of $Cdc42p^{Q61L}$ in cells lacking Bem1p failed to induce Cdc24p hyperphosphorylation (Fig. 2*E*). Thus, Bem1p is also important for the normal phosphorylation of Cdc24p.

Interactions among Bem1p, Cdc42p, Cla4p, and Cdc24p in Yeast Lysates—Bem1p is present in heterogeneous large complexes in yeast cell lysates, as judged by sucrose density gradient centrifugation (48). Multiple interactions have been reported (through a variety of methods) between Bem1p and other proteins, including Cdc24p (46), Boi1p and Boi2p (49, 50), Ste20p and Ste5p (48), Far1p and Cdc42p (51), and actin (48). These findings suggested that Bem1p may be present in a variety of complexes within yeast cells, and we were particularly intrigued by the possibility that the proteins required for Cdc24p phosphorylation (Bem1p, Cla4p, and GTP-Cdc42p) might form a complex together with Cdc24p in yeast. To test this hypothesis, we expressed functional epitope-tagged proteins in yeast and examined whether pairwise associations could be detected by coimmunoprecipitation (see "Experimental Procedures" for details). Indeed, pairwise associations between these proteins were readily detectable, suggesting that these interactions occur in yeast cells (Fig. 3). Specifically, we found that Myc-tagged Bem1p associated with FLAG-tagged Cdc42p (Fig. 3*A*), with HA-tagged Cla4p (Fig. 3*B*), and with

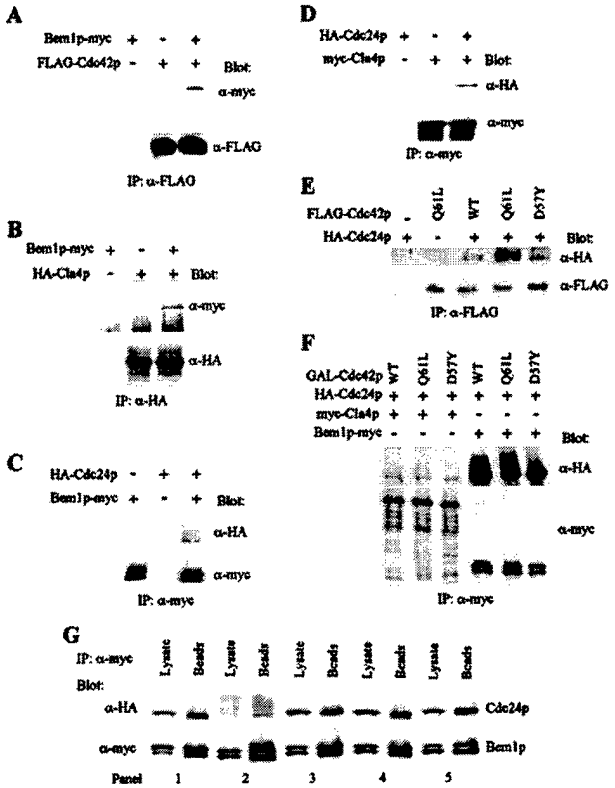

FIG. 3. **Coimmunoprecipitation of Cdc24p, Bem1p, and Cla4p from yeast lysates.** Cell growth, lysate preparation, immunoprecipitation, and Western blotting procedures were as described under "Experimental Procedures." *A*, Cdc42p-Bem1p complexes. Anti-FLAG immunoprecipitates from lysates of cells expressing FLAG-Cdc42p (DLY4554), Bem1p-Myc (DLY4000), or both (DLY4781) were analyzed by Western blotting with the indicated antibodies. *B*, Cla4p-Bem1p complexes. Anti-HA immunoprecipitates from lysates of cells expressing HA-Cla4p (DLY4738), Bem1p-Myc (DLY4000), or both (DLY4741) were analyzed by Western blotting with the indicated antibodies. *C*, Bem1p-Cdc24p complexes. Anti-Myc immunoprecipitates from lysates of cells expressing Bem1p-Myc (DLY4000), HA-Cdc24p (DLY4246), or both (DLY4248) were analyzed by Western blotting with the indicated antibodies. *D*, Cla4p-Cdc24p complexes. Anti-Myc immunoprecipitates from lysates of cells expressing Myc-Cla4p (DLY4739), HA-Cdc24p (DLY4246), or both (DLY2767) were analyzed by Western blotting with the indicated antibodies. *E*, Cdc42p-Cdc24p complexes. Anti-FLAG immunoprecipitates from lysates of cells expressing HA-Cdc24p (DLY4246), FLAG-$Cdc42p^{Q61L}$ (DLY4556), or HA-Cdc24p together with FLAG-Cdc42p (DLY2771), FLAG-$Cdc42p^{Q61L}$ (DLY2772), or FLAG-$Cdc42p^{D57Y}$ (DLY2773) were analyzed by Western blotting with the indicated antibodies. *F*, complex formation between Cdc24p and either Cla4p or Bem1p is unaffected by overexpression of GTP- or GDP-Cdc42p. Anti-Myc immunoprecipitates from lysates of cells expressing HA-Cdc24p and Myc-Cla4p (*1st three lanes*) or HA-Cdc24p and Bem1p-Myc (*last three lanes*) were analyzed by Western blotting with the indicated antibodies. Lysates were prepared from cells overexpressing wild-type Cdc42p, $Cdc42p^{Q61L}$, or $Cdc42p^{D57Y}$ as indicated. Immunoprecipitates from 750 μg of lysate were processed in parallel and analyzed on a single gel, which was probed first with anti-HA antibody, stripped, and reprobed with anti-Myc antibody. Strains (in same order as lanes) are DLY2768, DLY2769, DLY2770, DLY4299, DLY4300, and DLY4301. *G*, Bem1p-Cdc24p complex formation is unaffected by Cdc24p phosphorylation or cell cycle polarity cues. Cells expressing HA-Cdc24p and Bem1p-Myc (*panel 1*, DLY4248) and overexpressing either $Cdc42p^{Q61L}$ (*panel 2*, DLY4300), $Cdc42p^{D57Y}$ (*panel 3*, DLY4301), Swe1p (*panel 4*, DLY4308), or Clb1pΔ152 (*panel 5*, DLY4298) were lysed, and 20 μg of total lysate was loaded next to anti-Myc immunoprecipitate from 500 μg of starting lysate to compare the relative amount of Bem1p-Cdc24p complex in each case. The same blots were probed first with anti-HA antibody, stripped, and reprobed with anti-Myc antibody.

HA-tagged Cdc24p (Fig. 3*C*). In addition, HA-tagged Cdc24p associated with Myc-tagged Cla4p (Fig. 3*D*) and with FLAG-tagged Cdc42p (Fig. 3*E*). An association between Cdc24p and

GDP-Cdc42p is expected because GDP-Cdc42p is the substrate for Cdc24p, but surprisingly we observed a *stronger* association of Cdc24p with the GTP-locked mutant, Cdc42p^{Q61L}, than with wild-type Cdc42p or the GDP-locked mutant, Cdc42p^{D57Y} (Fig. 3*E*), suggesting that some other factor mediates interaction of GTP-bound Cdc42p with Cdc24p (see below). These results extend previous findings by identifying novel interactions (Bem1p-Cla4p, Cdc24p-Cla4p, and GTP-Cdc42p-Cdc24p) and are consistent with the existence of complexes containing Cdc24p, Cla4p, GTP-Cdc42p, and Bem1p in yeast.

As overexpression of Cdc42p^{Q61L} induces hyperphosphorylation of Cdc24p, we wondered whether it would also affect complex formation between Cdc24p and either Cla4p or Bem1p. However, the amount of Cdc24p associated with these proteins was unaffected regardless of whether wild-type, GTP-locked, or GDP-locked Cdc42p was expressed in the cells (Fig. 3*F*). However, this side-by-side comparison using epitope-tagged proteins (thereby evading possible antibody efficacy issues) unexpectedly revealed that Bem1p-Cdc24p complexes were recovered with much greater efficiency from cell lysates than were Cla4p-Cdc24p complexes (Fig. 3*F*), suggesting that Bem1p-Cdc24p complexes are more abundant than Cla4p-Cdc24p complexes *in vivo*. To examine whether Bem1p-Cdc24p complex formation was affected by Cdc24p phosphorylation, we compared the Cdc24p in the total cell lysate with the pool that was coimmunoprecipitated with Bem1p (after adjusting for the Myc-Bem1p immunoprecipitation efficiency; see Fig. 3*G*). All phospho-forms of Cdc24p were present in similar proportions in the starting lysate and in the Bem1p complexes, even in cells overexpressing Cdc42p^{Q61L} where phosphorylated forms of Cdc24p predominate (Fig. 3*G, panel 2*), suggesting that Cdc24p phosphorylation does not affect Bem1p binding.

We also examined whether the abundance of Bem1p-Cdc24p complexes was responsive to cell cycle cues causing polarized or depolarized growth. During normal bud growth, Clb-Cdc28p complexes trigger the apical-isotropic switch, causing a depolarization of growth within the bud (13). Inhibition of Clb-Cdc28p complexes by the kinase Swe1p (52) prevents this switch and causes a G_2 arrest associated with highly polarized growth, whereas expression of the stabilized Clb1pΔ152 (53) induces the switch prematurely and causes a telophase arrest associated with depolarized growth (13). Comparison of cells overexpressing Swe1p or Clb1pΔ152 did not reveal any change in the amount of Cdc24p associated with Bem1p (Fig. 3*G*), indicating that formation of these complexes is not tightly correlated with growth polarity.

Direct Interaction between Bem1p and Both GTP-Cdc42p and Cla4p—Previous studies found that two of the known interactions, Bem1p-Cdc24p (46) and GTP-Cdc42p-Cla4p (44), occurred between recombinant, bacterially expressed proteins, indicating direct binding. However, it was unclear whether the GTP-Cdc42p-Bem1p interaction (which was previously reported based on two-hybrid data (51) and which we detected by coimmunoprecipitation) or the novel interactions reported above are direct or mediated by bridging factors. To address this question, we performed *in vitro* binding assays between recombinant Bem1p, Cdc24p, Cla4p, Cdc42p^{Q61L}, and Cdc42p^{D57Y} expressed in bacteria (see "Experimental Procedures" for details). Myc-tagged Bem1p bound efficiently to GST-Cdc42p^{Q61L}, but not GST-Cdc42p^{D57Y}, *in vitro* (Fig. 4*A*). In the converse experiment, Myc-tagged Cdc42p^{Q61L} bound efficiently to GST-Bem1p, whereas Myc-tagged Cdc42p^{D57Y} did not (Fig. 4*B*). In addition, Myc-tagged Bem1p bound to GST-Cla4p (but not GST alone; see Fig. 4*C*), and Myc-tagged Cla4p bound to GST-Bem1p (but not GST alone; see Fig. 4*D*). Thus, the GTP-Cdc42p-Bem1p and Cla4p-Bem1p interactions occur

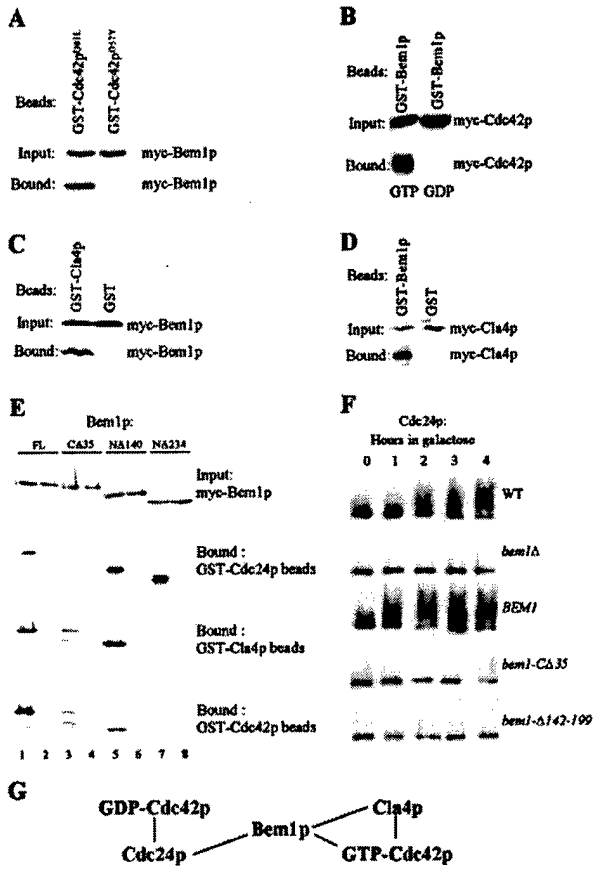

FIG. 4. **Direct interaction of Bem1p with GTP-Cdc42p and with Cla4p.** Recombinant GST-tagged and Myc-tagged proteins were made as described under "Experimental Procedures." *A,* GST-Cdc42p^{Q61L} (GTP-bound form) or GST-Cdc42p^{D57Y} (GDP-bound form) was bound to glutathione-Sepharose beads and incubated with bacterial lysates containing Myc-Bem1p, after which the beads were washed and the bound proteins analyzed by SDS-PAGE and Western blotting with anti-Myc antibodies as described under "Experimental Procedures." *Top lanes* show 10% of the input Myc-Bem1p, and *bottom lanes* show Myc-Bem1p bound to the beads. *B,* binding of Myc-Cdc42p to GST-Bem1p was assayed as above. GTP and GDP indicate Cdc42p^{Q61L} and Cdc42p^{D57Y}, respectively. *C,* binding of Myc-Bem1p to GST-Cla4p or GST alone was assayed as above. *D,* binding of Myc-Cla4p to GST-Bem1p or GST alone was assayed as above. *E,* Bem1p domains required for interaction with Cdc24p, Cla4p, and Cdc42p. Bacterial lysates containing Myc-tagged full-length Bem1p (*FL*), truncated Bem1p lacking the C-terminal 35 residues (*CΔ35*), truncated Bem1p lacking the N-terminal 140 residues (*NΔ140*), or truncated Bem1p lacking the N-terminal 234 residues (*NΔ234*) were indicated with the indicated beads. The *top panel* shows a Western blot of the input lysates; the *2nd panel* shows Myc-Bem1p variants bound to either GST-Cdc24p (*lanes 1, 3, 5,* and *7*) or GST alone (*lanes 2, 4, 6,* and *8*); the *3rd panel* shows Myc-Bem1p variants bound to either GST-Cla4p (*lanes 1, 3, 5,* and *7*) or GST alone (*lanes 2, 4, 6,* and *8*); and the *4th panel* shows Myc-Bem1p variants bound to either GST-Cdc42p^{Q61L} (*lanes 1, 3, 5,* and *7*) or GST-Cdc42p^{D57Y} (*lanes 2, 4, 6,* and *8*). *F,* the Bem1p C terminus and second SH3 domain are required for GTP-Cdc42p-induced Cdc24p phosphorylation in yeast. Wild-type (*WT*) (DLY3003), *bem1*Δ (DLY2827), *bem1*Δ with integrated full-length *BEM1* (DLY2781), *bem1*Δ with integrated *bem1-ΔC35* (DLY2782), and *bem1*Δ with integrated *bem1-Δ142-199* (DLY2844) strains were induced to express Cdc42p^{Q61L} by addition of galactose and processed to detect Cdc24p as described for Fig. 2*A* above. *G,* summary of direct interactions among this set of polarity establishment proteins.

in the absence of other proteins of eukaryotic origin, suggesting that these interactions are direct. In contrast, we did not detect interactions between recombinant Cdc24p and either Cla4p or GTP-Cdc42p (see below), suggesting that those interactions are indirect. These findings are summarized in Fig. 4*G*.

Consistent with a previous report (46), we found that the

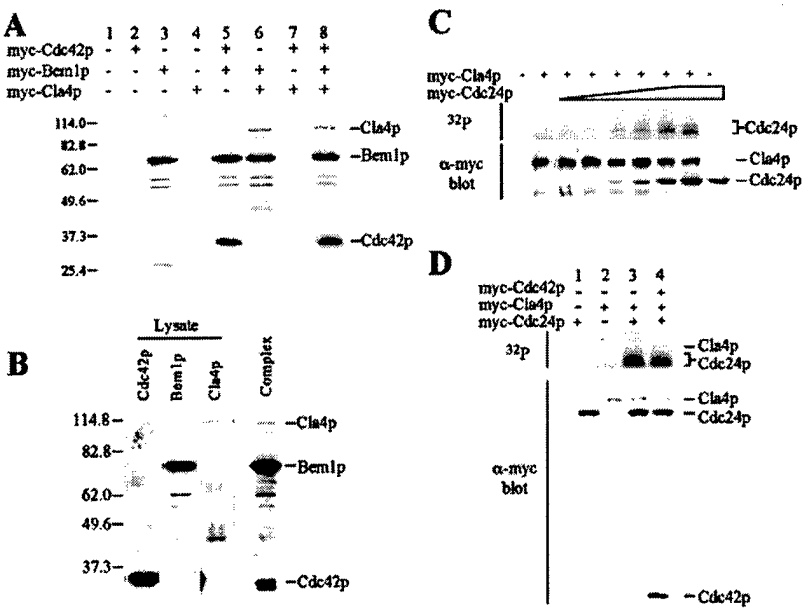

FIG. 5. **Phosphorylation of Cdc24p by Cla4p-containing complexes *in vitro*.** *A*, complex formation between recombinant Cdc24p, Bem1p, Cla4p, and GTP-Cdc42p. Bacterial lysates containing Myc-Cdc42p^{Q61L} (*lanes 2, 5, 7,* and *8*), Myc-Bem1p (*lanes 3, 5, 6,* and *8*), or Myc-Cla4p (*lanes 4, 6, 7,* and *8*) were incubated together with GST-Cdc24p bound to glutathione-Sepharose beads. Following repeated washes, bead-bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-Myc antibody. Molecular mass markers (kDa) are shown on the *left*. *B*, Western blots showing 10% of the input lysates containing the indicated Myc-tagged proteins (*left 3 lanes*) and 100% of the bead-bound complex formed as described in (*A*). Molecular mass markers (kDa) are shown on the *left*. *C*, phosphorylation of Cdc24p by Cla4p. Bacterial lysates containing Myc-Cla4p and increasing amounts of Myc-Cdc24p (0, 2.5, 5, 10, 20, 50, and 100 μl of bacterial lysate) were incubated together with GST-Bem1p bound to glutathione-Sepharose beads. Following repeated washes, bead-bound proteins were either incubated together with [γ-^{32}P]ATP in a standard kinase reaction (*top*) or resolved by SDS-PAGE and analyzed by Western blotting with anti-Myc antibody (*bottom*). *D*, phosphorylation of Cdc24p *in vitro* is unaffected by Cdc42p. Bacterial lysates containing Myc-Cdc42p^{Q61L} (*lane 4*), Myc-Cdc24p (*lanes 1, 3,* and *4*), or Myc-Cla4p (*lanes 2, 3,* and *4*) were incubated together with GST-Bem1p bound to glutathione-Sepharose beads. Following repeated washes, bead-bound proteins were either incubated together with [γ-^{32}P]ATP in a standard kinase reaction (*top*) or resolved by SDS-PAGE and analyzed by Western blotting with anti-Myc antibody (*bottom*).

interaction between Bem1p and Cdc24p was abrogated upon removal of the C-terminal 35 amino acids from Bem1p (Fig. 4*E*). In contrast, the interaction of Bem1p with Cla4p was largely unaffected by the C-terminal truncation, and the interaction of Bem1p with GTP-Cdc42p was decreased but not abolished (Fig. 4*E*). Removal of the N-terminal 140 amino acids from Bem1p also decreased GTP-Cdc42p interaction but did not affect binding to Cdc24p or Cla4p, and removal of the N-terminal 234 amino acids from Bem1p largely eliminated binding to either Cla4p or GTP-Cdc42p without affecting binding to Cdc24p (Fig. 4*E*). These results suggest that Bem1p residues 140–234, encompassing the second SH3 domain, are important for Cla4p interaction, whereas the interaction of Bem1p with GTP-Cdc42p is more complex and is perturbed upon truncation of either end of Bem1p. We then generated yeast strains in which the sole copy of Bem1p lacked either the C-terminal 35 residues or the second SH3 domain (residues 142–199). These strains did not exhibit detectable Cdc24p phosphorylation, even upon Cdc42p^{Q61L} overexpression (Fig. 4*F*). This suggests that the interactions of Bem1p with Cdc24p, Cla4p, and Cdc42p are important for the normal phosphorylation of Cdc24p.

In Vitro Assembly of Cdc24p-Bem1p-Cla4p-Cdc42p Complexes and Phosphorylation of Cdc24p—The findings that the proteins required for efficient Cdc24p phosphorylation *in vivo* were able to interact with each other *in vitro* prompted us to examine whether they could form hetero-oligomeric complexes and whether Cla4p could directly phosphorylate Cdc24p. Whereas Bem1p bound efficiently to GST-Cdc24p immobilized on glutathione-Sepharose beads (Fig. 5*A*, *lane 3*), neither Cla4p nor Cdc42p^{Q61L} bound to GST-Cdc24p, individually or in combination (Fig. 5*A*, *lanes 2, 4,* and *7*). However, Bem1p was able to recruit either Cla4p, Cdc42p^{Q61L}, or both proteins into complexes with GST-Cdc24p (Fig. 5*A*, *lanes 5, 6,* and *8*). Control experiments confirmed that none of these proteins bound detectably to GST alone (Fig. 4 and data not shown). Thus, Bem1p binds to Cdc24p, Cla4p, and Cdc42p directly and can mediate the assembly of hetero-oligomeric complexes containing these proteins.

There is some ambiguity in the details of the architecture of this complex, stemming from the potential for direct GTP-Cdc42p-Cla4p interactions (44) as well as direct interactions of each of these proteins with Bem1p. In principle, GTP-Cdc42p might be tethered to the complex via Cla4p or vice versa. However, recruitment of GTP-Cdc42p to the complex was unaffected by the presence or absence of Cla4p (Fig. 5*A*, compare *lanes 5* and *8*), and recruitment of Cla4p to the complex was unaffected by the presence or absence of GTP-Cdc42p (Fig. 5*A*, compare *lanes 6* and *8*). In addition, the stoichiometry of Bem1p and Cdc42p^{Q61L} in the complexes was ~1:1, as judged by the anti-Myc signal (each protein was tagged with 3 Myc epitopes at the N terminus), but Cla4p was present at considerably lower levels (Fig. 5*B*). This suggests that Bem1p recruitment of GTP-Cdc42p is very efficient and does not require Cla4p. We have been unable to obtain high level production of bacterial Myc-Cla4p using our vectors, and comparison of the levels of recombinant proteins in the GST-Cdc24p complex with those in the starting lysates (Fig. 5*B*) showed that a similar fraction of the starting material was bound in all cases (Bem1p, Cla4p, and Cdc42p^{Q61L}). In aggregate, these findings suggest that the complexes are assembled through direct pairwise interactions between Bem1p and each of the other components.

Initial attempts to assess whether Cla4p could directly phosphorylate GST-Cdc24p were complicated by the finding that

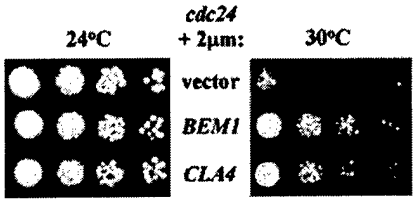

FIG. 6. **Suppression of *cdc24-4* temperature sensitivity by overexpressed *BEM1* and *CLA4*.** Strain Y147 (*cdc24-4*) was transformed with 2-μm plasmids containing either no insert (vector, YEplac195), *BEM1* (pDLB678), or *CLA4* (pDLB722). Cells were counted and spotted onto YEPD plates at 24 °C (*left*) or dextrose-Ura plates at 30 °C (*right*).

Cla4p efficiently phosphorylated the GST moiety (data not shown). We therefore assembled complexes containing Myc-tagged Cdc24p and Myc-tagged Cla4p on GST-Bem1p-coated beads (Fig. 5*C*). Incubation of these Cla4p-Bem1p-Cdc24p complexes with [γ-^{32}P]ATP promoted ^{32}P incorporation into the Myc-Cdc24p but not into the Myc-Cla4p (Fig. 5*C*). Cdc24p phosphorylation was dependent on Cla4p (Fig. 5, *C* and *D*) and was not stimulated by the further addition of Cdc42p^{Q61L} (Fig. 5*D*, *lanes 3* and *4*). It is not clear whether this reflects a physiological Bem1p-dependent activation pathway for Cla4p that does not require Cdc42p (as has been suggested for *Schizosaccharomyces pombe* shk1 (54)) or whether unphysiological activation of Cla4p occurs upon bacterial expression (perhaps due to misfolding that relieves autoinhibition, as apparently occurs for some mammalian PAKs).[3] A subset of the ^{32}P-labeled Cdc24p showed distinctly slower mobility on SDS-PAGE (Fig. 5, *C* and *D*), suggesting that more than one site was phosphorylated. Because all of these components were generated as recombinant proteins in bacteria, the observed phosphorylations cannot be due to contaminating kinases of eukaryotic origin. We conclude that Cla4p can directly phosphorylate Cdc24p, in complexes assembled through interactions with Bem1p.

Suppression of cdc24 by Overexpression of Bem1p or Cla4p—Many genetic interactions have been reported between components of the complex described above. In particular, synthetic lethality was observed between *cdc24* and *bem1* mutants (46), between *bem1* and *cla4* (55), and between *cdc42* and *cla4* mutants (44); in addition, high copy *CDC42* plasmids partially suppressed the temperature sensitivity of *cdc24* mutants (56). We found that high copy *BEM1* and *CLA4* plasmids could also partially suppress the temperature sensitivity of *cdc24* mutants (Fig. 6). These data are consistent with the hypothesis that complex formation between Bem1p, Cla4p, Cdc42p, and Cdc24p helps to promote Cdc24p function, at least in strains with mutationally compromised Cdc24p.

DISCUSSION

Phosphorylation of Cdc24p—Cdc24p phosphorylation state changes during cell cycle progression, with phosphorylated forms largely absent during early G_1 and accumulating concomitant with bud formation. Cdc24p phosphorylation was triggered by G_1 cyclin-Cdc28p activation, but the role of Cdc28p is probably indirect, because Cdc24p phosphorylation *in vivo* required other proteins (Cdc42p, Bem1p, and Cla4p) and could be stimulated upon overexpression of GTP-Cdc42p even in the absence of Cdc28p activity. These findings suggest that G_1 cyclin-Cdc28p initiates a regulatory pathway that culminates in Cdc24p phosphorylation by Cla4p (and probably also to a some extent by the related kinase Ste20p) during the cell cycle. The stimulation of Cdc24p phosphorylation by GTP-Cdc42p raises the possibility that the GTP-Cdc42p generated as a result of Cdc24p-catalyzed GDP/GTP exchange can feed back to regulate Cdc24p.

While this manuscript was being prepared for submission, another report was published (57) documenting Cdc24p phosphorylation and proposing that Cdc24p phosphorylation causes dissociation of Cdc24p from Bem1p, cessation of GTP-Cdc42p production, and depolarization of growth. This intriguing hypothesis was supported by three lines of evidence, none of which appear to be compatible with our data, as detailed below. First, Gulli *et al.* (57) detected only the hypophosphorylated form of Cdc24p in immune complexes with Bem1p, leading them to conclude that Cdc24p phosphorylation causes its dissociation from Bem1p. In contrast, we found that phosphorylated and unphosphorylated species of Cdc24p were proportionately represented in Bem1p complexes (Fig. 3*G*). This difference is likely due to technical aspects of the experiment; we used Myc-tagged Bem1p and HA-tagged Cdc24p, whereas they used GFP-Bem1p and untagged Cdc24p. Given the faintness of even the hypophosphorylated Cdc24p signal observed by Gulli *et al.* (57), their failure to detect phosphorylated species in the immune complex may simply reflect insufficient sensitivity (since each separate phospho-form would be present at low abundance). Second, Gulli *et al.* (57) found that gross overexpression of Cla4p was toxic to cells and caused both Cdc24p hyperphosphorylation and depolarized growth. This toxicity was apparently more severe in *cdc24* and *cdc42* mutants than in wild-type cells, leading the authors to conclude that the observed depolarization was caused by Cdc24p phosphorylation. However, with the less extreme overexpression attained by multicopy plasmids, we found that excess Cla4p was actually *beneficial*, both for *cdc24* mutants (Fig. 6) and for a variety of *cdc42* mutants.[2] In any case, it is far from clear whether these phenotypes are due to phosphorylation of Cdc24p or of other Cla4p targets. Third, Gulli *et al.* (57) reported that cells expressing a catalytically inactive Cla4p displayed reduced Cdc24p phosphorylation and hyperpolarized bud growth, leading them to conclude that failure to phosphorylate Cdc24p leads to its continued association with Bem1p, causing a defect in the apical-isotropic switch triggered by Clb/Cdc28p. However, we found that overexpression of Clb1pΔ152, which is known to trigger effectively the depolarizing switch (13), did not alter the association of Cdc24p with Bem1p (Fig. 3*G*). Furthermore, *cla4* mutants display defects in septin organization (44, 58), which in turn can trigger Swe1p-dependent cell cycle delays associated with hyperpolarized bud growth (58, 59), suggesting a plausible alternative explanation for the elongated buds they observed. In conclusion, the model of Gulli *et al.* (57) rests on their interpretation of a correlation between the effects of Cla4p manipulation on Cdc24p phosphorylation and on cell polarity as a direct cause and effect relationship. Our data do not support this correlation and indeed conflict directly with central tenets of the model. Distinguishing the true role of Cdc24p phosphorylation in cell polarity will require mapping of the phosphorylation sites and mutagenesis to generate nonphosphorylatable Cdc24p variants.

A Bem1p-dependent Complex Containing Cdc24p, Cdc42p, and Cla4p—We report that Bem1p binds directly to Cla4p and to GTP-Cdc42p as well as to Cdc24p and can mediate the assembly of complexes containing each of these proteins *in vitro*. This scaffold-mediated complex between an exchange factor, its target G protein, and an effector of the G protein is reminiscent of the complexes described for mitogen-activated protein kinase cascades (60), in that three signaling proteins thought to act sequentially in a linear pathway are physically joined by a scaffold that binds each component individually. It is commonly believed that one role of the scaffold proteins that

[3] Alan Hall, personal communication.

link mitogen-activated protein kinase cascades is to "insulate" distinct signaling cascades from cross-talk, thereby allowing individual kinases to participate in several separate signaling pathways (61). However, Bem1p-scaffolded complexes seem unlikely to play an analogous role, because the scaffold appears to be quite promiscuous in its interactions (48–50, 62) and may bring together many different combinations of polarity proteins. We found that Cla4p was only present in a small proportion of the Bem1p-Cdc24p complexes recovered from cell lysates and that the second SH3 domain of Bem1p, previously shown to mediate binding of Bem1p to Boi1p and Boi2p (49, 50), also mediated interaction with Cla4p. These findings suggest that Bem1p may participate in the formation of multiple complexes, rather than insulating one particular complex from the influence of other factors.

Our finding of a direct, GTP-dependent interaction between Cdc42p and Bem1p indicates that Bem1p fulfills the biochemical criteria for being a Cdc42p effector. Indeed, mutagenesis of the Cdc42p effector loop showed that residues in that region were critical for Bem1p interaction.[2] It seems possible, therefore, that GTP-Cdc42p may induce Bem1p to perform specific downstream polarization functions. However, the interaction of Bem1p with Cdc24p and Cla4p suggests that Bem1p may act at the same level as Cdc42p rather than (or perhaps in addition to) acting downstream. Overexpression of either Bem1p or Cla4p (but not other Cdc42p effectors) was able to ameliorate a variety of distinct phenotypic defects exhibited by a panel of *cdc42* effector-loop mutants (32),[2] consistent with the hypothesis that these proteins enhance *all* Cdc42p functions, possibly by improving the localization, GTP loading, or signaling efficacy of Cdc42p.

Polarization of growth in yeast is associated with the concentration of Cdc42p and Cdc24p together with scaffold proteins and effectors at a patch just beneath the plasma membrane, toward which they direct cytoskeletal polarization and hence membrane and cell wall expansion (11, 26). Intriguingly, Cdc42p and/or Rac have been found to play roles in cytoskeletal reorganization during chemotaxis (63, 64), T cell polarization toward antigen-presenting cells (7), phagocytosis (65, 66), and cell adhesion (67, 68), all of which involve the local clustering of cell surface receptors, potentially promoting local concentration of GTP-Cdc42p. In one study, recruitment of GTP-Cdc42p to the cell surface was ineffective in promoting cytoskeletal reorganization unless the GTP-Cdc42p was concentrated in a discrete patch by receptor clustering (69). Thus, many (if not most) Cdc42p functions may involve the local concentration of GTP-Cdc42p and its partners at a discrete site. In many cases this could be mediated by clustering of receptors or polarity cues such as the bud site selection proteins in yeast. However, yeast bud site selection mutants can still polarize (24), and we suggest that clustering of scaffold proteins (including Bem1p) into a patch at the internal face of the plasma membrane provides a mechanism to achieve the local concentration of polarity proteins even in the absence of polarity cues. In this way, Bem1p may contribute to the creation of a local "landing pad" with multiple binding sites for other polarity proteins, helping to maintain productively high local concentrations of these components. This hypothesis provides an attractive rationale for why Bem1p binds to so many polarity proteins (including GTP-Cdc42p) directly, and for why overexpression of Bem1p can enhance distinct functions of Cdc42p (including roles in actin organization, septin organization, and pheromone-responsive signal transduction (32, 62).[2]

Conclusions—We have documented several novel interactions among polarity establishment proteins in yeast. In complexes assembled through the scaffold protein Bem1p, the exchange factor Cdc24p was phosphorylated by the Cdc42p effector Cla4p. Although the function of this phosphorylation remains to be determined, the fact that it is regulated during the cell cycle suggests that either complex formation or the activity of Cla4p in the complex is responsive to cell cycle cues. Further studies will be required to address the basis for regulation of these proteins by the cell cycle machinery and its role in establishment and modulation of cell polarity in yeast.

Intriguingly, a family of "Pix" proteins (70) have been described in mammalian cells that possess domains homologous to both Cdc24p (DH and PH domain) and Bem1p (SH3 domain). These proteins also bind directly to the mammalian Cla4p homologue, PAK, potentially generating a complex with architecture similar to the Cdc24p-Bem1p-Cla4p complex described here. It will be interesting to determine whether these conserved structural features reflect conserved functional strategies.

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