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A two-tiered mechanism by which Cdc42 controls the localization and activation of an Arp2/3-activating motor complex in yeast

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The establishment of cell polarity in budding yeast involves assembly of actin filaments at specified cortical domains. Elucidation of the underlying mechanism requires an understanding of the machinery that controls actin polymerization and how this machinery is in turn controlled by signaling proteins that respond to polarity cues. We showed previously that the yeast orthologue of the Wiskott-Aldrich Syndrome protein, Bee1p/Las17p, and the type I myosins are key regulators of cortical actin polymerization. Here, we demonstrate further that these proteins together with Vrp1p form a multivalent Arp2/3-activating complex. During cell polarization, a bifurcated signaling pathway downstream of the Rho-type GTPase Cdc42p recruits and activates this complex, leading to local assembly of actin filaments. One branch, which requires formin homologues, mediates the recruitment of the Bee1p complex to the cortical site where the activated Cdc42p resides. The other is mediated by the p21-activated kinases, which activate the motor activity of myosin-I through phosphorylation. Together, these findings provide insights into the essential processes leading to polarization of the actin cytoskeleton.

Introduction

The generation of cell polarity is a fascinating problem in biology. Although this process is fundamental to many physiological functions, it remains poorly understood. This is due largely to the complexity of the cellular rearrangements that are involved and the diversity of the signals that may trigger cell polarization (Drubin and Nelson, 1996). For these reasons, the budding yeast Saccharomyces cerevisiae has been a useful model organism for studying the generation of cell polarity because of its simple and reproducible morphological changes during the cell cycle and powerful genetic tools. In yeast, cell polarization occurs at START immediately following activation of the Cdc28p cell cycle kinase. This involves major structural rearrangements, including polarization of the secretory pathway, rearrangements of the actin cytoskeleton, and assembly of bud neck structures (for reviews see Chant, 1999; Pruyne and Bretscher, 2000a,b). A large number of genes have been identified through genetic, biochemical, and interaction trap approaches. A recent large scale two-hybrid interaction study has linked many of these genes into highly intricate molecular networks (Drees et al., 2001). However, the fundamental mechanisms underlying polarity establishment have yet to be deciphered.

We have been taking a reductionist approach to the problem of cell polarity by focusing on one of the important and commonly occurring events, that is, the assembly of localized actin cytoskeletal elements. With the knowledge that activation of Cdc42p at the presumptive bud site leads to accumulation of F-actin at this site (for review see Gulli and Peter, 2001), the problem has been reduced to understanding the link between the activated Cdc42 and actin polymerization. An elegant example of such a link in metazoan organisms has been revealed by in vitro experiments using cell extracts. Activated Cdc42p and phosphatidylinositol 4,5-bisphosphate bind to Wiskott-Aldrich syndrome protein (WASP)* or neural WASP (N-WASP), exposing a COOH-terminal domain of the latter proteins, which could activate the Arp2/3 complex, leading to nucleation of new actin filaments.

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Key words: polarity; Cdc42; actin polymerization; formin; Arp2/3 complex

*Abbreviations used in this paper: Lat-A, latrunculin A; N-WASP, neural WASP; PAK, p21-activating kinase; WASP, Wiskott-Aldrich syndrome protein; WH, WASP homology; WIP, WASP-interacting protein.
ments (Rohatgi et al., 1999, 2000; Higgs and Pollard, 2000). However, it is unclear in which physiological context this signaling pathway functions, and there is no evidence suggesting that activation of WASP or N-WASP by Cdc42p is sufficient for the establishment of a polarized actin cortex in vivo.

In yeast, assembly of cortical actin structures, the actin patches, also depends on the yeast WASP orthologue Bee1p (Lechler and Li, 1997; Li, 1997) and the Arp2/3 complex (Winter et al., 1999). Additionally, the two type I myosins, Myo3p and Myo5p, have been shown to play an important role in cortical actin assembly, and intriguingly this function seems to require the myosin motor activity (Anderson et al., 1998; Evangelista et al., 2000; Geli et al., 2000; Lechler et al., 2000). These key actin assembly factors must somehow respond to the activated Cdc42p, but this connection has not been established at the molecular level, since none of the above proteins are known to interact directly with Cdc42 in contrast to WASP and N-WASP. The aim of the present study is to define a simple set of reactions downstream of Cdc42p that could result in polarized assembly of cortical F-actin in vivo with a belief that a simple central mechanism exists underneath all the molecular complexity. We provide additional evidence that a complex containing Bee1p, Vrp1p, and type I myosins has the functional premise to be an important target of Cdc42p in the induction of local actin polymerization. We show that the localization and activity of this complex are regulated by Cdc42p through concerted actions of two Cdc42 effectors.

Results

Bee1p, Vrp1p, and type I myosins form a complex that contains two activators of the Arp2/3 complex

The approach that we have taken to understand the link between Cdc42 and actin polymerization is to first identify protein factors that are critical for actin polymerization and then determine how these proteins are controlled by Cdc42p during cell polarization. Previous studies identified two interacting proteins important for actin patch assembly and organization, Bee1p and Vrp1p, a homologue of mammalian WASP-interacting protein (WIP) (Lechler and Li, 1997; Ramesh et al., 1997; Vaduva et al., 1997; Naqvi et al., 1998). To determine whether Vrp1p and Bee1p actually form a stable biochemical association in vivo, we constructed a strain that expresses Vrp1p tagged at the COOH terminus with (myc) epitope under the native VRP1 promoter as the sole source of Vrp1p. Gel filtration analysis indicated that Bee1p and Vrp1p cofractionate completely in a protein complex that migrates at a rate corresponding to a 1,000 kD globular protein (Fig. 1 A). Coimmunoprecipitation experiments showed that the Bee1p–Vrp1p interaction is resistant to 1 M KCl (unpublished data). This result suggest that Bee1p and Vrp1p exist in vivo as a stable protein complex.

We and others showed previously that the two type I myosins, Myo3p and Myo5p, bind both Vrp1p and Bee1p in a variety of assays (Anderson et al., 1998; Evangelista et al., 2000; Lechler et al., 2000). However, the myosins did not cofractionate with Bee1p and Vrp1p on the gel filtration column probably due to the low affinity of their interaction with Bee1p and Vrp1p. Since all Bee1p and Vrp1p in the native extract appeared to exist in a tight protein complex, it is reasonable to assume that at least a fraction of the myosin-I associates with the Bee1p–Vrp1p complex. An intriguing feature of this complex is the presence of at least two acidic motifs (A domains) that bind to the Arp2/3 complex: one in

Figure 1. Bee1p, Vrp1p, and type I myosins form a complex that contains two activators of the Arp2/3 complex. (A) Bee1p and Vrp1p exist in a stable protein complex. Bee1p and Vrp1p in a native extract were analyzed on a Superose-6 gel filtration column. The positions of markers, blue dextran (2000 kD) and thyroglobulin (670 kD), are indicated. (B) Activation of the Arp2/3 complex by combined action of the A domain of Myo3p and the WH2 domain of Vrp1p. Pyrene actin assembly assays were performed at 1.75 μM actin, 0.25 μM Arp2/3, and 0.5 μM activator. (a) Activation of Arp2/3 by chimeric proteins containing the WH2 domain of Bee1p or Vrp1p fused to the A domain of Myo3p. The graphs are as follows: actin alone (no symbol); actin with Arp2/3 complex, WH2(Vrp1p)-A(Vrp1p) (○); actin with Arp2/3 complex and WH2(Vrp1p)-A(Vrp1p) (□); and actin with Arp2/3 complex and WH2(Vrp1p)-A(Vrp1p) (■). (b) Activation of Arp2/3 complex by a dimer of WH2(Vrp1p) and A(Myo3p). The graph shows the following: actin alone (no symbol); actin with Arp2/3 complex (○); actin with Arp2/3 complex and WH2(A-Bee1p) (□); and actin with Arp2/3 complex, WH2(Vrp1p)-FRB, A(Myo3p)-FKBP12 with (●) and without (●) rapamycin.
Bee1p and the other in myosin-I. WASP family proteins have been shown to require an A domain and an adjacent G-actin–binding WASP homology (WH)2 domain to activate the Arp2/3 complex (Higgs and Pollard, 1999). Vrp1p contains a WH2 domain, raising the possibility that a complex of myosin-I and Vrp1p activates the Arp2/3 complex. To test biochemically whether the A domain of Myo3p can work together in a protein complex to activate the Arp2/3 complex, we used an inducible dimerization system, consisting of the proteins FRB and FKBP12, which form a tight dimer in the presence of rapamycin (Chen et al., 1995; Choi et al., 1996). The WH2 domain of Vrp1p was fused to FRB and the A domain of Myo3p to FKBP12. These two chimeras can together activate the Arp2/3 complex in the presence of rapamycin but not in its absence (Fig. 1 B). The dimer did not appear to be as potent as the Bee1p WH2-A fragment, possibly due to suboptimal orientations of the WH2 and A domains relative to each other within the dimer. Nonetheless, this result suggests that the WH2 domain of Vrp1p and the A domain of Myo3p are able to activate the Arp2/3 complex when present in a protein complex.

Activation of Cdc42p leads to recruitment of the Bee1p–Vrp1p complex to the site of cell polarization

The above data suggest that the Bee1p–Vrp1p–myosin-I complex plays an important role in the assembly of cortical actin filaments by activating the Arp2/3 complex. During cell polarization, the localization and/or activity of this complex is likely to be controlled by the activated Cdc42p. To test whether Bee1p is recruited to the site of cell polarization upon Cdc42p activation before actin polymerization, cells expressing Bee1p-GFP and bearing the cdc28-13 mutation were synchronized in G1 at 37°C and then released from the arrest in the presence of latrunculin A (Lat-A), a compound that prevents actin polymerization (Ascough et al., 1997). When these cells were analyzed for Bee1p-GFP localization by fluorescence microscopy, we observed a striking polarized distribution of Bee1p-GFP at the cell cortex in the absence of F-actin (Fig. 2 A). Similar results were also obtained for Vrp1p. By comparing the kinetics of Bee1p-GFP polarization with the kinetics of bud emergence in control-treated (DMSO) cells, it appears that Bee1p localization slightly precedes bud emergence, consistent with the timing of actin polarization in cells (Fig. 2 B).

To determine whether Bee1p-GFP polarization requires Cdc42p, a dominant negative allele of Cdc42p under the control of the GAL1 promoter (GAL-hsCDC42A118) was introduced into the above strain, and its expression was induced in G1-arrested cells. When these cells were released from the G1 arrest, essentially no Bee1p polarization was observed, indicating that polarized recruitment of Bee1p is Cdc42-dependent (Table I). Also, we tested whether Bee1p recruitment was dependent on Cdc24p, a guanine-nucleotide exchange factor required for activation of Cdc42p (Zheng et al., 1994). In the temperature-sensitive cdc24-1 strain, Bee1p-GFP polarization was impaired at the restrictive but not permissive temperature (Table I). Finally, to determine whether activated Cdc42p is sufficient for Bee1p polarization in the absence of cell cycle activation, we expressed Bee1p-GFP in a cdc28-13 strain containing the constitutively active allele of Cdc42p controlled by the GAL1 promoter (GAL-CDC42L61-HA). Cells arrested in G1 at the restrictive temperature were induced to express Cdc42L61 in the presence of Lat-A. Cdc42L61 was able to induce polarization of Bee1p-GFP (Table I), which colocalized with Cdc42L61 (Fig. 2 C). Therefore, Cdc42p is necessary and
sufficient for recruiting Bee1p to the site of cell polarization, and this process can occur in the absence of F-actin.

The Bee1p–Vrp1p complex marks the site of actin polymerization

Results described above suggest that Cdc42p induces actin cytoskeleton polarization in part through polarized recruitment of the Bee1–Vrp1 complex, which then locally activates the Arp2/3 complex. Under this hypothesis, actin polymerization should occur at the site of Bee1p/Vrp1p recruitment upon washout of Lat-A in the experiments described in Fig. 2. Therefore, we examined the regeneration of F-actin in cells that have recruited Bee1p–Vrp1p complex to the presumptive bud site.

\[\text{cdc28-13 cells expressing Bee1-GFP were released from a G1 arrest in the presence of Lat-A. After 1 h, Lat-A was washed away, and cells were fixed at varying times and stained for both F-actin and Bee1-GFP.} \]

\[\text{At the zero time point after washout, no actin structures were visible in cells, but many contained polarized Bee1-GFP. By 1 min after washout, a small fraction of cells contained very low but detectable levels of F-actin. In all cases, this actin colocalized with Bee1-GFP. By 2 min time point, most cells had polarized Bee1-GFP that colocalized with F-actin, although the levels of F-actin were still low compared with cells without Lat-A treatment. This result suggests that cortical actin assembly is initiated at the site of Bee1p/Vrp1p recruitment. As a control, when the same experiment was done in a cdc24-1 mutant background in which there is no Bee1p polarization we did not observe the regeneration of F-actin at the presumptive bud site, but rather we observed the regeneration in a depolarized manner (unpublished data).}\]

Cdc42p-induced myosin-I phosphorylation is required for actin polymerization at the site of cell polarization in vivo

Although the above data suggest that localization of the Bee1p–Vrp1p complex is an important step in polarized assembly of actin patches, it does not appear to be sufficient. This is because we observed that in cdc42-1 (a temperature-
sensitive allele of $CDC42$) cells grown at the nonpermissive temperature polarized recruitment of Bee1p and Vrp1p can still occur in a significant percentage of cells (Fig. 4 A), but the mutant cells are still defective in establishing a polarized actin distribution in vivo (Adams et al., 1990). Previous work showed that Cdc42p-induced actin polymerization in permeabilized yeast cells required phosphorylation of type I myosins by p21-activating kinase (PAK) kinases (Lechler et al., 2000). To determine whether myosin-I phosphorylation is the missing event required for establishing a polarized actin patch distribution in $cde42$-1 cells, we introduced a constitutively active allele of Myo3p, $MYO3S357D$, into $cde42$-1 cells (Wu et al., 1997). When $cde42$-1 cells were grown at the restrictive temperature, they accumulated as large unbudded cells with a depolarized actin patch distribution (Fig. 4 B). In contrast, $cde42$-1 $MYO3S357D$ cells accumulated as unbudded cells, a significant fraction of which had actin patches polarized to one end of the cell (Fig. 4 B). Quantitation revealed that 36% of $cde42$-1 $MYO3S357D$ cells had a polarized actin distribution as opposed to only 5% of $cde42$-1 cells ($n = 200$). This level of polarization in $cde42$-1 $MYO3S357D$ cells was comparable to that in an exponentially growing population of wild-type cells where 41% displayed a polarized actin patch distribution. This comparison is valid as the $cde42$-1 mutation, although preventing budding, does not block progression of the cell cycle (Adams et al., 1990). $MYO3S357D$ was not able to rescue the defect in actin polarization caused by the $cde24-1$ allele (Fig. 4 B) or by the dominant negative allele of $CDC42$ (unpublished data), probably due to a lack of polarized recruitment of the Bee1p–Vrp1p complex in these cells.

Polarized recruitment of the Bee1p–Vrp1p complex is mediated through formin-like proteins

Above results suggest that two distinct pathways downstream of Cdc42 are required for polarized actin polymerization: one is mediated through PAKs, leading to myosin-I phosphorylation, and the other leads to recruitment of the Bee1p–Vrp1p complex to the polarization site. The latter pathway requires further investigation, since unlike its mammalian homologues, WASP and N-WASP, Bee1p lacks an obvious Cdc42p-binding motif. Consistently, we have not been able to detect a direct biochemical interaction of Bee1p or Vrp1p with Cdc42p (unpublished data). Thus, Cdc42p must recruit the Bee1p–Vrp1p complex to the site of cell polarization through intermediary proteins. Since known Cdc42 effectors are obvious candidates for this function, we tested the ability of mutant cells deficient in each of the Cdc42p effectors to establish polarized Bee1p distribution. We observed no defects in Bee1p polarization in cells lacking both Gic1p and Gic2p, two redundant and homologous Cdc42 effectors (Brown et al., 1997; Chen et al., 1997) (Fig. 5 A). To test if PAK kinases play a role in recruiting the Bee1p–Vrp1p complex, we used a strain expressing a temperature-dependent degron allele of Cla4p as its sole source of PAK kinase (Holly and Blumer, 1999). We first confirmed that the shutoff phenotype in this strain can be rescued by a plasmid carrying the $CLA4$ gene, unlike a previous observation (Weiss et al., 2000). Similar to the $Gic1$ $Gic2$ double mutant, $CLA4$ shutoff did not affect polarized Bee1p recruitment (Table I).
To test the role of the two redundant formin-like proteins, Bni1p and Bnr1p (Evangelista et al., 1997; Imamura et al., 1997), we took advantage of a temperature-sensitive allele generated by alanine-scanning mutagenesis of the FH2 domain of BNII. This mutant (R1528A, R1530A) shows temperature-dependent cell lethality and depolarization of the actin cytoskeleton in the ΔbnlΔbnr1 double mutant background (Fig. 5, B and C). When a G1-enriched population of this mutant was allowed to enter the cell cycle at room temperature for 2 h, this strain showed normal polarized Bee1p recruitment in the presence of Lat-A. However, when cells were incubated at 37°C for 2 h, Bee1p, although still cortically associated, was not polarized in a majority of the mutant cells (Fig. 5 D and Table I).

To ensure that the temperature shift itself did not contribute to the failure to polarize Bee1p, we also examined the effect of Δbnl mutation during the mating factor response where Bni1p is essential for the formation of the mating projection (Lee et al., 1999; unpublished data). In α-factor-treated wild-type cells, Bee1-GFP is polarized to the shmoo tip, and this polarization is further enhanced after treatment with Lat-A for 15 min (Fig. 5 E), probably due to inhibition of actin patch movement away from the shmoo tip. Consistent with a requirement of Bni1p in Bee1p polarization, Bee1-GFP, although still cortically associated, is distributed in a nonpolarized pattern in the majority of the Bni1p cells (Fig. 5 E and Table I). Polarization was also disrupted in mating cells carrying the temperature-sensitive allele of BNII as their sole source of formins (Table I). The results above strongly suggest that the formin-like proteins are required for Bee1p recruitment to polarization sites in response to both intracellular and extracellular signals.

We also tested whether it is Bee1p or Vrp1p that is primarily responsible for their polarized recruitment. If Bee1p is recruited through its interaction with Vrp1p, then polarized cells carrying the vrp1ΔC mutation, which abrogates interaction with Bee1p (Fig. 6 A). This is indeed the case, since Bee1-GFP is not polarized in the vrp1ΔC strain (Fig. 6 B, a, and Table I). In contrast, Vrp1p recruitment occurred normally in cells bearing the bee1ΔWH1 mutation, which abrogates binding to Vrp1p (Fig. 6, A and B, b, and Table I), suggesting that polarized recruitment of Vrp1p is independent of Bee1p. This result indicates that an important function of Vrp1p in polarized actin assembly is to target the Bee1p–Vrp1p complex to the site of polarization, consistent with the actin polarization defect observed in Δvrp1p mutant cells.

### Discussion

In the study described above, we have investigated the biochemical events required for the establishment of a polarized actin cytoskeleton. Several findings are of significance, the foremost though least addressed being the observation that a constitutively activated form of Cdc42p, when expressed globally in completely nonpolarized cells can itself adopt a polarized distribution, recruit actin assembly factors, and induce local actin polymerization. This result is in contrast to the model where localization of Cdc42 is dependent upon a localized guanine-nucleotide exchange factor, Cdc24p, which in turn is recruited by intra- and extracellular landmarks (Gulli and Peter, 2001). We hypothesize that the activated Cdc42p, possibly together with its associated proteins and membrane components, has an intrinsic ability to achieve an asymmetric distribution independent of any localized cue. The latter only serves to restrict the direction of polarization, consistent with the observation that in the absence of the bud site selection function yeast cells can still bud efficiently, albeit in random directions, relative to previous buds (Chant and Pringle, 1991). The other findings concern the pathways downstream of Cdc42p activation that are necessary for the induction of actin polymerization as discussed below.

### Role of the Bee1p, Vrp1p, and myosin-I complex in actin nucleation

Although there is strong evidence that myosin-I interacts directly with Bee1p and Vrp1p (Anderson et al., 1998; Evangelista et al., 2000; Lechler et al., 2000), myosin-I is not present in the stable Bee1p–Vrp1p complex after fractionation (Lee et al., 1999; unpublished data). In contrast, myosin-I does not coprecipitate with the active Cdc42p-Cdc24p complex from extracts of wild-type cells and cells in which the temperature-sensitive allele of WH1 (H9004) was deleted. Instead, myosin-I appears to be associated with the formin-like proteins in a large complex that is required for the induction of actin polymerization downstream of Cdc42p activation. The pathway leading to the localization of this complex is not yet clear, but it is likely to involve signals that are necessary for the induction of actin polymerization as discussed below.
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FIGURE 7. A model depicting a bifurcated pathway downstream of Cdc42p required for polarized actin polymerization. In a formin protein-dependent manner, Cdc42p recruits a Bee1p/ Vrp1p/myosin-I-containing complex to the polarization site to activate the Arp2/3 complex. The double headed arrow between myosin-I and Vrp1p/Bee1p represents a dynamic interaction. In parallel, Cdc42 recruits the PAKs, which phosphorylate and activate the motor activity of type I myosins.

Mechanism by which the Bee1–Vrp1–myosin-I complex is recruited to the site of cell polarization

A polarized actin cytoskeleton may potentially be established in two ways: (a) by polar movement of existing actin filament and (b) by local induction of actin polymerization. The latter is more likely because of the highly dynamic and responsive nature of the cortical actin cytoskeleton. If this were true, then the proteins that control actin assembly, in particular those that control de novo filament nucleation, should be targeted to the polarized front through interaction with polarity regulators such as Cdc42p. Indeed, we have found that the Bee1p–Vrp1p complex is recruited to the polarization site upon induction of the activated Cdc42p. Significantly, this process can occur in the absence of F-actin (that is, in the presence of Lat-A). Upon Lat-A washout, F-actin assembles at the site where Cdc42p and Bee1p/Vrp1p accumulate. This result supports the notion that establishment of a polarized actin distribution occurs through Cdc42 recruitment of actin polymerization factors, which locally nucleate actin filaments. This result also suggests that localization of the machinery that controls cortical actin assembly does not depend upon the actin cable-based transport system.

To elucidate the mechanism of Bee1p/Vrp1p recruitment by the activated Cdc42, we systematically tested the role of known Cdc42 effectors in this process. Two of the three sets of Cdc42 effectors, the PAKs and Gic1p/Gic2p, have been eliminated by this strategy, but the formin-like proteins are found to be required for polarized Bee1p recruitment. On the complex side, Vrp1p rather than Bee1p appeared to be involved in targeting the complex. This activity is similar to the one attributed to WIP, which was shown to recruit N-WASP to intracellular vaccinia virus, leading to actin nucleation by the Arp2/3 complex (Moreau et al., 2000). In this system, the adapter protein Nck bridges the interaction of WIP with a tyrosine-phosphorylated viral surface protein. We have not yet detected a physical interaction between the Bni1p and Vrp1p complex by coimmunoprecipitation. Since there is evidence that formin-like proteins can exist in an autoinhibited form (Alberts, 2001), it is conceivable that Bni1p in the soluble extract requires an activation event in order to interact with Vrp1p, a possibility that is currently under investigation. Since mammalian formin family proteins have also been implicated in regulating actin dynamics downstream of small GTPases (Watanebe et al., 1997), it would be interesting to test whether these proteins also play a role in localizing WASP family proteins, in particular WAVE isoforms, which themselves do not contain small GTPase-binding domains.

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Myosin-I phosphorylation as a second event required for polarized actin polymerization

Our results indicate that even in a simple system such as yeast, polarized actin assembly does not occur by a linear pathway connecting signaling protein to actin assembly factors. In addition to the formin-dependent recruitment, the Bee1p–Vrp1p–myosin-I complex must also be activated through phosphorylation by PAKs, which is required for the motor activity of these myosins (Maruta and Korn, 1977; Wu et al., 1996) (Fig. 7). This finding is consistent with the result from actin patch reconstitution experiments in permeabilized yeast cells, which suggested an essential role for the motor activity in actin polymerization at cell cortex (Lechler et al., 2000). Motor phosphorylation is likely to occur after myosin-I is recruited to the polarization site, since the kinase responsible for this phosphorylation is recruited to the same site by Cdc42p (Peter et al., 1996). Mechanistically how the motor activity participates in polarized actin polymerization is an intensely interesting question. The motor activity may somehow be required for the activation or targeting of the Arp2/3 complex. Alternatively, it may be required after the nucleation step, for example, during filament elongation. In mammalian type I myosins, the PAK phosphorylation site is replaced by an acidic amino acid, resulting in “constitutively active” motors (Bement and Moosiker, 1995). Therefore, other activation mechanisms may exist to accompany the recruitment of actin nucleation factors in order to ensure tight spatial regulation of actin polymerization.

Materials and methods

Plasmid and strain construction

The Bee1-GFP construct (pTL58) was made by ligating the Bee1-containing BamHI fragment of pRL111 into pRL73. pTL58 was digested with Xhol-XbaI and ligated into the same sites of pBS306 to generate pTL80, an integration construct. The Bee1WH1-expressing ∆ amino acids 20–122 construct was made by ligating two fragments into pBS313: the first fragment (BamHI-XbaI) containing the Bee1 promoter and coding region up to amino acid 19 and the second containing the coding region from amino acid 123 and extending into the 3'UTR. To generate Vrp1p-myc-expressing plasmid, we PCR amplified the entire ORF (minus the stop codon) and 234 nucleotides upstream of the start site from yeast genomic DNA. This was ligated into the HindIII-BamHI sites of pTL48 to generate pTL48. Vrp1p-Myc plasmid was generated by ligating an EcoRI (blunted) to ApaI fragment of pTL48 into pRL222 (a HIS3, CEN, COOH-terminal 6-myc–tagging vector). This eliminates the CCOOH-terminal 56 amino acid of Vrp1p. GST-WH2(Vrp1p)-A(Myo3p) and GST-WH2(Bee1p)-A(Myo3p) were generated by ligating a PCR product encoding the WH2 domain of Vrp1p or Bee1p into the BamHI site of pTL87 (Lechler et al., 2000). The GST fusion construct A(Myo3p)-FKBP12 was made by subcloning the A domain of Myo3p from pTL87 (BamHI, Xhol) into pGEX-2T FKBP12 (obtained from Stuart Schrieber, Harvard University, Cambridge, MA). The GST fusion construct WH2(Vrp1p)-FRB was prepared by ligating a PCR product containing the Vrp1p WH2 domain into the BamHI site of pTL87 (Lechler et al., 2000). The GST fusion construct WH2(Bee1p)-FRB was prepared by ligating a PCR product containing the Bee1p WH2 domain into the BamHI Xhol sites of pGEX-2T FRB (Stuart Schrieber). The bni1 temperature-sensitive mutation containing plasmid KB19 was made by using PB1060 (CEN-TRP1-BNI1, pRS314) to perform an oligonucleotide-directed mutagenesis with primer “FH2#1alascan” (5'-AGCGTGAAGCAGTGCATCAGGACGAC-3'). This work
Protein work

Extracts were prepared from log phase cells by the liquid nitrogen grinding method (Sorger and Pelham, 1987). Cells were resuspended in UB (50 mM Hepes, pH 7.5, 100 mM KCl, 1 mM EGTA, 3 mM MgCl\(_2\), 1 mM DTT) supplemented with protease inhibitors and clarified at 300,000 g for 60 min. Immunoprecipitations were performed as described (Lechler et al., 2000). Gel filtration analysis was performed using a Superose-6 gel filtration column (Amersham Pharmacia Biotech) run on a BioLogic workstation (Bio-Rad Laboratories).

Arp2/3 complex purification

The Arp2/3 complex was purified by modification of a previously established method (Eggle et al., 1999). Yeast cells were lysed in UB, and an extract was made by centrifugation at 42 K for 1 h. A 55% ammonium sulfate precipitation was performed, and the pellet was dissolved and dialyzed overnight into buffer A (20 mM Tris, pH 7.5, 25 mM KCl, 1 mM MgCl\(_2\), 1 mM DTT, 0.5 mM EGTA, 0.1 mM ATP). This fraction was then incubated with GST-A(Bee1p) fragment coupled to Affigel-10 (Bio-Rad Laboratories). The beads were washed with buffer B (20 mM Tris, pH 7.5, 0.2 M KCl, 1 mM MgCl\(_2\), 1 mM DTT, 0.5 mM EGTA, 0.1 mM ATP) and eluted with buffer C (20 mM Tris, pH 7.5, 25 mM KCl, 0.2 M MgCl\(_2\), 1 mM DTT, 0.5 mM EGTA, 0.1 mM ATP). The Arp2/3 complex–containing eluate was dialyzed into UB supplemented with 0.2 mM ATP.

Pyrene actin assembly assays

Pyrene actin assembly assays were performed in UB with 0.5 mM ATP at 30°C in an SLM Aminco Bowman series 2 luminescence spectrophotometer. Actin (50% pyrene labeled) concentration was 1.75 μM, the Arp2/3 complex was at 0.25 μM, and the various activators at 0.5 μM. The dimerization experiments were performed by first mixing the dimerizing pair (25 μM each protein) with or without rapamycin (50 μM) (Sigma-Aldrich) at room temperature for 15 min. The mixture was diluted to a final concentration of 0.5 μM each protein and 1 μM rapamycin in the pyrene-actin assembly assays.

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